FORMATION OF NR1/NR2 AND NR1/NR3 HETERO-DIMERS CONSTITUTES THE INITIAL STEP IN N-METHYL-D-ASPARTATE RECEPTOR ASSEMBLY*

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Running Title: Assembly of the NMDA receptor
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N-methyl-d-aspartate (NMDA) receptors are tetrameric protein complexes composed of the glycine-binding NR1 subunit with a glutamate-binding NR2 and/or glycine-binding NR3 subunit. Tri-heteromeric receptors containing NR1, NR2 and NR3 subunits reconstitute channels, which differ strikingly in many properties from the respective glycine- and glutamate-gated NR1/NR2 complexes and the NR1/NR3 receptors gated by glycine alone. Therefore, an accurate oligomerization process of the different subunits has to assure proper NMDA receptor assembly which has been assumed to occur via the oligomerization of homo-dimers. Indeed, using fluorescence resonance energy transfer (FRET) analysis of differentially fluorescent-tagged subunits and blue native polyacrylamide gel electrophoresis after metabolic labelling and affinity purification revealed that the NR1 subunit is capable to form homo-oligomeric aggregates. In contrast, both the NR2 and the NR3 subunits formed homo- and hetero-oligomers only in the presence of the NR1 subunit indicating differential roles of the subunits in NMDA receptor assembly. However, expression of the NR3A subunit with a N-terminal domain-deleted NR1 subunit (NR14NSTD) abrogating NR1 homo-oligomerization did not affect NR1/NR3A receptor stoichiometry or function. Hence, homo-oligomerization of the NR1 subunit is not essential for proper NR1/NR3 receptor assembly. Since identical results were obtained for NR14NTP/NR2 NMDA receptors (Madry, C., Mesic, I., Betz, H, and Laube, B. (2007) Molecular Pharmacology, in press) and rather NR1-containing hetero-oligomers are readily formed, we assume that hetero-dimerization of the NR1 with a NR3 or NR2 subunit which is followed by the subsequent association of two hetero-dimers is the key step in determining proper NMDA receptor subunit assembly and stoichiometry.

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: (RS)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)-propionic acid (AMPA) receptors, kainate receptors, and N-methyl-D-aspartate (NMDA) receptors (1). All iGluR subunits share a common modular design of four distinct regions: i) an extracellular amino terminal domain (NTD) of about 400 amino acids sharing homology with the bacterial leucine-, isoleucine- and valine-binding protein (LIVBP), implicated to play a role in iGluR oligomerization and modulation, ii) an extracellular S1S2 ligand binding domain (LBD) sharing homology with the bacterial glutamine binding protein (QBP) that binds agonists in a Venus-flytrap like mechanism, iii) a membrane associated domain composed of 4 membrane segments forming the ion channel and iv) an intracellular carboxyterminal domain (CTD) involved in linking the receptor to the membrane scaffold and signal transduction proteins (2).

The NMDA subtype of iGluRs is an obligatory hetero-oligomeric membrane protein composed of homologous NR1, NR2 and/or NR3 subunits and plays a key role in brain development, synaptic plasticity and memory formation (1). Cloning of NMDA receptor subunits revealed that the glycine-binding NR1 subunit is a single gene product occurring in eight splice variants (a-h), while the glutamate-binding NR2 and the glycine-binding NR3 subunits are encoded by four (NR2A-NR2D) and two (NR3A,B) different genes, respectively (1). The so called „conventional“ type of NMDA receptors is a tetrameric membrane protein composed of two NR1 and NR2 subunits, each (3),
that requires both glutamate and the co-agonist glycine for channel activation (4). In contrast, receptors composed of NR1 and NR3 NMDA receptor subunits function as excitatory receptors activated exclusively by glycine (5,6) with a so far unknown subunit stoichiometry. However, tri-heteromeric receptors containing NR1, NR2 and NR3 subunits reconstitute glycine- and glutamate-gated channels, which strikingly differ in channel properties from the respective NR1/NR2 and NR1/NR3 di-heteromers (7,8). Hence, the proper assembly of the different subunits crucially determines NMDA receptor function and pharmacology.

Studies on native and recombinant iGluRs suggest that their subunits assemble in a two-step-mechanism as dimer-of-dimers (9-12). For the conventional NMDA receptor it has been suggested that receptor assembly occurs via dimerization of a NR1 and a NR2 homodimer (10,13,14). However, subunit oligomerization and assembly of NR3 containing NMDA receptor is not completely understood and many questions remain unanswered. Here, using fluorescently- and His-tagged NR1, NR2 and NR3 subunits in combination with fluorescence resonance energy transfer (FRET), Blue Native PAGE (BN-PAGE) and electrophysiological recordings, we analyzed oligomerization intermediates of the different subunits after heterologous expression in HEK cells and Xenopus oocytes. Our results support the idea that NR1/NR2 or NR1/NR3 hetero-dimers are intermediate states of NMDA receptor assembly and that subunit composition of tetrameric NMDA receptors is defined by a dimer-of-heterodimers.

EXPERIMENTAL PROCEDURES

Reagents – All reagents were obtained from RBI Sigma (Taufkirchen, Germany).

DNA constructs, oocyte expression and electrophysiology – The cDNAs of NMDA receptor subunits NR1-1a (rat), NR2A (mouse), NR2B (mouse)(15), NR1-1a (mouse) kindly provided by Dr. Seeburg and NR3A (rat) kindly provided by Dr. Woodward were subcloned into enhanced cyan fluorescent protein (ECFP) and into enhanced yellow fluorescent protein (EYFP) expression vectors, pECFP-N1 and pEYFP-N1 to generate the C-terminally tagged fusion constructs NR1-YFP, NR1-CFP, NR2A-YFP, NR2A-CFP, NR2B-YFP, NR2B-CFP, NR3A-YFP and NR3A-CFP (see also Fig. 1A). Appropriate restriction sites were introduced by site-directed mutagenesis (QuikChange XL Site-Directed Mutagenesis Kit, Stratagene, CA) and confirmed by DNA sequencing as follows: SacI/BamHI for NR1, NheI/BamHI for NR2A, KpnI/BglII for NR2B and SalI/BamHI for NR3A. Subsequently, the resulting fragments were ligated in-frame with the vectors. To generate N-terminally tagged fusion constructs CFP-NR1 and YFP-NR1, the NR1 subunit cDNA was subcloned into ECFP and EYFP expression vectors, pECFP-C1 and pEYFP-C1 (Clontech). A signal peptide sequence was introduced 5’ of the CFP and YFP coding sequences. All constructs were verified by DNA sequencing, electrophysiological recordings and imaging techniques. The NR1<sup>ANTI</sup> construct was generated by excising the nucleotide sequence encoding for amino acid 23 to 376 with the restriction endonuclease PvuI. For biochemical characterization in Xenopus oocytes, NR1, NR2 and NR3 cDNAs were provided with a C-terminal hexahistidyl-tag and subcloned into the pNKS2 oocyte expression vector (16). In vitro synthesis of cRNA (mCAP mRNA Capping Kit, Ambion) was performed as described (6). For the expression of NMDA receptors, 25 ng cRNA/oocyte was injected. Xenopus laevis oocytes were isolated and maintained as described (17). Two-electrode voltage-clamp recording of glutamate- and glycine-induced whole-cell currents was performed according to (18).

