Appropriate NR1-NR1 Disulfide-linked Homodimer Formation Is Requisite for Efficient Expression of Functional, Cell Surface N-Methyl-d-aspartate NR1/NR2 Receptors*

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A c-Myc epitope-tagged N-methyl-D-aspartate receptor NR1-2a subunit was generated, NR1-2a_c-Myc where the tag was inserted after amino acid 81, NR1-2a_c-Myc/NR2A receptors when expressed in mammalian cells are not trafficked to the cell surface nor do they yield cell cytotoxicity post-transfection. NR1-2a_c-Myc was, however, shown to assemble with NR2A subunits by immunoprecipitation and [3H]MK801 radioligand binding assays. Immunoblot analysis of cells co-transfected with wild-type NR1-2a/NR2A subunits yielded two NR1-2a immunoreactive species with molecular masses of 115 and 226 kDa. Two-dimensional electrophoresis under non-reducing and reducing conditions revealed that the 226-kDa band contained disulfide-linked NR1-2a subunits. Only the 115-kDa NR1-2a species was detected for NR1-2a_c-Myc/NR2A. The c-Myc epitope is inserted adjacent to cysteine 79 of the NR1-2a subunit; therefore, it is possible that the tag may prevent the formation of NR1 disulfide bridges. A series of cysteine ↔ alanine NR1-2a mutants was generated, and the NR1-2a mutants were co-expressed with NR2A or NR2B subunits in mammalian cells and characterized with respect to cell surface expression, cell cytotoxicity post-transfection, co-association by immunoprecipitation, and immunoblotting following SDS-PAGE under both reducing and non-reducing conditions. When co-expressed with NR2A in mammalian cells, NR1-2a_c-Myc/NR2A displayed similar properties to NR1-2a_c-Myc/NR2A in that the 226-kDa NR1 immunoreactive species was not detectable, and trafficking to the cell surface was impaired compared with wild-type NR1/NR2 receptors. These results provide the first biochemical evidence for the formation of NR1-NR1 intersubunit disulfide-linked homodimers involving cysteine 79. They suggest that disulfide bridging and structural integrity within the NR1 N-terminal domain is requisite for cell surface N-methyl-D-aspartate receptor expression.

N-Methyl-D-aspartate (NMDA) receptors are a subclass of excitatory, ionotropic glutamate neurotransmitter receptors.

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The abbreviations used are: NMDA, N-methyl-D-aspartate; AMPA, amino α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; HEK, human embryonic kidney; LAMP, lysosomal-associated membrane protein; MLDI105,159, (E)-3-(2-phenyl-2-carboxyethyl)-4,6-dichloro-1-indole-2-carboxylic acid; MK801, (+)-5-methyl-10,11-dihydro-dibenzo[c,d]cyclohepten-5,10-imine; NR1, NR2, etc., NMDA receptor subunit NR1, NR2; PSD-95, postsynaptic density-95; ELISA, enzyme-linked immunoassorbent assay; LIVBP, leucine-isoleucine-valine-binding protein; PBS, phosphate-buffered saline.

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NR1-NR1 Disulfide-linked Homodimer Formation

Mammalian Cell Transfections—Human embryonic kidney (HEK) 293 cells were cultured and transfected with either single subunit NR1 or NR2 NMDA receptor clones, co-transfected with both NR1/NR2 subunit combinations using the calcium phosphate method with a total of 10 μg of DNA and a ratio of 1:3 for NR1:NR2 transfections, respectively, or alternatively with NR1, NR2, and post-synaptic density 95 (PSD-95) clones using a total of 20 μg of DNA and a ratio of NMDA receptor clones/PSD-95 of 1:1 (20, 21). Cells were cultured in the presence of 1 μM ketamine post-transfection. They were harvested 24–36 h post-transfection by centrifugation at 3000 × g, and homogenates were prepared, adjusted to 0.5 mg protein/ml, and analyzed immediately for [3H]MK801 and [3H]MDL105,519 radioligand binding activities or alternatively frozen at −80 °C and later analyzed by quantitative immunoblotting.

Cell Cytotoxicity—HEK 293 cells were co-transfected with various pCISNR1-2a/pCISNR2B constructs with or without pGWIPSD-95,54FLAG. Twenty hours post-transfection, cell cytotoxicity was determined using the Promega CytoTox 96™ cytotoxicity assay according to the manufacturer’s instructions and as described previously (20).

