Molecular Pharmacology Fast Forward. Published on September 18, 2007 as doi:10.1124/mol.107.040071 MOL Manuscript # 40071

The N-terminal domains of both NR1 and NR2 subunits determine allosteric Zn²⁺ inhibition and glycine affinity of NMDA receptors

Christian Madry, Ivana Mesic, Heinrich Betz, and Bodo Laube

Abteilung Neurochemie, Max-Planck-Institut für Hirnforschung, Deutschordenstr. 46, 60528 Frankfurt am Main, Germany (C.M., I.M., H.B., B.L.)

AG Molekulare und zelluläre Neurophysiologie, Technische Universität Darmstadt, Schnittspahnstr. 3, 64287 Darmstadt, Germany (I.M., B.L.)

Running title: Role of NTDs in NMDA receptor function

Corresponding author: Bodo Laube, Technische Universität Darmstadt, AG Molekulare und zelluläre Neurophysiologie, Schnittspahnstr. 3, 64287 Darmstadt, Germany; Phone: ++49 69 96769 295, Fax: ++49 69 96769 441, e-mail: laube@mpih-frankfurt.mpg.de

Number of text pages:	33
Number of tables:	1
Number of figures:	7
Number of words in abstract:	222
Number of words in Introduction:	493
Number of words in Discussion:	1134

Abbreviations

iGluR, ionotropic glutamate receptor; NMDA, N-methyl-D-aspartic acid; NTD, N-terminal domain; LBD, ligand-binding domain; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid; ifenprodil, 4-[2-[4-(cyclohexylmethyl)-1-piperidinyl]-1-hydroxypropyl]phenol; DTT, dithiothreitol; LIVBP, leucine/isoleucine/valine binding protein, MK801, (+)-5-methyl-10,11-dihydro-5Hdibenzo[a,d]cyclohepten-5,10-imine maleate); D-APV, (D-(-)-2-amino-5-phosphonopentanoic acid; MDL-29951, 3-(2-Carboxyethyl)-4,6-dichloro-1H-indole-2-carboxylic acid; EGFP, enhanced green fluorescent protein; TCS, thrombin cleavage site.

Abstract

The N-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptors (iGluRs) is a tetrameric complex composed of homologous NR1 and NR2 subunits, which require the binding of glycine and glutamate, respectively, for efficient channel gating. The extracellular N-terminal domains (NTDs) of iGluR subunits show sequence homology to the bacterial periplasmic leucine/isoleucine/valine binding protein (LIVBP) and have been implicated in iGluR assembly, trafficking and function. Here, we investigated how deletion of the NR1- and NR2-NTDs affects the expression and function of NMDA receptors. Both proteolytic cleavage of the NR1-NTD from assembled NR1/NR2 receptors and co-expression of the NTD-deleted NR1 subunit with wildtype or NTD-deleted NR2 subunits resulted in agonist-gated channels that closely resembled wild-type receptors. This indicates that the NTDs of both NMDA receptor subunits are not essential for receptor assembly and function. However, deletion of either the NR1 or the NR2 NTD eliminated high-affinity, allosteric inhibition of agonist-induced currents by Zn²⁺ and ifenprodil, consistent with interdomain interactions between these domains being important for allosteric receptor modulation. Furthermore, by replacing the NR2A-NTD with the NR2B NTD, and vice versa, the different glycine affinities of NR1/NR2A and NR1/NR2B receptors were found to be determined by their respective NR2-NTDs. Together, these data show that the NTDs of both the NR1 and NR2 subunits determine allosteric inhibition and glycine potency but are not required for NMDA receptor assembly.

Introduction

Excitatory neurotransmission in the mammalian brain is mainly mediated by ionotropic glutamate receptors (iGluRs). Based on pharmacological studies, iGluRs have been grouped into three distinct subfamilies: AMPA receptors (GluR1-4), kainate receptors (GluR5-7, KA1, 2), and NMDA receptors (NR1, NR2A-D, NR3A, B) (overview in Dingledine et al., 1999, and Cull-Candy et al., 2001). All iGluR subunits share a common modular design characterized by: i) an extracellular N-terminal domain (NTD) of about 400 amino acids that shows sequence homology to the bacterial periplasmic leucine/isoleucine/valine binding protein (LIVBP) and has been implicated in iGluR subunit oligomerization, trafficking and function; ii) a S1S2 ligand binding domain (LBD) composed of an extracellular region preceding the first transmembrane domain (S1) and a second extracellular region (S2) connecting the transmembrane segments 1 and 2, which lines the ion channel; and iv) an intracellular carboxyterminal tail region that interacts with postsynaptic scaffolding and signal transduction proteins (reviewed in Madden, 2002).

Among iGluRs, NMDA receptors stand out with respect to both their molecular diversity and their particular pharmacological and functional properties (Dingeldine et al., 1999). Within the hetero-tetrameric receptor proteins, various splice variants of the glycine-binding NR1 subunit (Kuryatov et al., 1994) co-assemble with glutamate-binding NR2 (Laube et al., 1997) and/or glycine-binding NR3 subunits (Yao and Mayer, 2006). Activation of NMDA receptors is a complex process that requires ambient glycine and release of glutamate from presynaptic terminals in coincidence with postsynaptic membrane depolarization, which relieves the receptor channel from a voltage-dependent block by Mg²⁺ ions. NMDA receptor function is regulated by allosteric inhibitors, such as Zn²⁺ and the phenylethanolamine ifenprodil, which bind to the NTDs of NR2A and NR2B subunits (Herin and Aizenman, 2004) and enhance receptor desensitization (Krupp et al., 1998; Zheng et al., 2001). Presently, the molecular basis of allosteric NMDA receptor

inhibition is poorly understood but has been attributed to interactions between the NTD and the LBD of the NR2 subunits (Paoletti et al., 2000). Deletion of the NR2A and NR2B NTDs generates NMDA receptors that display a reduced inhibition by both Zn²⁺ and ifenprodil (Paoletti et al., 2000). The role of the NR1-NTD has not been investigated further, since N-terminal truncations within the NR1 subunits have been reported to impair receptor function upon co-expression with NR2 subunits (Meddows et al., 2001).