Transfection of HEK293 cells – The culture conditions for human embryonic kidney 293 (HEK293) cells (ATCC#CRL1537) and the protocols for transfection with Lipofectamine<sup>®</sup> 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s protocol have been described previously (19). HEK293 cells were grown in Minimal Essential Medium (MEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin (5000 U), 1% (v/v) glutamine and maintained at 37°C in a 5% CO<sub>2</sub> environment. For transfection cells were plated onto fibronectin (Roche, Mannheim, Germany) coated 1 cm glass cover slips for transfection placed in 24 well dishes and maintained in supplement free MEM. Unless stated otherwise, 0.25 μg DNA of each construct were used per transfection. Transfected cells were cultured in the presence of the NMDA receptor inhibitor MK801 (100 μM) for 48h. Cells were fixed 18 h after transfection with 4% (w/v) paraformaldehyde in PBS for 12 min at room temperature. Non-CFP or -YFP tagged NMDA receptor subunits (NR2A-PCIS, NR1-pCis) were
antibody labeled after fixation using primary antibodies to the NR1 subunit (1:200, BD Bioscience, Heidelberg, Germany) and NR2A/B (1:200, Chemicon, Temecula, USA) and secondary antibody to mouse and rabbit respectively, labeled with Alexa 546 fluorophore. After fixation cells were permeabilized in 0.01 (w/v) Triton X-100 (Serva, Heidelberg, Germany) for 3 min followed by incubation in 2% (w/v) serum albumin for 30 min before processing for immunofluorescence.

Detection and calculation of FRET efficiencies using acceptor photo-bleaching – Fluorescence signals were detected with a confocal laser scanning microscope type Leica TCS-SP (Wetzlar, Germany) using dual laser excitation at 458 nm (ECFP) and 514 nm (EYFP) and emission windows of 470-495 nm for ECFP and 565-580 nm for EYFP controlled by the image software Leica-TCS-NT version 1.6.551. All fluorescence data were collected with a 63x objective. Effective FRET efficiency (%) between eCFP (donor) and eYFP (acceptor) was measured with acceptor photo-bleaching methods and quantified using the equation as described previously (20). Images of the donor were obtained before and after bleaching of the acceptor in a selected square (see Fig. 1B). Fluorescence intensities of the donor in this square before and after bleaching were used to calculate the FRET efficiencies according to (20). The bleaching procedure was unchanged for all experiments. Briefly, 514 nm laser intensity was adjusted to a value not influencing donor emission as was confirmed in cells that were only transfected with CFP fusion constructs. The acceptor was bleached with 10 successive scans to 80 to 100 % to levels comparable to non-transfected neighboring cells.

Metabolic labelling and purification of hexahistidyl-tagged proteins from Xenopus oocytes – Xenopus laevis oocytes were injected with 50 nl aliquots of cRNA (0.5 µg/µl). cRNA-injected and non-injected control oocytes were metabolically labelled by overnight incubation with L-[35S]methionine (>40 TBq/mmol, Amersham Pharmacia) at about 100 MBq/ml (0.2 MBq per oocyte) in sterile ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2 and 5 mM HEPES, pH 7.4) at 18°C and chased with 10 mM unlabeled methionine after 24h as indicated. Histagged NR1 and NR3A proteins were then purified by Ni2+-NTA agarose (Qiagen) chromatography from n-dodecyl-ß-D-maltoside (0.5%, w/v) extracts of oocytes as detailed previously (21). Shortly, oocytes were homogenized in 0.1 M phosphate buffer (20 µl per oocyte) containing 0.4 mM Pefabloc SC (Fluka, Buchs, Switzerland) and the indicated detergent (n-dodecyl-ß-D-maltoside, ULTROL Grade, Calbiochem-Novabiochem GmBH, Bad Soden, Germany). The homogenate was incubated on ice for 15 min and the extract was then cleared by centrifugation (10 min at 15,000 rpm in a desktop centrifuge). 100 µl of the clear supernatant were diluted with 400 µl of the above buffer and supplemented with 30 µl of Ni2+-NTA agarose beads and 10 mM imidazole. After 30 min of incubation under continuous inversion, the agarose-bound proteins were washed 4-times with 1 ml phosphate buffer containing 0.1% n-dodecyl-ß-D-maltoside, 0.08 mM Pefabloc SC, and 30 mM imidazole. Subsequently, the proteins were released from the Ni2+-NTA agarose beads with non-denaturing elution buffer consisting of 0.5% (w/v) n-dodecyl-ß-D-maltoside, 100 mM imidazole/HCl (pH 7.8), 20 mM Tris-HCl and 10 mM EDTA and kept at 0°C until analyzed by PAGE. For the biochemical determination of subunit stoichiometry, oocytes injected with NR1 and NR3 (wt or His-tagged) subunit cRNAs at the ratios indicated were labelled and purified by Ni2+-NTA chromatography as described above. The purified protein was then separated on 10 % tricine-SDS-PAGE gels, which were fixed, dried, and exposed to PhosphorImager screens. The radioactivity of individual [35S]methionine-labelled polypeptide bands was quantified using a Phosphor Imager (Phosphor Imager 445 SI, Molecular Dynamics) and evaluated using the software package ImageQuant as described (16).