Immunoblotting—Immunoblotting was performed as described previously using 25–50 μg of protein/sample precipitated using the chloroform:methanol method and SDS-PAGE under both reducing and non-reducing conditions as specified in 7.5% polyacrylamide slab minigels (15). Affinity-purified anti-NR1 (C2 exon) and anti-NR2A (1381–1394) antibodies were used at final concentrations of 1–5 μg/ml; anti-c-Myc 9E10 antibodies were monoclonal antibodies used at a dilution of 1:2000. Rabbit and mouse horseradish-linked secondary antibodies (Amer sham Biosciences) were used at a final dilution of 1:2000, and immunoreactivities were detected by using the ECL Western blotting system. Immunoreactive bands were quantified by either molecular densitometry using a Personal Densitometer with ImageQuant (Amer sham Biosciences) in the linear range of the film (15) or using the GeneGnome Chemiluminescence Capture and Analysis System (Syngene, Cambridge, UK).

Two-dimensional Electrophoresis—Two-dimensional electrophoresis was carried out as described by Cadieux and Kadner (22) except that after the first dimension of SDS-PAGE under non-reducing conditions, the band of interest was excised from the gel, and the protein was subjected to trypsinization (50 μg/ml)-coated 24-well dishes, and 0.8 μg of total plasmid DNA was used per well. Cells were cultured in the presence of 1 μM ketamine for 24–36 h post-transfection. Cell culture media was aspirated, and each well was washed with 1 × 500 μl of phosphate-buffered saline (PBS). Cells were fixed by the addition of 4% (w/v) paraformaldehyde (250 μl) for 5 min at room temperature, washed once with 500 μl of PBS, and non-specific sites blocked by incubation with 4% (w/v) milk powder in PBS (500 μl) for 30 min. Cells were incubated for 1 h with the primary antibody, which was anti-NR2B (46–60) (0.5 μg/ml; 250 μl) because NR2B subunits are found on the cell surface only when co-expressed with an NR1 subunit (12). Cells were washed 4 × for 10 min with 4 × 500 μl of PBS, 0.5% (w/v) milk powder in PBS, mouse horseradish peroxidase-linked anti-rabbit IgG was added for 1 h (Amer sham Biosciences; 1:2000 dilution in 4% (w/v) milk powder in PBS, 250 μl). Cells were washed 3 times for 10 min with 500 μl of 4% (w/v) milk powder in PBS, 1 time with 500 μl of PBS for 10 min, and then K-blue substrate (Adgen Ltd., Ayr, UK) was added. Cells were then incubated for 25 min, and the OD450 nm values obtained were subtracted from the background values. For the detection of total immunoreactivity (e.g., the lysosome), the transfected cells, cells were fixed for 20 min with 4% (w/v) paraformaldehyde (250 μl), washed 1 time with 500 μl of PBS, and then permeabilized with 0.25% (v/v) Triton X-100 in PBS for 5 min. All subsequent steps were as above for the measurement of cell surface NMDA receptor subunit expression. For the determination of non-specfic binding of anti-NMDA receptor antibodies to transfected cells, HEK 293 cells were always transfected with the plasmid, pCIS, and the OD450 nm values obtained were subtracted to give specific NMDA receptor total and cell surface binding.

Radioligand Binding Assays—[3H]MDL105,519 (74 Ci/mmol, Amer...
Coexpression of NR1-2a,c-Myc with NR2A does not result in cell cytotoxicity and cell surface expression following co-transfection in HEK 293 cells. A, HEK 293 cells were transfected with different combinations of NMDA receptor subunits; cells were harvested 24 h post-transfection, and cell death was measured using the Promega Cytotox96TM assay as described under “Experimental Procedures.” The results are the normalized values ± S.E. for n = 6 independent transfections, where 100% is the percentage cytotoxicity measured for NR1-1a/NR2B receptors cultured post-transfection in the absence of ketamine (**, p < 0.005). B, HEK 293 cells were transfected with NR1-2a or NR1-2a,c-Myc in the presence and absence of NR2B. The resulting cell homogenates were subjected to analysis by quantitative immunoblotting all as described under “Experimental Procedures.” The figure is a representative immunoblot where lane 1 indicates NR1-2a alone; lane 2 indicates NR1-2a,c-Myc alone; lanes 3 and 5 and 6 are NR1-2a/NR2A and NR1-2a,c-Myc/NR2A, respectively, where lane 3 indicates NR1-2a; lane 4 indicates NR1-2a,c-Myc; and lanes 5 and 6 indicate NR2A. Arrows denote the NR1 115- and 226-kDa and NR2A 180-kDa species. Molecular weight standards (×10^3 kDa) are shown on the right. C, histogram summarizing the immunoblotting results. The values are normalized such that expression of wild-type subunits is 100%, and they are the means ± S.E. for n = 3 independent transfections (*, p < 0.05). D, HEK 293 cells were transfected with NR1-2a or NR1-2a,c-Myc, in the presence of NR2B. The cell surface expression of the respective subunits was determined 24 h post-transfection by an ELISA using antibodies directed against an NR2B extracellular epitope, all as described under “Experimental Procedures.” Results are expressed as the percentage of total expression for NR1-2a/NR2B wild-type receptors, where the total expression of NR1-2a/NR2B = 100% values are the means ± S.E. for n = 3 independent transfections (**, p < 0.005).