Here, we analyzed the role of the NTD of the NR1 subunit in NMDA receptor assembly and allosteric inhibition by both enzymatically cleaving this domain from properly assembled receptors and co-expressing a truncated NR1 subunit with wild-type or NTD-deleted NR2A and NR2B subunits. We find that, like the NR2-NTDs, the NR1-NTD is not required for receptor function and assembly, but importantly contributes to allosteric Zn²⁺ and ifenprodil inhibition. In addition, high-affinity glycine binding requires the NTDs of both NR1 and NR2B subunits. Our data suggest that direct interactions between the NR1 and NR2 NTDs determine the potency of allosteric inhibitors and the co-agonist glycine.

Materials and Methods

MK801, D-APV and MDL-29951 were purchased from Tocris (Biotrend, Cologne, Germany). All other chemicals used were obtained from Sigma (Taufkirchen, Germany).

DNA constructs, oocyte expression and electrophysiology

cDNAs of the NR1a, NR2A and NR2B subunits were subcloned into the pNKS2 vector. Mutations were introduced by site-directed mutagenesis (QuikChange XL Site-Directed Mutagenesis Kit, Stratagene, Amsterdam, Netherlands) and confirmed by DNA sequencing. The NR1^{ΔNTD} construct was generated by excising the nucleotide sequence encoding amino acids 5 -358 of the mature protein with Pvul. To enzymatically remove the NTD of NR1, a thrombin recognition sequence (LVPRGS; Madry et al., 2007) was inserted at position 358 of the NR1 subunit that had been fused to enhanced green fluorescent protein (EGFP-NR1^{TCS}) by subcloning into the pEGFP-C1 vector (Clontech, Mountain View, CA, USA). The NR2A^{ΔNTD}. NR2B^{ΔNTD}, NR2A^{NTD2B} and NR2B^{NTD2A} constructs (Paoletti et al., 2000; Rachline et al., 2005) were kindly provided by Dr. P. Paoletti (Ecole Normale Supérieure, Paris, France). The NR2A*-His construct was generated by replacing the C-terminal region from amino acid 930 with a 6x His tag (Madry et al., 2007). In vitro synthesis of cRNA (mCAP mRNA Capping Kit, Ambion, Austin, TX, USA) was performed as described (Madry et al., 2007). For heterologous expression of NMDA receptors, 25 ng of cRNA was injected at a NR1:NR2 ratio of 1:2 into Xenopus laevis oocytes. Oocytes were isolated and maintained as described previously (Laube et al., 1997). Two-electrode voltage-clamp recording of whole-cell currents was performed according to Laube et al. (1995). To monitor the voltage dependence of NR1/NR2B NTD-deleted receptor combinations, 2 sec -80/+40 mV voltage ramps were used. Leakage currents were recorded prior to agonist/Zn²⁺ application and subtracted from the agonist/Zn²⁺ -induced currents. To measure desensitization of receptor responses, we recorded currents upon application of saturating concentrations of glycine and glutamate (100 μ M, each) until a steady-state plateau was reached. On the basis of steady-state (I_{ss}) and peak (I_p) current amplitudes recorded in the same solution, we calculated the extend of desensitization as the percentage (%) of current decay in the continuous presence of the agonists. For thrombin treatment oocytes were incubated with 30 U/ml of the protease for 60 min at room temperature. Same oocytes were measured prior to and after thrombin exposure.

Transfection of HEK293 cells and thrombin treatment

Culture conditions for human embryonic kidney 293 (HEK293) cells (ATCC#CRL1537) have been described previously (Laube et al., 1995). Transfection with Lipofectamine[®] 2000 was performed according to the manufacturer's protocol. HEK293 cells were co-transfected with either EGFP-NR1 (wt) or EGFP-NR1^{TCS} plasmid together with the NR2A construct, using 20 μ g of total DNA at a NR1:NR2 ratio of 1:3. Transfected cells were cultured in the presence of the NMDA inhibitors MK801, D-APV and MDL-29951 (all 100 μ M) for 48 h. Then new medium without Ca²⁺ and bovine serum albumine was added, and the cells were incubated with 30 U/ml thrombin for 30 min at 37°C followed by harvesting and homogenization in a Polytron[®]. After centrifugation at 1000 x g, the supernatant was centrifuged at 10.000 x g for 20 min at 4°C to obtain the membrane pellet, which then was suspended in SDS sample buffer.

Metabolic labeling, purification and SDS-PAGE of NMDA receptor complexes

Injected oocytes were metabolically labelled by overnight incubation with [³⁵S]methionine as described (Madry et al., 2007). After an additional 24 h chase interval, labelled receptor complexes were purified by Ni²⁺-NTA chromatography from 0.5% (w/v) dodecylmaltoside extracts of the labelled occytes as detailed previously (Sadtler et al., 2003). For SDS-PAGE, protein samples were solubilized in SDS sample buffer containing 20 mM dithiotreitol (DTT) and electrophoresed in parallel with molecular mass markers (Precision Plus Protein All Blue

Standard, Biorad, Munich, Germany) on 10% tricine/SDS-polyacrylamide gels. Radioactive gels were dried and exposed to BioMax MR films (Kodak, Stuttgart, Germany) at 80°C or to a phosphor-imaging plate for quantification purposes. Phosphor plates were scanned on a Typhoon Trio fluorescence scanner and analyzed with Image Quant TL software (GE Healthcare).

Antibodies

Anti-NR1 (generated against amino acids 660-811 of the rat NR1 subunit) and anti-EGFP primary antibodies were purchased from BD Biosciences (Heidelberg, Germany) and used at dilutions of 1:500 (NR1) and 1:1000 (EGFP), respectively. Goat anti-mouse horseradish peroxidase-linked secondary antibody (Dianova, Hamburg, Germany) was employed at a final dilution of 1:10,000, and immunoreactive bands were detected with the ECL Western blotting system (Amersham - GE Healthcare, Munich, Germany).

Statistical analyses

Values given represent means \pm SE. Statistical significance was determined at the p < 0.01 (*) and p < 0.001 (**) levels using a Student's two-tailed, unpaired t-test.

Results

To investigate the role of the NTD of the glycine-binding NR1 subunit in NMDA receptor assembly and function, we designed two different NR1 cDNA constructs. First, by inserting a thrombin cleavage site (TCS) sequence at amino acid position 358 of the NR1 subunit (EGFP-NR1^{TCS}; Fig. 1A), we generated a NR1 subunit, which should allow proteolytic cleavage of the NR1-NTD from surface-located receptors upon thrombin treatment. Visualization and immunological detection of the respective NR1-NTD fragment was achieved by an N-terminal EGFP tag (see Fig. 1A, and Material and Methods). Second, a truncated NR1 subunit (NR1 $^{\Delta NTD}$; Fig. 1A) was generated by deleting the nucleotide sequence encoding residues 5-358.