Blue native PAGE and SDS-PAGE - Blue native PAGE was carried out as described (22). Just before gel loading, purified proteins were supplemented with blue native sample buffer to a final concentration of 10% glycerol, 0.2% Serva blue G and 20 mM sodium 6-amino-n-capronate, and applied onto polyacrylamide gradient gels. As a size standard, molecular mass markers (Pharmacia) were run in two different lanes on both borders of the gel. This resulted in incomplete separation and thus poor correlation with the molecular mass marker but enabled separation of a wide range of protein sizes in a short time. For SDS-PAGE, protein samples were supplemented with SDS sample buffer containing 20 mM dithiotreitol (DTT) and electrophoresed in parallel with molecular mass markers (Precision Plus Protein All Blue Standard, Biorad) on 10%
tricine/SDS-polyacrylamide gels. Gels were blotted, fixed, dried and exposed to BioMax MR films (Kodak, Stuttgart, Germany) at -80°C.

Surface protein labelling using fluorescent dyes - After 4 days in culture, oocytes expressing NR1 and NR3A-His subunits were incubated for 20 min. at 4°C in 65 nmol/oocyte of the Cy-NHS-ester dye Cy5 (Amersham Biosciences) in phosphate buffer (20 mM Na2HPO4/NaH2PO4 pH 8.5; 110 mM NaCl; 1 mM MgCl2). After washing NMDA receptor complexes were purified as described above. Gels containing Cy5 labelled protein samples were scanned with a gel imager (Typhoon 9400, Amersham) as described (6).

Glycosylation analysis - For analysis of the glycosylation status, 10 µl aliquots of affinity-purified receptor protein were supplemented with reducing (20 mM DTT) SDS sample buffer and 1% (w/v) octylglucoside (Calbiochem, La Jolla, CA, USA) and incubated for 2 h at 37°C with either 5IUB milliunits endoglycosidase H (Endo H) or PNGase F (New England Biolabs GmBH, Frankfurt, Germany) as indicated.

Statistical analysis - Results represent means ± SD and were analyzed using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA). Statistical significance was assessed using one-way analysis of variance (ANOVA) with Newman-Keul's multiple comparison test for post hoc comparison of the results obtained from the FRET analyses and considered to be statistically significant at P<0.05 (n.s., not significant). Statistical significance of electrophysiological data was determined at the P<0.05 (*) and P<0.01 (**) levels using a Student's two-tailed t-test.

RESULTS

In this study, a biophysical and biochemical approach based on fluorescence resonance energy transfer (FRET) and blue native polyacrylamide gel electrophoresis (BN-PAGE) was used to investigate the role of the NR1, NR2 and NR3 subunits in NMDA receptor oligomerization. For this purpose, spectral variants of the green fluorescent protein (GFP) were fused to either the C- or N-Terminus of NMDA receptor subunits and analyzed by measuring FRET after acceptor photo-bleaching combined with confocal microscopy (23). Furthermore, BN- and SDS-PAGE analysis of metabolically labelled and under non-denaturating conditions purified NMDA receptor complexes were performed to verify the FRET data by independent biochemical approaches.

Differentially tagged-NR1 subunits show a FRET signal, indicative for homo-oligomerization - In an initial set of experiments we calibrated our FRET detection system by analyzing two appropriate controls i) to quantify the maximal detectable FRET efficiency and ii) to avoid false positives of FRET signals by acceptor bleaching (24). We expressed two types of constructs for our controls: i) a negative control expressing only the soluble enhanced cyan fluorescence (CFP) and enhanced yellow fluorescent (YFP) constructs and ii) a positive control expressing a CFP-linker-YFP tandem construct, known to provide a strong energy transfer (20,24). For the CFP-YFP tandem construct, we detected a FRET efficiency of E = 17.8 ± 2.1 %, which is similar to results found in the literature (12,20,24). However, co-expression of the soluble CFP and YFP constructs as a negative control yielded a FRET efficiency of E = 5.1 ± 0.6 %, a phenomenon previously also described as pseudo-FRET (24). FRET efficiencies similar to this background signal were subsequently defined as non-specific association of the respective constructs.

Based on recent biochemical and biophysical results indicating a homo-oligomerization of the recombinant NR1 subunit upon single expression (12,13), we first analyzed the FRET efficiency of combinations of two differentially tagged NR1 subunits with opposing intra- and extracellular localized fluorophores (Fig. 1). Co-expression of CFP- and YFP-N-terminally tagged NR1 subunits (CFP-NR1 and YFP-NR1, respectively) with an extracellular orientation of the respective fluorophores revealed a highly significant FRET efficiency of E = 8.7 ± 1.0 % compared to our negative control (Fig. 1; P<0.001). Co-expression of NR1 subunits with both fluorophores fused to the intracellular oriented C-terminus (NR1-CFP and NR1-YFP), again resulted in a strong FRET efficiency of E = 10.2 ± 0.7 % (p<0.001; Table 1; Fig. 1,2). In contrast, combining the extracellularly tagged CFP-NR1 with the intracellularly tagged NR1-YFP subunit or vice versa resulted in highly significantly reduced FRET efficiencies (NR1-CFP/YFP-NR1: E = 4.7 ± 0.4 %; CFP-NR1/NR1-YFP: E = 3.0 ± 1.1 %; Table 1; Fig. 1), which were similar to the results obtained with the soluble constructs of CFP and YFP (P>0.05 for both). These data are i) consistent with a proper insertion and orientation of our fluorophores relative to the...
membrane and ii) in line with recent data showing an homo-oligomerization of the NR1 subunit in heterologous expression systems (12,13).

To confirm that our method indeed detects specific subunit interactions mediated by subunit assembly, we measured the FRET efficiency between a N-terminally tagged GluR2 subunit, a member of the AMPA-subtype iGluR family, and the NR1 subunit. Both iGluR subunits co-localize in postsynaptic densities, but do not co-assemble (1). Consistent with an efficient homo-oligomerization of the GluR2 subunit, co-expression of the N-terminally tagged CFP-GluR2 and YFP-GluR2 subunits revealed a FRET efficiency of $E = 8.5 \pm 0.9 \%$, which is similar to the signal obtained with the respective CFP-NR1 and YFP-NR1 subunits. In contrast, co-expression of the CFP-NR1 and the YFP-GluR2 subunit resulted in a highly significant reduced FRET efficiency of $E = 4.0 \pm 1.3 \% \ (P<0.01)$, a value not statistically different from our negative control ($P>0.5$). These data showed that i) NR1 and GluR2 subunits do not oligomerize and ii) our method allows to discriminate between randomly co-localized and properly assembled subunits of AMPA- and NMDA-receptor subtypes.