Immuno precipitation—HEK 293 cells were co-transfected with either pCISNR1-2a/pCISNR2A, pCISNR1-2a,c-Myc/NR2A (1:3 ratio with 10 μg of total DNA), or wild-type and mutant combinations ± pG-WIPS-D95,-M95. Cells were harvested 24 h after transfection, and cell homogenates were solubilized with 10 μg of total DNA, or wild-type and mutant combinations ± pG-WIPS-D95,-M95. Cells were harvested 24 h after transfection, and cell homogenates were solubilized by 1 h at 4 °C with 50 mM Tris-HCl, pH 7.4, 240 mM NaCl, 5 mM EDTA, 5 mM EGA, 1% (v/v) Triton X-100, containing benzamidine (1 μg/ml), bacitracin (1 μg/ml), soybean trypsin inhibitor (1 μg/ml), chicken egg trypsin inhibitor (1 μg/ml), and phenylmethylsulfonyl fluoride (1 mM). Samples were diluted to 1 mg of protein/ml, and the solubilized material was collected by centrifugation at 100,000 × g. Aliquots (500 μl) were incubated with affinity-purified anti-NR2A-(1381–1394), anti-NR1 C2, or protein A purified non-immune Ig (5 μg) overnight at 4 °C. Protein A-Sepharose (Sigma; 2.5 mg) was added, and samples were incubated for 1 h at 4 °C. Immune pellets were collected by centrifugation at 3000 × g for 10 s, washed with 3 × solubilization buffer as above (3 × 1 ml), solubilized with SDS-PAGE sample buffer, and analyzed by immunoblotting.

Preparation of Rat Forebrain Membranes—Membranes from adult rat forebrains were prepared as described previously and frozen at −80 °C until used (18). Subcellular Fractionation—HEK 293 cells were co-transfected with NR1-2a/NR2A or NR1-2a,c-Myc/NR2A and cultured for 24 h post-transfection in the presence of 1 mM ketamine, and cells were collected by centrifugation at 3000 × g for 10 min at 4 °C. The pellet was suspended by homogenization using a Dounce (glass/glass) homogenizer (10 passes) followed by four passes through a 25-gauge needle in 1.5 ml of 60 mM HEPES, pH 7.4, 5 mM EDTA benzamidine (1 μM/ml), bacitracin (1 μg/ml), soybean trypsin inhibitor (1 μg/ml), chicken egg trypsin inhibitor (1 μg/ml), and phenylmethylsulfonyl fluoride (1 mM) containing 0.25 mM sucrose. The resuspended material was centrifuged at 3000 × g for 10 min at 4 °C. The supernatant (1 ml) was applied to a
HEK 293 cells were transfected with NR1-2a/NR2A (f) or NR1-2a c-Myc/NR2A (1394) antibodies; lanes 3 immunoprecipitated with anti-NR2-(1381–H11006) experiments where each point is the mean and lanes 4 and 5 immunoblotting all as described under “Experimental Procedures.” A is immunoblotting with anti-NR2A-(1381–1394) antibodies; B is immunoblotting with anti-NR1 C2 antibodies. The lane layout is the same for both A and B, where lanes 1–3 indicate cells transfected with NR1-2a/NR2A and lanes 4–6 indicate cells transfected with NR1-2a c-Myc/NR2A. Lanes 1 and 4 indicate solubilized, transfected cells; lanes 2 and 5 indicate cells immunoprecipitated with anti-NR2-(1381–1394) antibodies; lanes 3–6 indicate cells immunoprecipitated with non-immune Ig. The positions of molecular weight standards (×10^3 kDa) are shown on the right. The immunoblot is representative of n = 5 independent immunoprecipitations. C, HEK 293 cells were transfected with NR1-2a/NR2A (■) or NR1-2a c-Myc/NR2A (□), and cell homogenates were assayed in parallel 24 h post-transfection for [3H]MK801-specific binding using a concentration range from 0 to 15 nM. Both curves are representative of n = 3 independent experiments where each point is the mean ± S.E. for triplicate measurements. D, HEK 293 cells were transfected with NR1-2a (■) or NR1-2a c-Myc (□), and cell homogenates were assayed in parallel 24 h post-transfection for [3H]MDL105,519-specific binding using 0–15 nM [3H]MDL105,519 concentrations. Each binding curve is representative of n = 3 independent experiments, with each point being the mean ± S.E. of triplicate values.