Biochemical and functional characterization of NMDA receptors containing the thrombincleavable EGFP-NR1^{TCS} subunit

To examine whether the NR1^{TCS} construct is cleaved by thrombin, we co-expressed both the EGFP-NR1 and the EGFP-NR1^{TCS} subunits with the NR2A subunit in HEK 293 cells. Western blot analysis of membrane fractions prepared from the transfected cells revealed a single band of apparent molecular mass of about 130 kDa with both the wild-type (wt) EGFP-NR1 and the EGFP-NR1^{TCS} DNAs upon staining with an anti-EGFP antibody (Fig. 1B, lanes 1 and 2). Upon thrombin treatment of the intact cells, membranes prepared from wt EGFP-NR1 and NR2A transfected cells again contained a 130 kDa NR1 protein band that was recognized by both anti–NR1 and anti-EGFP antibodies (Fig. 1B, lanes 3 and 4). In contrast, treatment of EGFP-NR1^{TCS} and NR2A subunit expressing cells with thrombin generated in addition to the 130 kDa band two prominent fragments of about 60 kDa and 70 kDa that were stained by anti-NR1 and anti-EGFP, respectively (Fig. 1B, lanes 5 and 6). These fragment sizes are consistent with the calculated masses of the membrane-bound "core" NR1 subunit and the truncated EGFP-tagged NTD of the EGFP-NR1^{TCS}/NR2A receptors, whereas the non-cleaved NR1^{TCS} 130 kDa protein most likely

corresponds to thrombin-inaccessible intracellularly located subunits. Furthermore, copurification of the truncated EGFP-tagged NTD in the membrane fraction shows that cleavage of the NR1-NTD by thrombin does not necessarily result in a separation of this domain from the "core" receptor implying strong non-covalent interactions with the remaining protein.

The consequences of thrombin-mediated cleavage of the NR1-NTD on apparent agonist affinities and maximal inducible currents (I_{max}) were analyzed by two-electrode voltage clamping after co-expression of EGFP-NR1^{TCS} with the NR2B subunit in *Xenopus laevis* oocytes. The resulting glycine and glutamate dose-response curves were indistinguishable to those of the wt NR1/NR2B receptor in the absence and presence of thrombin. In contrast, after thrombin treatment the EC₅₀ value of the EGFP-NR1^{TCS}/NR2B receptor showed a significant decrease in apparent glycine affinity (0.30±0.04 µM vs. 0.80±0.14 µM; p<0.01, n=4), whereas the glutamate EC₅₀ value (1.2±0.4 µM vs. 1.3±0.3 µM) and the maximal inducible currents were not significantly changed (Fig. 2A, left). Since a similar result was also obtained for EGFP-NR1^{TCS}/NR2A receptors (Fig. 2B, left), we conclude that thrombin-mediated cleavage of the NR1 NTD does not impair receptor function.

Cleavage of the NR1-NTD eliminates high-affinity Zn²⁺ inhibition of NR1/NR2 receptors

Since the NTDs of the NR2 subunits have been found to mediate the allosteric inhibition of NMDA receptors (overview in Herin and Aizenman, 2004), we also examined the effect of thrombin-mediated NR1-NTD deletion on Zn^{2+} inhibition of both EGFP-NR1^{TCS}/NR2B and EGFP-NR1^{TCS}/NR2A receptor currents. NR2B containing NMDA receptors are inhibited by micromolar concentrations of Zn^{2+} (Rachline et al., 2005). Upon thrombin treatment of oocytes expressing the EGFP-NR1^{TCS}/NR2B combination, the IC₅₀ value of Zn^{2+} increased 19-fold, from 13±3 µM before to 256±34 µM after incubation with the protease (p<0.01, n=3; Fig. 2A, right). This

suggested that the NTD of the NR1 subunit is not essential for receptor function but contributes to allosteric Zn²⁺ inhibition.

To examine whether the NR1-NTD is also required for the biphasic mode of Zn²⁺ inhibition seen with NR1/NR2A receptors (Williams, 1996; Paoletti et al., 1997), we determined the effects of Zn²⁺ on agonist-induced currents of EGFP-NR1^{TCS}/NR2A expressing oocytes before and after thrombin treatment (Fig. 2B). Recordings from untreated oocytes disclosed the typical biphasic Zn²⁺ inhibition curve with IC₅₀ values of 0.028±0.005 μ M for the high-affinity, and 75±8 μ M for the low-affinity Zn²⁺-binding sites (n=5). After an 1 h incubation with thrombin, the high-affinity component of Zn²⁺ inhibition was reduced by >80 %, with low-affinity Zn²⁺ inhibition predominating (259±64 μ M, n=5; Fig. 2B right). In conclusion, thrombin efficiently cleaves surface-localized EGFP-NR1^{TCS} subunits and thereby strongly reduces the affinity of Zn²⁺ inhibition at both NR1/NR2A and NR1/NR2B receptors.

N-terminally truncated NR1 subunits assemble efficiently into functional NMDA receptors

To investigate the importance of the NR1-NTD for receptor assembly, we examined whether an N-terminally truncated NR1 subunit, which lacks amino acids 5 to 358 of the mature NR1 subunit (NR1^{ΔNTD}, Fig.1A), forms heteromeric NMDA receptors after heterologous expression in *Xenopus* oocytes. To this end, we co-expressed the wt and the NR1^{ΔNTD} construct with the tagged NR2A^{*}-His subunit (Madry et al., 2007) in oocytes that were metabolically labelled with [³⁵S]methionine. The NR2A^{*}-His subunit was then purified under non-denaturating conditions by metal affinity chromatography from digitonin extracts of the oocytes and analyzed by reducing SDS-PAGE and autoradiography (Sadtler et al., 2003). Fig. 3A, lane 1, shows that two ³⁵S-labelled bands with apparent molecular weights of about 116 kDa and 105 kDa corresponding to those of the NR1 and NR2A^{*}-His subunits, respectively, were co-isolated by this protocol. Similarly, co-expression of the NR1^{ΔNTD} with the NR2A^{*}-His construct resulted in co-isolation of