NR2 and NR3 constructs display FRET signals only after co-expression with the NR1 subunit - After demonstrating self-association of the NR1 subunit and based on the assumption that NMDA receptor assembly occurs in a dimer-of-homodimer fashion (10,12), we expected that co-expression of NR3A-YFP and NR3A-CFP constructs would result in a strong FRET signal. However, co-expression of these constructs did not result in a significant FRET signal ($E = 4.8 \pm 0.7 \% \ (P>0.5)$; Fig. 2; Table 1), suggesting that i) our C-terminal constructs of the NR3A subunit may not be capable to induce FRET, ii) the expressed NR3A subunits became rapidly degraded or iii) an additional subunit is required for efficient oligomerization.

To test the assumptions mentioned above, we first wanted to know whether our fluorescently-tagged NR1 and NR3A constructs are capable of forming functional hetero-oligomeric channels (6). To this end we performed whole-cell recordings of combinations of the tagged NR1 and NR3A subunits after heterologous expression in HEK293 cells and *Xenopus* oocytes. Indeed, co-expression of the tagged NR1-CFP and NR3A-YFP constructs provided glycine-induced whole-cell inward-currents that were similar to the results obtained with the corresponding wt channels (data not shown, see 6).

Since we had compelling evidence that our fluorescence tags do not exert significant effects on subunit assembly and/or ion channel function, we tested the ability of the tagged NR3A subunit to induce FRET upon co-expression with the respective fluorescently-tagged NR1 construct. Consistent with our functional analyses, co-expression of the NR1-YFP and NR3A-CFP constructs yielded a strong FRET efficiency of $E = 10.3 \pm 0.6 \% \ (Fig. 2; Table 1)$, which was highly significantly increased as compared to the FRET efficiency of co-expressed NR3A-CFP and NR3A-YFP constructs and our control ($P<0.001$ for both; Table 1). Furthermore, the NR1-YFP and NR3A-CFP FRET signal was similar to that obtained after co-expressing fluorescently-tagged NR1 or GluR2-constructs ($P>0.5$), which is consistent with an efficient hetero-oligomerization of our tagged NR1 and NR3A subunits.

In order to test whether homo-oligomerization between NR3A subunits might occur in the presence of the NR1 subunit, we analyzed FRET signals upon co-expression of the NR3A-CFP and YFP constructs with wt NR1. To our surprise, we measured a robust FRET signal ($E = 9.1 \pm 1.6 \% \ (Fig. 2; Table 1)$, which was significantly increased to that obtained in the absence of NR1 ($P<0.01$; see Table 1). This result clearly shows that i) our tagged NR3A constructs can give rise to pronounced FRET signals and ii) co-expression of the NR1 subunit is necessary to promote NR3A interactions. To examine whether NR1 interactions are affected by overexpressing the NR3A subunit, we co-expressed the NR1-CFP and NR1-YFP constructs together with a 5-fold excess of wt NR3A. The resulting FRET signal was slightly, but not significantly reduced ($E = 8.1 \pm 0.6 \% \ (P>0.05)$; Table 1) compared to that obtained with the respective NR1 constructs alone. These data indicate that overexpression of the NR3A subunit does not substantially interfere with NR1-NR1 interactions and/or the relative amount of NR1 subunits in the receptor complex, implicating a fixed ratio of NR1/NR3 subunits within the excitatory glycine receptor.

Formation of tri-heterooligomeric receptors composed of NR1, NR2 and NR3 subunits - As previous studies suggest a dimer-of-homodimer assembly of both NR1- and NR2-homoooligomers to “conventional” NMDA receptors (10,12,14), we wondered how the NR3 subunits can be incorporated into tri-heteromeric NMDA receptors composed of...
NR1, NR2 and NR3 subunits. We therefore tested the association of the NR3A subunit with either the NR2A or the NR2B subunits. Although co-expression of our NR2-CFP constructs with NR1-YFP yielded functional receptors with strong FRET efficiencies (Table 1), co-expression of the tagged NR3A construct with both, tagged-NR2A or -NR2B subunits resulted only in FRET efficiencies not significantly different from our control (E = 3.5 ± 1.1 % for NR3A-CFP/NR2A-YFP and E = 3.1 ± 0.8 % for NR3A-CFP/NR2B-YFP; Fig. 2; Table 1). Thus, hetero-oligomerization between NR2 and NR3 subunits seems not to exist. Next, we analyzed the capability of NR2 and NR3 subunits to form hetero-oligomers in the presence of NR1. Similar to the results obtained for tagged NR3A subunits in the presence of wt NR1, co-expression of NR3A-CFP and NR2B-YFP, together with wt NR1 generated a significant FRET efficiency of E = 8.1 ± 1.1 % (Fig. 2; Table 1; P<0.01 compared to co-expression studies in the absence of NR1). Accordingly, exchanging NR2B-YFP with NR2A-YFP also provided a comparable high FRET efficiency (NR1/NR2A-YFP/NR3A-CFP E = 7.1 ± 0.9 %; Fig. 2; Table 1). Therefore, our data substantiate i) a lack of NR2/NR3 hetero-oligomerization in the absence of NR1 but show ii) an efficient incorporation of the NR3 subunit into tri-hetero-oligomeric NMDA receptor complexes composed of NR1, NR2 and NR3 subunits (see also 8). These findings are however difficult to interprete within the context of homo-oligomeric NR1 and NR2 dimers as a prerequisite of NMDA receptor assembly (10,12; overview in 14). We therefore investigated the capability of the NR2 subunits to form homo-oligomers. Surprisingly, neither co-expression of the respective YFP- and CFP-tagged NR2A- (E = 5.2 ± 0.8 %) nor NR2B- (E = 3.4 ± 1.3 %) subunits in the absence of NR1 resulted in significant FRET efficiencies (Table 1), indicative for inefficient homo-oligomerization of the respective subunits. This result is, however, not in agreement with the study by (12), but consistent with the result we obtained with the fluorecently-tagged NR3A subunit in the absence of NR1 and a recent study showing in a biochemical assay that NR2 subunits do not form homo-oligomeric aggregates (13). So far, our data indicate that oligomerization of both NR2 and NR3 subunits occur only in the presence of NR1, whereas the NR1 subunit shows a tendency to form homo-oligomeric aggregates.

Analyses of the excitatory glycine receptor by BN-PAGE indicate a tetrameric assembly of 2NR1 and 2NR3 subunits - Our FRET analyses have shown that the NR1 subunit is capable to form homo-oligomers, whereas the NR2 and NR3 subunits do not associate and require the NR1 subunit for efficient homo- and/or hetero-oligomerization. Since the oligomerization process and stoichiometry of excitatory NR1/NR3 glycine receptor is far from clear, we continued to assess the oligomerization of the NR1 and NR3 subunit with an additional independent assay to identify putative assembly intermediates. We therefore applied the BN-PAGE system (22) after heterologous expression of the NR1 and NR3 subunit in Xenopus laevis oocytes. By using [35S]methionine metabolic labelling of C-terminally hexahistidyl tagged NR1 and NR3A subunits (NR1-His and NR3A-His, respectively), metal affinity chromatography was performed to purify the respective subunits from digitonin extracts after heterologous expression (6).

The NR1-His subunit isolated under non-denaturing conditions (21) migrated predominantly as a single subunit and two defined oligomers, which likely correspond to monomeric, dimeric and tetrameric NR1 subunits (Fig. 3A, lane 1). However, the occurrence and the relative ratio of the respective bands detected turned out to be variable (see also Fig. 4A and 13,25). To investigate further the number of NR1-His subunits incorporated in the oligomers, 1% SDS was used to weaken non-covalent subunit interactions, in order to induce dissociation into lower order intermediates. SDS treatment did not lead to dissociation into monomers (see also Fig. 4A) whereas additional treatment with 100 mM DTT resulted in well-defined monomeric NR1 subunits (Fig. 3A, lane 5). In contrast, under non-denaturing conditions isolated NR3-His protein existed in an unspecific aggregate as indicated by the high molecular mass aggregations that migrated at a broad range of masses above that of the NR1 oligomers (Fig. 3A, lane 2). This broad band likely contains an undefined number of NR3 subunits. By comparing the band pattern produced after treating the NR1-His protein with SDS/DTT (lane 5), the discrete protein band obtained corresponds clearly to monomeric NR3 subunits (Fig 3A, lane 6), confirming that the NR3 subunit does not form defined homo-oligomers under non-denaturing conditions. Similar results were obtained using a NR2A construct where the C-terminal region from amino acid 930 was replaced by a 6xHis tag.
...resulted in a mass shift to about 15 kDa (Fig. 3C, bottom, first lane) shows two bands corresponding to NR1-His and NR3A subunits with apparent molecular weights of 116 kDa and 128 kDa (25,26), respectively. Affinity purification of both subunits after single cRNA injection and pulse-chase revealed remarkable differences in the stability of the respective subunits. Although similar expression levels of both subunits occurred one day after injection (Fig. 3C), a successive decline of the NR3A band intensity was already observed at day 3 (Fig. 3C, middle) whereas band intensities of the NR1 subunit were absolutely stable at day 3 (Fig. 3C). Co-expression of both subunits dramatically increased the stability of the former “instable” NR3A subunit to levels similar to NR1 (Fig. 3C, bottom and Fig. 4). Thus, our data show that stability of NR1 and NR3A subunits are remarkably different upon single expression and that oligomerization with the NR1 increases the stability of the NR3A subunit.

To analyze the subcellular localization of the NR1 and NR3 subunit, we monitored the glycosylation status of the respective subunits by analyzing the sensitivity to treatment with different glycosydases. De-glycosylation of the NR1-His subunit with both Endo H or peptide:N-glycosidase F (PNGase F) resulted in a mass shift to about 15 kDa (Fig. 3C, top) consistent with the release of high-mannose type N-glycans and a retention of the mature NR1-His polypeptide in the endoplasmic reticulum (ER). Similar, the NR3-His subunit turned out to be entirely Endo H and PNGase F sensitive, resulting in a reduced molecular weight (see Fig. 3C, middle). Taken together our data demonstrate that both the NR1 and NR3A subunits are retained in the ER after single expression, although the ability to form homo-oligomeric intermediates is different.

Mutation of the cysteines 61 and 290 of the NR1 subunit affects protein stability rather than NR1/NR3 oligomerization - Different investigators have shown that the NTDs of iGluR subunits are capable of forming interdomain interactions (27-29) which have been implicated in receptor assembly (9,14,25,30). In line with the proposed model of a dimer-of-homodimer arrangement of conventional NR1/NR2 NMDA receptors (10), Papadakis et al. (13) reported...
about an initial disulfide bond formation by two cysteines (Cys61 and Cys290) within the NTD of the NR1 subunit important for NMDA receptor oligomerization. We therefore analyzed the consequences of mutating both cysteines to alanines (NR1C61A and NR1C290A) for NR1/NR3A receptor assembly and subunit stoichiometry. Expression of the NR1 mutants isolated in the presence of 1% SDS, but in the absence of DTT, revealed a complete loss of both NR1 mutants to form homo-oligomers (Fig. 4A). Analysis of the NR1C61A and NR1C290A mutants upon co-expression with the NR3A subunit by SDS-PAGE revealed a reduction of the [35S]methionine-labelled subunit bands, but no change in the respective relative ratios compared to wt NR1/NR3A receptors (Fig. 4B, data not shown). However, functional analysis of wt NR1/NR3, NR1C61A/NR3A and NR1C290A/NR3A receptors upon expression in Xenopus oocytes revealed a significant reduction in maximal inducible glycine currents for both cysteine mutants (>65% reduction, P<0.01; Fig. 4C). To investigate whether the reduced currents seen for the NR1 mutants are due to a reduced plasma membrane insertion and/or altered stoichiometry of the NR1/NR3A surface receptors, we performed surface-labelling experiments by using a Cy5-NHS-ester based protocol (6). Consistent with the electrophysiological data, affinity purification of NR1/NR3A-His receptors from Cy5 surface-labeled oocytes demonstrated that the assembled mutant receptors were present to a lesser extent at the cell surface (Fig. 4C). Analyses of the fluorescence intensities of the NR1 cysteine-mutants compared to the respective NR3A-His band revealed again similar subunit stoichiometries as obtained upon co-expression of the wt NR1/NR3A-His subunits (Fig. 4C). These data show that surface localized NR1C61A/NR3A-His and NR1C290A/NR3A-His receptors maintain an invariant 2NR1:2NR3 stoichiometry.