two ³⁵S-labelled bands with molecular weights of about 78 kDa and 105 kDa, showing that the NR1^{ΔNTD} subunit also assembles with NR2A^{*}-His (Fig. 3A, lane 2). Quantification of the subunit bands by phosphor imaging revealed a ratio of ³⁵S-radioactivities of the wt NR1 subunit to the NR2A^{*}-His polypeptide of 1.09±0.16 (n=3). This value is in good agreement with the theoretical ratio of 0.93, calculated from the determined subunit stoichiometry of 2NR1:2NR2 (Laube et al., 1998) and the known numbers of 28 and 30 methionine residues per mature NR1 and NR2A^{*}-His subunit, respectively. Analysis of NR1^{ΔNTD}/NR2A^{*}-His receptors yielded a ratio of 0.60±0.09 (n=3) of NR1^{Δ NTD} to NR2A^{*} subunit radioactivities. This is consistent with a lower number (18) of methionine residues in NR1^{ΔNTD}, which predicts a theoretical ratio of 0.60 for a receptor complex containing two NR1^{ΔNTD} and two NR2A^{*}-His subunits. Since the intensities of the NR2A^{*}-His polypeptide bands were not different in the affinity-purified NR1/NR2A*-His and NR1^{ΔNTD}/NR2A*-His receptors, the close to theoretical NR1:NR2A ratio of 1:1 obtained values for both preparations indicate that i) both the wt NR1 and NR1^{ΔNTD} subunits assemble at a 2:2 stoichiometry with NR2A^{*}-His, and that ii) both NR1 polypeptides show comparable assembly efficiencies. In conclusion, NMDA receptor formation seems not to depend on the NTD of the NR1 subunit.

NTD-deleted NR1 and NR2 subunits generate functional NMDA receptors

A previous study has shown that co-expression of NTD-deleted NR2A and NR2B subunits with wt NR1 generates functional NMDA receptors (Paoletti et al., 2000). To analyze whether the NTD deleted NR1 subunit NR1^{Δ NTD} assembles into functional receptors upon co-expression with the NR2A or NR2B subunit, we applied saturating glutamate and glycine concentrations (100 μ M each) to recombinant NR1/NR2B, NR1^{Δ NTD}/NR2B, NR1/NR2B^{Δ NTD} and NR1^{Δ NTD}/NR2B^{Δ NTD} receptors. All subunit combinations mentioned above were found to produce robust currents with I_{max} values that were not significantly different from each other (Fig. 3B; Table 1). Similarly, receptors composed of NTD-deleted NR1 and NR2A subunits displayed robust agonist

responses in the presence of saturating agonist concentrations, with I_{max} values similar to those of wt NR1/NR2A receptors (Table 1). Furthermore, we determined the extent of current decay of NR1/NR2A, NR1NR2B, NR1^{ΔNTD}/NR2A^{ΔNTD} and NR1^{ΔNTD}/NR2B^{ΔNTD} expressing oocytes in the continuous presence of saturating glycine and glutamate (100 µM, each) concentrations by measuring the ratio of the peak (I_p) and steady-state (I_{ss}) current as an estimate for receptor desensitization. Fig. 3C, left, shows typical traces recorded from wt NR1/NR2A and mutant NR1^{ΔNTD}/NR2A^{ΔNTD} receptors, which rapidly reached peak amplitude and then strongly decayed to steady-state currents in the presence of agonists. For wt NR1/NR2A channels the extent of desensitization expressed as a percentage of the peak current was 83±2.4 % (n=13), whereas mutant receptors showed a significant decreased extent of desensitization (51±1,3 %; n=13) (Fig. 3D). Analysis of wt NR1/NR2B and mutant NR1^{ΔNTD}/NR2B^{ΔNTD} receptors revealed no differences in the desensitization ratios with values of 54±1,2 and 54±5,5 % (n=13), respectively (Fig. 3C, right traces and Fig. 3D). Overall, these data clearly show that the NTDs of both the NR1 and NR2 subunits are not required for NMDA receptor assembly and membrane insertion but may play a role in determining receptor kinetics.

The NR1-NTD is required for high-affinity Zn²⁺ inhibition of NR1/NR2A receptors

The NTD of the NR2A subunit is known to harbour crucial determinants of the voltageindependent, high-affinity inhibition by Zn^{2+} (Choi and Lipton, 1999; Fayyazuddin et al., 2000; Paoletti et al., 2000). Recordings of wt NR1/NR2A receptors exhibited a biphasic Zn^{2+} inhibitionresponse curve, with IC₅₀ values in the nanomolar and micromolar range (Table 1) and a maximal inhibition of about 60 % exerted via the high-affinity site (Fig. 4A). To examine whether co-assembly with the NR1^{Δ NTD} construct would result in a similar reduction of Zn^{2+} inhibition as seen upon thrombin treatment of NR1^{TCS}/NR2A receptors, we co-expressed different combinations of wt and NTD-deleted NR1 and NR2A subunits. With the NR1^{Δ NTD}/NR2A, NR1/NR2A^{Δ NTD} and NR1^{Δ NTD}/NR2A^{Δ NTD} combinations, we found a complete loss of high-affinity Zn^{2+} inhibition; only a low-affinity inhibitory component persisted at all these truncated receptors cases (Fig. 4A, Table 1). Thus, not only the NR2A-NTD but also the NR1-NTD is crucially required for high-affinity Zn^{2+} inhibition of NR1/NR2A receptors.

Both NR1- and NR2B-NTDs contribute to Zn²⁺ and ifenprodil inhibition

To unravel possible roles of the NTDs also in NR1/NR2B receptor modulation, we first analyzed the effects of Zn^{2+} on all possible combinations of wt and NTD-deleted NR1 and NR2B subunits, i.e. NR1/NR2B, NR1^{ΔNTD}/NR2B, NR1/NR2B^{ΔNTD} and NR1^{ΔNTD}/NR2B^{ΔNTD}. Analysis of the respective inhibition curves revealed significant differences in Zn^{2+} sensitivity (Table 1). Both single and double deletions of the NTDs of either the NR1 and/or NR2B subunits markedly increased to a similar extent the concentration of Zn^{2+} required to half-maximally inhibit NR1/NR2B receptors (Fig. 4B; Table 1). We furthermore examined the role of the NTDs for inhibition by the synthetic neuroprotective compound ifenprodil, which has been reported to allosterically inhibit NMDA receptors via the NTD of the NR2B subunit (Perin-Dureau et al., 2002). At wt NR1/NR2B receptors, ifenprodil displayed an IC₅₀ value of 0.89±0.08 µM (Fig. 4C). Again single as well as double deletions of the NTDs for NR1 and NR2B subunits caused a >100-fold reduction in inhibitory potency (Fig. 4C, Table 1). Overall, our data emphasize the importance of both the NR1- and NR2-NTDs for high-affinity allosteric Zn^{2+} and ifenprodil inhibition of NR1/NR2A and NR1/NR2B receptors.