Since the cysteines NR1C61 and NR1C290 in the NTD of the NR1 seem not to be crucial for NR1/NR3A receptor assembly and/or stoichiometry, we speculated whether these cysteines may contribute to protein stability of the NR1 subunit. We therefore investigated the expression pattern of singly expressed NR1C61A and NR1C290A subunits for three days after cRNA injection into Xenopus oocytes. Consistent with this assumption, the NR1C61A subunit showed a reduction and decline of protein amount already after the first day (compare Fig. 3B (top) and 4D) whereas the expression pattern of the NR1C290A subunit was not remarkably affected (Fig. 4D). Thus, based on our electrophysiological, metabolical- and surface-labelling data, the cysteines NR1C61 and NR1C290 seem to be most important for NR1 protein stability, folding or surface targeting rather than determining subunit stoichiometry and assembly of the NR1/NR3 protein complex.

Deletion of the entire NR1 NTD does not impair NR1/NR3A receptor assembly and function - To further investigate the importance of the NR1-NTD in mediating NR1 homo-oligomerization and subsequent receptor assembly, we examined whether a N-terminally truncated NR1 subunit, which lacks the entire NR1-NTD (NR1ΔNTD, amino acids 5 to 358 of the mature NR1 subunit), forms functional hetero-oligmeric NR1/NR3A NMDA receptors. Co-expression of NR1ΔNTD and NR3A subunits in Xenopus oocytes resulted in functional receptors with maximal inducible glycine currents similar to wt NR1/NR3A receptors (Fig. 4E, left; see also 6) indicating an efficient assembly of the NR3A with the NR1ΔNTD subunit. To analyze the stoichiometry of the NR1ΔNTD and NR3A subunits in the receptor complex, we co-purified the NR1ΔNTD subunit with a His-tagged NR3A subunit. Fig. 4E (right) shows two 35S-labelled subunit bands corresponding to the NR1ΔNTD and NR3A subunit with a molecular weight of 70 and 128 kDa, respectively. Quantification of the respective subunit bands revealed a ratio of 0.6±0.1 (n=3) of NR1ΔNTD to NR3A subunit radioactivities that was not affected by increasing amounts of NR1ΔNTD cRNA injected (data not shown). Due to a lower number of 18 methionine residues in NR1ΔNTD as compared to wt NR1, a ratio of 0.6±0.1 is consistent with a 1:1 ratio of NR1ΔNTD to NR3A-His subunits (theoretical ratio 0.55). Since both the absolute intensities of the NR3A-His and the relative ratios of the NR1 and NR3A polypeptide bands were not different in NR1/NR3A-His and NR1ΔNTD/NR3A-His expressing oocytes (Fig. 4E, right), our data strongly indicate that i) both the wt NR1 and NR1ΔNTD subunits assemble at an invariant 1:1 stoichiometry with the NR3A-His, and that ii) both NR1 polypeptides have similar assembly efficiencies. In conclusion, NR1/NR3A receptor formation does not depend on the NTD of the NR1 subunit. Thus, our results show that neither mutating the cysteines 61 and 290 in the NR1-NTD nor deleting the entire L1VBP-homology domain of the NR1 subunit does interfere with both oligomerization and stoichiometry of the excitatory
NR1/NR3A glycine receptor. Since the deletion of the entire NR1-NTD did not affect expression efficiency and function of NR1/NR3A receptors, we interpret this finding as an indication that the reduced NR1/NR3 receptor cell surface expression seen upon mutating the cysteines 61 or 290 is likely explained by incorrect folding of the polypeptide chain or impaired membrane targeting.

Conclusions - Our data allows us to propose a model of subunit assembly for the excitatory NR1/NR3 glycine receptor (see also Discussion). Following association of a NR1 subunit with a NR3 subunit, two heteromeric dimers can then associate to complete the formation of a tetrameric ion channel composed of 2NR1 and 2NR3 subunits. However, NTD-mediated homo-oligomerization of the NR1 subunit previously assumed to be a prerequisite for conventional NMDA receptor assembly is not essential for subunit association of the NR1/NR3 (this study) and NR1/NR2 (31) receptors, but may increase the stability of the NR1 subunit in the absence of other NMDA receptor subunits.

DISCUSSION

Here, by using a combination of fluorescence resonance energy transfer (FRET) and native PAGE (polyacrylamide gel electrophoresis), we elucidated key steps controlling NMDA receptor oligomerization and subunit stoichiometry. As expected from the hetero-oligomeric nature of NR1/NR2 and NR1/NR3 receptors, co-expression of NR1-YFP and NR2-CFP or NR3A-CFP constructs resulted in strong FRET signals and functional channels with the expected pharmacology. However, only single expression of the NR1 subunit yielded in a strong FRET efficiency, indicating different homo-oligomerization behaviour of the NR1 versus the NR2 and NR3A subunits. Analysis of BN-PAGE of NR1, NR2 and NR3 subunits revealed that neither the NR2 (25,32) nor the NR3 (this study) subunits are capable of forming homo-oligomers, whereas the NR1 subunit showed a tendency to associate to higher ordered complexes (13,25,32, this study). However, deletion of the entire NR1-NTD abolishing NR1 homo-oligomerization, did neither impair receptor assembly nor function (31, this study). Assuming a tetrameric structure of NR1/NR3 receptors, we propose the formation of an initial hetero-dimeric NR1/NR3 intermediate assembly complex enabling the subsequent association of an additional heterodimer, which can be also composed of a NR1/NR2 dimer, since homo-oligomerization of the NR1 subunit seems also not required for the assembly of conventional NMDA receptors composed of NR1 and NR2 subunits (31).