Residual Zn²⁺ and ifenprodil inhibition of NTD-deleted receptors is mediated by both voltagedependent and -independent low-affinity components

To reveal whether the residual low-affinity Zn^{2+} and ifenprodil inhibition seen with NTD-deleted NR1/NR2B receptors (see Table 1) is mediated by either a channel-blocking effect or a voltageindependent low-affinity site, we analyzed the current-voltage relationship of agonist currents recorded in the presence of Zn^{2+} . Whereas in Mg²⁺-free medium the current-voltage relation of

wt NR1/NR2B receptors was linear in the presence of 10 μ M Zn²⁺ (Fig. 5A), the inhibition of NTD-deleted NR1^{Δ NTD}/NR2B, NR1/NR2B^{Δ NTD} and NR1^{Δ NTD}/NR2B^{Δ NTD} receptors seen in the presence of 100 μ M Zn²⁺ was found to be composed of a voltage-dependent and -independent component (Fig. 5B-D). The latter, detected at positive holding potentials, is likely mediated via a separate Zn²⁺ binding site located within domains distinct from the NTDs (see Fayyazuddin et al., 2000; Rachline et al., 2005). Similar to Zn²⁺ inhibition, the remaining ifenprodil effect observed with the NTD-deleted receptors displayed voltage dependency at negative holding potentials (not shown). We therefore conclude that besides a voltage-dependent Zn²⁺-binding site outside the NTDs responsible for voltage-independent low-affinity Zn²⁺ inhibition.

Removal of NR1- and/or NR2B-NTDs reduces glycine affinity

Initially we had observed that thrombin cleavage of EGFP-NR1^{TCS}/NR2B receptors reduced not only their Zn²⁺ sensitivity but also increased the EC₅₀ value of glycine (0.30±0.04 μ M vs. 0.80±0.14 μ M). This prompted us to determine the apparent glutamate and glycine affinities of NTD-deleted NR1/NR2A and NR1/NR2B receptors. In agreement with previous studies (Laurie and Seeburg, 1994; Priestley et al., 1995), the glycine affinities of NR1/NR2A and NR1/NR2B receptors were found to be significantly different (Fig. 6A), with EC₅₀ values of 1.7±0.2 μ M vs. of 0.39±0.04 μ M, respectively (p<0.001; Table1). We found significant changes in glycine EC₅₀ values only with NTD-deleted NR1/NR2B receptors remained unaltered upon NTD removal (Fig. 6B, Table 1). No changes in glutamate affinities were obtained for both NR1/NR2A and NR1/NR2B receptors after NTD-deletion (Table 1). Hence, both the NR1- and the NR2B-NTDs are essential for high-affinity glycine binding to NR1/NR2B receptors. To further examine whether the NR2-NTDs play a role in determining the different glycine affinities of distinct NMDA receptor subtypes, we used two chimeric constructs in which the NTD of NR2A was replaced by the corresponding NR2B-NTD (NR2A^{NTD2B}), and *vice versa* (NR2B^{NTD2A}), as detailed previously (Paoletti et al., 2000). NR1/NR2A^{NTD2B} receptors were found to have the same glycine EC₅₀ value than wt NR1/NR2B receptors (Fig. 6A, B; 0.28±0.09 vs. 0.39±0.04 μ M; p>0.05, n=4), which was significantly different from the respective wt NR1/NR2A receptor (p<0.01, n=5). Inversely, NR1/NR2B^{NTD2A} receptors (Fig. 6A, B; 2.4±0.4 μ M vs. 1.7±0.3 μ M, p>0.05, n=3). In contrast, no significant differences in glutamate affinities between wt NR1/NR2A and NR1/NR2B receptors and the respective chimeric receptors were observed (Fig. 6C). Thus, the NTDs of NR2A and NR2B determine not only allosteric inhibition but also the glycine affinity of different NMDA receptor subtypes.

Discussion

In this paper, we examined the contributions of the N-terminal LIVBP-homology domains of the NR1 and the NR2 subunits to NMDA receptor assembly, function and allosteric inhibition. We show that the NTDs are not required for subunit assembly and channel function. However, high-affinity inhibition by Zn²⁺ or ifenprodil was abolished upon NTD deletion of either the NR1 or NR2 subunit, indicating that both NTDs are required for allosteric receptor inhibition. Furthermore, the different apparent glycine affinities of NR1/NR2A vs. NR1/NR2B receptors were found to be determined by their respective NR2-NTDs.

Role of the NTDs in NMDA receptor modulation

Several studies have shown that the LIVBP-like domains in both ionotropic and metabotropic GluRs are capable of specifically forming dimers or higher-order oligomers via interdomain interactions (Kuusinen et al., 1999; Kunishima et al., 2000). In non-NMDA receptors of the iGluR family, these interactions have been implicated in subunit assembly (Ayalon and Stern-Bach, 2001; Matsuda et al., 2005). Here, we show that NTD-deleted NMDA receptor subunits form functional channels with agonist-induced currents similar to those of wt receptors; this clearly excludes an essential role of the NTDs in the assembly of NR1/NR2 receptors. This finding is consistent with the data demonstrated by others (Fayyazuddin et al., 2001; Hu and Zheng, 2005) where deletion of the NR2 NTDs resulted in functional NMDA receptors. However, Meddows et al. (2001) reported that deletion of the first 380 amino acid residues of the NR1 subunit impairs subunit oligomerization. We attribute this different result to the longer deletion used by these authors than that studied here. Our data are also in agreement with studies obtained for other members of the iGluR family, which demonstrate proper assembly of natural and recombinant subunits lacking an NTD (Chen et al., 1999; Pasternack et al., 2002).

Although interactions between the NTDs of the NMDA receptor subunits are not required for receptor assembly, both thrombin-mediated cleavage of the NR1-NTD and deletion of the NR1or NR2-NTDs abrogated voltage-independent high-affinity Zn²⁺ and ifenprodil inhibition. This clearly demonstrates that the NR1-NTD is required for the inhibitory effects exerted by these allosteric inhibitors, although both have shown to bind to the NR2-NTDs (overview in Herin and Aizenman, 2004). The residual low-affinity voltage-independent and dependent inhibition observed upon NTD deletion are likely due to additional binding sites located outside the NTDs and within the channel region, respectively (Paoletti et al., 1997, Traynelis et al., 1998; Rachline et al., 2005).

Model of NTD-mediated inhibition

Previous studies indicate that both ifenprodil and Zn²⁺ share common binding sites and mechanisms, which result in increased NMDA receptor desensitization upon binding-induced domain closure of the LIVBP-homology region (Chen et al., 1997; Paoletti et al., 1997, 2000; Krupp et al., 1998; Low et al., 2000; Zheng et al., 2001). This is also consistent with our finding that removal of the NTDs of the NR1/NR2A receptor slows receptor desensitization. Based on these data, we favor a mechanism of NTD-mediated NMDA receptor inhibition that is adapted from a recent model of AMPA receptor activation (Mayer, 2006) and relies on i) the crystallographically demonstrated heterodimeric arrangement of NR1 and NR2 subunits (Furukawa et al., 2005) and ii) iGluR desensitization resulting from a disruption of LBD interdomain-interactions (Armstrong et al., 2006). Accordingly, binding of an allosteric inhibitor to the NR2-NTD is proposed to induce closure of the LIVBP-homology domain and to thereby produce a conformational strain, which weakens interdomain interactions between NR1- and NR2-LBDs (Fig. 7). This facilitates receptor desensitization upon agonist binding. An important feature of our model is that only binding of an allosteric modulator to an NR2-NTD stabilized by an adjacent NR1-NTD would be able to sufficiently weaken the interactions between NR1 and

NR2 LBDs (Fig. 7; see Armstrong et al., 2006). This implies that a hetero-dimer is formed by the NR1 and NR2 LIVBP homology domains, an idea which is entirely consistent with both the heterodimeric arrangement of NR1 and NR2 subunits (Furukawa et al., 2005) and our data showing that both the NR1 and NR2 NTDs equally contribute to high-affinity Zn²⁺ and ifenprodil inhibition. Our model assigning an important role to the NTD heterodimer (Fig. 7) is also consistent with the observation that the glycine affinity of NMDA receptors containing chimeric NR2 subunits is determined by their respective NR2-NTDs.

Contribution of NTDs in determining agonist affinity

The pharmacological profile of NMDA receptors is known to crucially depend on the NR2 subunit isoform incorporated (Laurie and Seeburg, 1994; overview in Cull-Candy et al., 2001). For example, NR1/NR2B receptors have a 10-fold higher glycine affinity than NR1/NR2A receptors (Laurie and Seeburg, 1994; Priestley et al., 1995; this study), although both receptors share the same glycine-binding NR1 subunit. Here we show that upon co-expression with NR1 a chimeric NR2A subunit containing the NTD of NR2B generates receptors displaying the high glycine affinity characteristic of wt NR1/NR2B receptors. *Vice versa*, the EC₅₀ value of glycine at NR1/NR2B^{NTD2A} receptors was similar to that determined for wt NR1/NR2A receptors. These results are consistent with the observation that mutations within NR2-NTDs can affect apparent glycine affinity (Choi et al., 2001). All these findings can be explained by allosteric interactions between the NTDs of the NMDA receptor subunits, which determine both the affinity of glycine binding to the NR1 subunit and the efficay of allosteric inhibitors at the NR2 subunits.

Implications on the pathology and therapy of t-PA triggered neurotoxicity

Excessive stimulation of NMDA receptors is known to cause neuronal cell death by apoptosis or necrosis due to enhanced Ca²⁺ influx (overview in Cull-Candy et al., 2001). NMDA receptors are tonically inhibited by Zn²⁺; a mechanism, which has been shown to protect neurons against

NMDA receptor-mediated glutamate toxicity in vitro (Chen et al., 1997). Here, we demonstrate that deletion of the NR1-NTD by thrombin abolishes high-affinity Zn^{2+} inhibition of NR1/NR2A receptors. Tissue-type plasminogen activator (t-PA), an endogeneous serine protease, has been found to potentiate NMDA receptor currents through cleavage of the NR1-NTD, which has been implicated in pathophysiological aspects of glutamatergic neurotransmission (Nicole et al., 2001; Fernández-Monreal et al., 2004). After focal cerebral ischemia, t-PA triggers the neurotoxic cascade mediated by elevated concentrations of glutamate (Tsirka et al., 1995). Blockade of this serine protease in cortical neuron cultures has been reported to reduce NMDA-induced excitotoxic cell death (Nicole et al., 2001). As we found a loss in Zn^{2+} inhibition of both NR1/NR2A and NR1/NR2B receptors upon thrombin cleavage and deletion of the NR1-NTD, our results might provide an explanation for the enhanced NMDA receptor activity seen in the presence of t-PA. Accordingly, relief of NMDA receptors from tonic Zn^{2+} inhibition (Rachline et al., 2005) by t-PA-mediated cleavage of the NR1-NTD would result in enhanced Ca^{2+} influx and thereby cause neuronal cell death. This mechanism should be particularly effective at synaptically localized NR1/NR2A receptors, due to their high-affinity Zn²⁺-binding site.

Acknowledgements

We thank Dr. P. Paoletti for providing the NR2A^{Δ NTD}, NR2B^{Δ NTD}, NR2B^{NTD2A} and NR2A^{NTD2B} cDNAs, Dr. A. Nicke for technical advice on metabolic labelling and Drs. J.R.P. Geiger and B. Mathias-Costa for critical reading of the manuscript.

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Footnotes

This study was supported by the Max-Planck-Gesellschaft (H.B.), Gemeinnützige Hertie-Stiftung (B.L.), Dr. Robert Pfleger Stiftung (B.L.), Deutsche Forschungsgemeinschaft (grant LA 1086/4-1, B.L.) and Fonds der Chemischen Industrie (H.B.). C.M. received a predoctoral fellowship from the Graduiertenkolleg Neuronale Plastizität, University of Frankfurt.