Role of the NR1-NTD in NMDA receptor assembly - It has been shown that the conventional tetrameric NMDA receptor is composed of two glycine-binding NR1 and two glutamate-binding NR2 subunits (3,33), which are proposed to be oriented in a homodimeric arrangement (10,14). The initial phase of tetramerization of these NMDA receptors is thought to be guided by a N-terminal recognition domain (NTD)(13,25), a domain also implicated in NMDA receptor modulation (34). The function of the NTDs in assembly process would therefore be twofold, i) providing stabilizing interactions for some, and ii) prohibiting the interaction of other subunits. Since all conventional NMDA receptors characterized so far contain two NR1 subunits, the formation of a N-terminal NR1-NR1 disulfide-linked homodimer by the cysteines Cys61 and Cys290 (13) would be mechanistically an attractive stable intermediate to initiate the subsequent recruitment of a NR2 homodimer (12). Dimerization of a NR1 and a NR2 homo-dimer was therefore thought to be the prerequisites for proper NMDA receptor assembly (14). However, we can show that when i) both cysteines are mutated and ii) the entire NTD of the NR1 subunit is removed, functional NR1/NR3 channels are still formed. In addition, we can show that homo-oligomerization of the NR1 subunit is not involved in NR1/NR3 subunit assembly and that the loss of the NTD of the NR1 subunit does not affect NR1/NR3 subunit stoichiometry, indicating a different role of the NR1-NTD within excitatory glycine receptor function. This is also true for conventional NMDA receptors, since co-expression of i) NR1C61A or NR1C290A mutants with wt NR2 subunits (35,36) and ii) NTD-deleted NR1 and NR2 subunits (28,31) results in functional conventional NMDA receptor channels. As mutating the cysteine residues Cys61 and Cys290 impair the stability and membrane targeting of singly expressed NR1 subunits, respectively, we speculate that disulfide-bridges mediating NR1 homo-oligomerization might reduce protein degradation in the ER resulting in a stable NR1 subunit pool in the absence of other NMDA receptor subunits (32,37).

Model for the assembly of triheteromeric NMDA receptors - Here we can show, that in the absence of the NR1 subunit the NR2 and NR3 subunits do not associate in a homo-oligomeric fashion. These
findings imply that the formation of NR2 and NR3 hetero-dimers is very unlikely (see also 25,32), whereas hetero-oligomeric NR1/NR2 and NR1/NR3 complexes are predicted to form readily. In line with our results, Gouaux and colleagues have shown that the functional entity of conventional NR1/NR2 NMDA receptors is a heterodimeric arrangement of NR1 and NR2 subunits (33), which seems to be also true for the excitatory glycine receptor composed of NR1/NR3 subunits (6). However, since the NR2 and NR3 subunits do not homo-oligomerize and homo-oligomerization of the NR1 subunit is not essential for NMDA receptor assembly, we provide evidence for a two-step assembly pathway of NMDA receptors by association of a NR1 subunit with a NR2 or NR3 subunit into an initial hetero-dimer, which is followed by the subsequent association of an additional hetero-dimer to complete the tetrameric subunit arrangement (Fig. 5). These NR1/NR2 and NR1/NR3 hetero-dimers would also display the optimal configuration for the assembly to a tetrameric receptor complex composed of all three subunits, which i) restricts the number of the glycine-binding NR1 subunits to two and ii) regulates the differential incorporation of NR2 and NR3 subunits. The intact tri-hetero-oligomeric channel would therefore have a stoichiometry of 2NR1:1NR2:1NR3, which is consistent with the findings of hetero-oligomeric NR1/NR2/NR3 NMDA receptor combinations in vivo (8).

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FOOTNOTES

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The abbreviations used are: AMPA, (RS)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)-propionic acid; BN-PAGE, blue native poly acrylamid gel electrophoresis; CFP, cyan fluorescent protein; CTD, carboxyterminal domain; E, effective FRET efficiency; FRET, fluorescence resonance energy transfer; HEK, human embryonic kidney; iGluR, ionotropic glutamate receptor; I_{max}, maximal current; LBD, ligand-binding domain; LIVBP, leucine-, isoleucine- and valine binding protein; NMDA, N-methyl-D-aspartate; NTD, N-terminal domain; QBP, glutamine binding protein; YFP, yellow fluorescent protein; wt, wild-type.
FIGURE LEGENDS

Figure 1: Schematic representation of differentially fluorophore-tagged NR1 subunits of the NMDA receptor and an acceptor photobleaching FRET measurement of the NR1-CFP and NR1-YFP construct upon heterologous expression in HEK cells. (A) Schematic representation of the N- and C-terminal fused YFP and CFP constructs of the NR1 subunit of the NMDA receptor illustrating the extracellular and intracellular orientation of the respective fluorophores. Position of the fluorophore in the NR1 subunit construct is indicated by black or white boxes. Co-expression of CFP- and YFP-N-terminally tagged NR1 subunits (CFP-NR1 and YFP-NR1, respectively) revealed a robust significant FRET efficiency of \(E = 8.7 \pm 1.0\%\) (left). Co-expression of NR1 subunits with both fluorophores fused to the intracellular oriented C-terminus (NR1-CFP and NR1-YFP), resulted in a slightly stronger FRET efficiency of \(E = 10.2 \pm 0.7\%\) (middle). In contrast, combining the extracellularly tagged CFP-NR1 with the intracellularly tagged NR1-YFP subunit resulted in a highly significantly reduced FRET efficiency (CFP-NR1/NR1-YFP: \(E = 3.0 \pm 1.1\%\); right). Transmembrane domains of the NR1 subunit are indicated by shaded columns. (B) Images of the NR1-CFP donor before and after photo-bleaching of the NR1-YFP acceptor in a square of the image (indicated by rectangle) after heterologous expression in HEK cells. Inside the bleached region the NR1-CFP donor is unquenched resulting in an increased intensity indicative of FRET. Note that singly expressed NR1 constructs are retained in the ER. Based on the robust and reliable FRET signals of our C-terminal tagged NR1 constructs, we used only C-terminally tagged subunits in our subsequent analyses. Scale bar = 10 \(\mu m\).

Figure 2: FRET efficiencies of different homo- and hetero-oligomeric combinations of NMDA receptor subunits. Mean FRET efficiencies and SD (%) measured from HEK cells expressing the different combinations of CFP and YFP-tagged subunits after acceptor photobleaching. Note that neither the NR2 nor NR3 subunits are capable of forming homo- or hetero-oligomers in the absence of the NR1 subunit. Statistical data mentioned in the text are indicated by horizontal bars. Non-specific pseudo-FRET of the control construct (mean ± SD) is indicated by dotted lines. *** highly significant different compared to control (\(P<0.001\)); ### highly significantly different compared to the respective subunit combination in the absence of the NR1 subunit (\(P<0.001\)).