Figure Legends

Figure 1. Biochemical characterization of a thrombin-cleavable NR1^{TCS} subunit. **A**, Schematic representations of i) an NR1 construct harboring a thrombin clevage site (LVPRGS) at amino acid position 358 and an N-terminal EGFP-tag (EGFP-NR1^{TCS}, top) and ii) an NTD-deleted NR1 subunit lacking amino acids 5-358 (NR1^{ΔNTD}, bottom). S, signal peptide (18 amino acids); TCS, thrombin cleavage site; NTD, N-terminal domain; S1S2, glycine binding domains; EGFP, enhanced green fluorescence protein. Hydrophobic intramembrane regions are indicated as vertical boxes. Amino acid numbering starts with the first amino acid of the mature protein. **B**, Left, Western blot analysis of wt EGFP-NR1 and EGFP-NR1^{TCS} proteins generated upon co-expression with the NR2A subunit in HEK 293 cells. A single band of about 130 kDa molecular mass is detected using the anti-EGFP antibody (lanes 1 & 2). Right, thrombin treatment of EGFP-NR1^{TCS} expressing HEK 293 cells for 30 min resulted in the appearance of 70 kDa N-terminal and 60 kDa C-terminal fragments that reacted with the anti-EGFP and anti-NR1 antibodies, respectively (lanes 5 & 6). In contrast, the wt EGFP-NR1 subunit was not cleaved by thrombin under the same conditions (lanes 3 & 4).

Figure 2. Functional characterization of NR1^{TCS}/NR2A and NR1^{TCS}/NR2B receptors prior to and after thrombin cleavage. Dose-response analysis of receptors formed by the EGFP-NR1^{TCS} subunit upon co-expression with either the NR2B (**A**) or the NR2A (**B**) subunits in *Xenopus* oocytes before ($\mathbf{\nabla}$, broken line) and after ($\mathbf{\blacksquare}$, full line) thrombin treatment by two-electrode voltage clamping. Left panels, comparison of agonist-induced currents of EGFP-NR1^{TCS}/NR2A and –NR2B expressing cells elicited by application of glutamate and glycine (100 µM, each) prior to and after a 1h exposure to thrombin. Right panels, Zn²⁺ inhibition curves determined prior to and after thrombin cleavage revealed an about 19-fold reduction in the apparent Zn²⁺

affinity of EGFP-NR1^{TCS}/NR2B (A) receptors and an almost complete loss of high-affinity Zn²⁺ inhibition for EGFP-NR1^{TCS}/NR2A (B) receptors upon proteolytic cleavage of the NR1 NTD.

Figure 3. Assembly and functional properties of NMDA receptors containing NTD-deleted NR1 and/or NR2 subunits. A, The NR1^{ΔNTD} subunit forms hetero-oligomers with the NR2A subunit. Xenopus laevis oocytes co-expressing a His-tagged NR2A* with non-tagged NR1 or NR1^{ΔNTD} subunits were metabolically labelled with [³⁵S]methionine, and the receptor complexes formed were isolated by affinity purification and analyzed by SDS-PAGE. Lane 1 shows two bands with apparent molecular weights of about 116 kDa and 105 kDa, which represent the co-isolated wt NR1 and NR2A*-His subunits. Co-expression of the NR1^{ΔNTD} with the NR2A^{*}-His construct similarly resulted in co-isolation of two ³⁵S-labelled bands with molecular weights of about 78 and 105 kDa, which correspond to the NR1^{ΔNTD} and NR2A*-His subunits (lane 2). Lane 3, isolate from non-injected oocytes. **B**, Quantitative analysis of wt NR1/NR2B, NR1^{ΔNTD}/NR2B. NR1/NR2B^{ΔNTD} and NR1^{ΔNTD}/NR2B^{ΔNTD} expressing oocytes showed no significant differences in the mean maximal agonist-inducible whole-cell currents (1 s application of 100 µM glutamate and glycine, each) compared to wt NR1/NR2B receptors (Table 1). C, Examples of current traces showing the extent of desensitization of NR1/NR2A, NR1^{ΔNTD}/NR2A^{ΔNTD}, NR1/NR2B and NR1^{ΔNTD}/NR2B^{ΔNTD} receptor combinations to sustained application of glutamate and glycine (100 μ M, each). **D**, Relative ratios of steady-state (I_{ss}) vs. peak (I_p) currents of NR1/NR2A, NR1/NR2B, NR1^{ΔNTD}/NR2A^{ΔNTD} and NR1^{ΔNTD}/NR2B^{ΔNTD} receptors. Note a significant decrease in the extent of receptor desensitization (%) for the NR1^{ΔNTD}/NR2A^{ΔNTD} combination compared to wt.

Fig. 4. Allosteric inhibition by Zn^{2+} and ifenprodil of NMDA receptors containing NTD-deleted NR1 and/or NR2 subunits. **A**, **B**, Inhibition of agonist-evoked currents by Zn^{2+} at wt and NTD-deleted NR1/NR2 receptors. Agonist concentrations were 100 µM glycine and glutamate, each.

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A, Zn²⁺ inhibition of wt NR1/NR2A (♥), NR1^{ΔNTD}/NR2A (■), NR1/NR2A^{ΔNTD} (●) and NR1^{ΔNTD}/NR2A^{ΔNTD} (♦) receptors. Note biphasic inhibition of the wt receptor, with high (HA) and low-affinity (LA) sites dispaying IC₅₀ values of 0.012±0.004 µM (60% inhibition) and 225±19 µM (40% inhibition), respectively. HA Zn²⁺-inhibition was eliminated in all mutant combinations, whereas LA inhibition was not affected. *B*, Zn²⁺ inhibition of wt NR1/NR2B (♥), NR1^{ΔNTD}/NR2B (■), NR1/NR2B^{ΔNTD} (●) and NR1^{ΔNTD}/NR2B^{ΔNTD} (♦) receptors. *C*, Ifenprodil inhibition of the NR1/NR2B receptor combinations described under (*B*). Note similar residual inhibition of NR1^{ΔNTD}/NR2B, NR1/NR2B^{ΔNTD} and NR1^{ΔNTD}/NR2B^{ΔNTD} receptors for Zn²⁺ and ifenprodil. For IC₅₀ values, see Table 1.