Figure 3: Characterization of the oligomerization behaviour and subunit ratio of the NR1 and NR3A subunits using BN- and SDS-PAGE. (A) Oocytes membranes expressing NR1-His, NR3A-His, or NR1-His/NR3A were analyzed after purification using BN-PAGE in the absence and presence of SDS/DTT. The experiment was performed three times with comparable results. The data suggest that the NR1 and NR3A subunits assemble in an invariant 1:1 stoichiometry (lane 7) to form a tetrameric complex (lane 3) that is not present when either the NR1 or NR3A subunit is expressed alone (lanes 1 and 2). However, the NR1-His subunit tends to migrate in defined complexes, whereas the NR3A-His shows no homo-assoziation. (B) Subunit ratio stoichiometry of the heterooligomeric NR1/NR3 NMDA receptor. Xenopus oocytes co-expressing a His-tagged NR1 with a non-tagged NR3A subunit with His-NR1 with His-NR3A resulted in a highly significantly reduced FRET of the control construct (mean ± SD) is indicated by dotted lines. *** highly significant different compared to control (\(P<0.001\)); ### highly significantly different compared to the respective subunit combination in the absence of the NR1 subunit (\(P<0.001\)).

Figure 4: Biochemical and electrophysiological characterization of the mutants NR1\(^{C61A}\) and NR1\(^{C290A}\) and the N-terminal deletion construct NR1\(^{AStd}\) upon co-expression with the NR3A subunit. (A) Oocytes membranes expressing NR1-His, NR1\(^{C61A}\)-His, or NR1\(^{C290A}\)-His subunits were analyzed after purification using BN-PAGE. The experiment was performed three times with comparable results. The data suggest that mutating cysteine residues C61 or C290 to alanine in the NTD of the NR1 subunit impair NR1 homo-oligomerization. (B) SDS-PAGE of metabolically labelled and affinity-purified receptors generated by co-expressing wt NR1 with His-tagged NR3A, NR1\(^{C61A}\) with His-tagged NR3A and NR1\(^{C290A}\) with His-tagged NR3A subunits, respectively.
Sensitivity of labeled subunits to Endo H and PNGase F treatment as indicated below the gel. (C) SDS-PAGE of NR1<sup>C61A</sup>/NR3A-His and NR1<sup>C290A</sup>/NR3A-His receptors affinity purified after Cy5 surface labelling and maximal inducible glycine currents as compared to NR1/NR3A-His wt receptors. Note, that mutation of cysteines 61 and 290 in the NTD of NR1 to alanine impairs NR1/NR3A receptor surface expression (left) and maximal inducible glycine currents (middle) but does not alter relative subunit ratios (right). (D) Expression pattern of the singly expressed NR1<sup>C61A</sup> (top) subunit displayed a remarkable reduction of protein amount (see for comparison Fig. 3B) whereas the NR1<sup>C290A</sup> (bottom) subunit showed only a marginal decline. (E) Comparison of glycine-evoked currents and protein expression of NR1/NR3A and NR1<sup>ΔNTD</sup>/NR3A receptors. (left) In contrast to NR1<sup>C61A</sup> and NR1<sup>C290A</sup> mutants, the NR1<sup>ΔNTD</sup> construct showed no difference in maximal inducible glycine currents compared to the wt NR1/NR3A receptor. (right) Similar amounts of receptor protein for NR1/NR3A and NR1<sup>ΔNTD</sup>/NR3A were purified for up to five days (lanes 1-5, each) of <sup>35</sup>S-labeled oocytes as revealed by SDS-PAGE.

Figure 5: **Schematic model for the dimer of hetero-dimers assembly mechanism of the NMDA receptor.** Our model is based on i) that neither the NR2 nor the NR3 subunits do form homo- and hetero-oligomers in the absence of NR1 (25,32, this study), ii) homo-oligomerization of the NR1 subunit is not required for proper receptor assembly (31, this study) and iii) the finding that the LBDs of NMDA receptors function as a hetero-dimer (6,33). We therefore suggest two basic steps in NMDA receptor assembly: a) hetero-dimerization of a NR1 subunit with a NR2 or NR3 subunit; b) dimerization of two NR1/NR2 or NR1/NR3 hetero-dimers to the tetrameric complex resulting in diheteromeric NR1/NR2 and NR1/NR3 or triheteromeric NR1/NR2/NR3 receptors. The intermediate dimers (b) are assumed to assemble in a symmetric fashion (c). The pairs of fused circles (c) represent functional dimers (6,33) within the tetrameric NMDA receptor complex.
**TABLES**

Table 1: FRET efficiencies of tagged NMDA receptor subunit constructs after acceptor photo-bleaching.

|                        | E [%]       |
|------------------------|------------|
| **Control**            |            |
| NR1-CFP / YFP-NR1      | 4.7 ± 0.4  |
| NR1-YFP / CFP-NR1      | 3.0 ± 1.1  |
| **Homo-oligomeric combinations** |          |
| NR1-CFP / NR1-YFP      | 10.2 ± 0.7*** |
| NR1-CFP / NR1-YFP + NR3A | 8.1 ± 0.6*** |
| NR3A-CFP / NR3A-YFP    | 4.8 ± 0.7  |
| NR3A-CFP / NR3A-YFP + NR1 | 9.1 ± 1.6### |
| NR2A-CFP / NR2A-YFP    | 5.2 ± 0.8  |
| NR2A-CFP / NR2A-YFP + NR1 | 9.0 ± 1.8### |
| **Hetero-oligomeric combinations** |        |
| NR3A-CFP / NR1-YFP     | 10.3 ± 0.6*** |
| NR3A-CFP / NR2A-YFP    | 3.5 ± 1.1  |
| NR3A-CFP / NR2A-YFP + NR1 | 7.1 ± 0.9### |
| NR3A-CFP / NR2B-YFP    | 3.1 ± 0.8  |
| NR3A-CFP / NR2B-YFP + NR1 | 8.1 ± 1.1### |
| NR1-CFP / NR2A-YFP     | 9.2 ± 0.8*** |

FRET efficiencies values (E) of the different fluorophore-tagged constructs upon co-expression in HEK293 cells represent means ± SD from 10-24 independent measurements. *P* values were determined by ANOVA with multiple comparison test for post hoc pairwise comparison of the results. *** *P*<0.001 compared to control. ### *P*<0.001 highly significantly different from the respective subunit combination in the absence of the wt NR1 subunit (Student’s t-test).
FIGURES

Figure 1
Figure 2
Figure 3
Figure 4
Formation of NR1/NR2 and NR1/NR3 hetero-dimers constitutes the initial step in N-methyl-D-aspartate receptor assembly
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