Fig. 5. Effect of the NTD-deletions of the NR1 and/or NR2B subunits on the voltage dependence of Zn^{2+} inhibition. Current-voltage (I-V) relationships for oocytes expressing wt NR1/NR2B (**A**), NR1^{ΔNTD}/NR2B (**B**), NR1/NR2B^{ΔNTD} (**C**) and NR1^{ΔNTD}/NR2B^{ΔNTD} (**D**) receptors in the absence (-) and presence (+) of Zn^{2+} at the respective IC₅₀ value (see Table 1). Note that I-V curves for wt NR1/NR2B (**A**) receptors in the presence of 10 µM Zn^{2+} exhibit only a high-affinity voltageindependent inhibition whereas NR1^{ΔNTD}/NR2B (**B**), NR1/NR2B^{ΔNTD} (**C**) and NR1^{ΔNTD}/NR2B^{ΔNTD} (**D**) receptors display a combination of a low-affinity voltage-independent and dependent inhibition in the presence of 100 µM Zn^{2+} .

Figure 6. Agonist response properties of NMDA receptors containing N-terminally deleted and chimeric NR2A or NR2B subunits. **A**, Dose-response curves for glycine determined in the presence of saturating concentrations of glutamate (100 μ M) at wt NR1/NR2A (\bullet , 1.7±0.3 μ M) and NR1/NR2B ($\mathbf{\nabla}$, 0.39±0.04 μ M), and at chimeric NR1/NR2A^{NTD2B} (∇ , 0.28±0.09 μ M) and NR1/NR2B^{NTD2A} (O, 2.4±0.9 μ M), receptors. **B**, Comparison of the glycine EC₅₀ values of NR1/NR2 receptors containing NTD-deleted and chimeric NR2 subunits as compared to the respective wt proteins. Apparent glycine affinities of wt NR1/NR2A and NR1/NR2B receptors (±

SE) are indicated by dotted lines. *(C)* Dose-response curves for glutamate determined in the presence of saturating concentrations of glycine (100 μ M) for NR1/NR2A (\bullet , 2.6±0.4 μ M), NR1/NR2B ($\mathbf{\nabla}$, 1.8±0.4 μ M), NR1/NR2A^{NTD2B} ($\mathbf{\nabla}$, 2.0±0.3 μ M), and NR1/NR2B^{NTD2A} (O, 1.6±0.5 μ M) receptors.

Figure 7. Model illustrating the conformational changes proposed to occur upon Zn^{2+} or ifenprodil binding to wt and NTD deleted NMDA receptors. Binding of Zn²⁺ or ifenprodil to the open, agonist-bound ion channel is thought to cause a closure of the NR2-NTD. This results in weakening of NR1-NR2-LBD interactions and thereby promotes closure of the channel by enhanced desensitization (see Armstrong et al., 2006). For simplicity, only one NR1-NR2 dimer of the tetrameric receptor is shown. NTD, N-terminal domain; LBD, ligand-binding domain; CD, channel domain. Yellow arrow indicates ion flux through the open channel. A, Gating scheme for the wt NR1/NR2 receptor showing the hetero-dimeric organization of the LBDs and the NTDs of the NR1 and NR2 subunits (left, closed unliganded receptor). Binding of glycine (blue circle) to the NR1-LBD and of glutamate (red circle) to the NR2-LBD results in channel opening (middle). Weakening of NR1-NR2-LBD interactions by binding of Zn²⁺ (green rectangle) or ifenprodil to the respective NR2-NTD leads to a conformational strain, which disrupts the LBD interface and thus drives the receptor into the desensitzed closed state (right). B. Reaction scheme for the NR1-NTD-truncated receptor. Here, deletion or enzymatic cleavage of the NR1-NTD results in a loss of conformational strain deriving from Zn²⁺ binding, and thereby prevents weakening of LBD interactions. Consequently, the Zn²⁺ occupied receptors resides in its open state. The truncated NR1-NTD still associated with the "core" receptor after thrombin cleavage is indicated by lucent drawing.

 Table 1. Pharmacology of NMDA receptors assembled from wt and NTD-deleted
 NR1/NR2A

 and NR1/NR2B subunits
 NR1/NR2B

A: NR1/NR2A subunit combination

Subunit composition	I _{max}	EC_{50} glu	EC_{50} gly	$IC_{50} Zn^{2+}$ (high-, low affinity)	
	[µA]	[µM]	[µM]	[µM]	
NR1 / NR2A	5.4±0.3	2.6±0.4	1.7±0.2	0.012 ± 0.004; 225 ± 19	
$NR1^{\Delta NTD} / NR2A$	8.5±1.0	1.7±0.3	1.8±0.2	n.d. ; 305 ± 79	
NR1 / NR2A ^{ΔNTD}	5.0±1.9	3.6±0.7	2.1±0.4	n.d. ; 319 ± 99	
$NR1^{\Delta NTD} / NR2A^{\Delta NTD}$	6.4±0.8	4.6±1.1	2.7±0.5	n.d. ; 302 ± 61	

B: NR1/NR2B subunit combination

Subunit composition	I _{max}	EC ₅₀ glu	EC_{50} gly	IC ₅₀ Zn ²⁺	IC ₅₀ ifenprodil
	[µA]	[µM]	[µM]	[µM]	[µM]
NR1 / NR2B	3.5±0.4	1.8±0.4	0.39±0.04	6.8 ± 1.7	0.89 ± 0.08
$NR1^{\Delta NTD} / NR2B$	3.3±0.5	4.0±1.0	6.6±1.6**	51 ± 7 **	129 ± 14 **
NR1 / NR2B $^{\Delta NTD}$	3.7±0.8	0.95±0.1	1.8±0.1**	37 ± 3 **	140 ± 14 **
$NR1^{\Delta NTD} / NR2B^{\Delta NTD}$	4.3±0.3	2.3±0.4	2.8±0.6*	126 ± 29 **	124 ± 29 **

Glycine and glutamate EC_{50} values were determined in the presence of 100 µM glutamate or glycine, respectively. IC_{50} values of Zn^{2+} and ifenprodil were obtained by pre-incubating the cells with the allosteric inhibitor followed by co-applying the inhibitor with 100 µM glutamate and glycine, each. cRNAs were injected at a NR1:NR2 ratio of 1:2, and recordings were performed after 2-3 days of expression. Values represent means \pm SE. Stars indicate highly significant differences (*, p<0.01; and **, p<0.001) as compared to the respective values obtained with wild-type receptors. n.d., not detectable. Number of experiments was between 5 and 21.





В



NR NR28







С





В







В

D

С

 $NR1^{\Delta NTD}/NR2B^{\Delta NTD}$



NR1^{ΔNTD}/NR2B











A. wild-type



B. NR1-NTD-truncated