RESULTS

Expression of NR1-2a c-Myc/NR2A Heteromeric NMDA Receptors Does Not Result in the Cell Surface Expression of Functional NMDA Receptors—We have reported previously that co-expression of wild-type NR1/NR2 heteromeric receptors results in cell cytotoxicity post-transfection (20). This NMDA receptor-induced cell death was eliminated by the inclusion of NMDA receptor antagonists in the cell culture medium post-transfection. Cytotoxicity was attributed to activation of functional cell surface NMDA receptors by L-glutamate and glycine in the cell culture media with a subsequent unregulated influx of Ca^{2+}. The lack of observed cell death post-transfection was not because of a reduced expression of the NR1-2a c-Myc polypeptide, because immunoblots of transfected cell homogenates showed that when expressed alone, there was no significant difference in molecular weight and expression levels between wild-type and epitope-tagged subunits (Fig. 1, A and B). When NR1-2a c-Myc was co-expressed with NR2A, however, the subunit level was reduced 2.8 ± 0.8-fold compared with wild-type NR1-2a/NR2A combinations (Fig. 1, B and C). Cell surface ELISAs confirmed the cell cytotoxicity observations in that no significant cell surface anti-NR2B antibody reactivity was detected following the co-expression of NR1-2a c-Myc/NR2B receptors in HEK 293 cells (Fig. 1D). This contrasted with ketamine-sensitive cell cytotoxicity post-transfection and cell surface expression for wild-type NR1-2a/NR2A and NR1-2a c-Myc/NR2B receptors, respectively.

NR1-2a c-Myc Co-assembles with NR2A Subunits, Demonstration by Immunoprecipitation, and Radioligand Binding Studies—From the above results, it was evident that although the c-Myc tagged NR1-2a subunit was expressed, it was not trafficked to the cell surface when co-expressed with an NR2 subunit. Further experiments were carried out to characterize the properties of the mutant. In the first instance, immunoprecipitation studies were used to establish whether NR1-2a c-Myc subunits were able to associate with NR2 subunits. Wild-type and mutant NR1-2a were co-expressed with NR2A in HEK 293 cells; transfected cells were detergent-extracted, and immunoprecipitation assays were carried out with anti-NR2A antibodies. The results are shown in Fig. 2, A and B. It can be seen that both NR1-2a and NR1-2a c-Myc subunits were specifically immunoprecipitated by anti-NR2A antibodies demonstrating that c-Myc-tagged NR1-2a subunits do co-assemble with NR2A.
Further proof of their co-association was obtained by radioligand binding studies. [3H]MK801 is a use-dependent, non-competitive antagonist of NMDA receptors. It binds with high affinity only to assembled NR1/NR2 receptors (25, 26). [3H]MK801 radioligand binding assays were carried out on cells transfected with NR1-2a/NR2A and NR1-2a-c-Myc/NR2A. The saturation binding curves are shown in Fig. 2C. Both curves were best fit by a single high affinity site with no significant difference in \( K_D \) values between wild-type and mutant heteromers. Values are as follows: \( K_D = 11.4 \pm 4.3 \) (n = 3) nM (NR1-2a/NR2A) and \( K_D = 7.7 \pm 1.9 \) (n = 3) nM (NR1-2a-c-Myc/NR2A). There was, however, a significant decrease in the number of binding sites, \( B_{max} \). Values are as follows: 0.6 \pm 0.2 (n = 3) pmol/mg protein (NR1-2a/NR2A) and 0.1 \pm 0.05 (n = 3) pmol/mg protein (NR1-2a-c-Myc/NR2A). This percentage decrease correlates with that found by immunoblotting for the expression of NR1-2a-c-Myc compared with NR1-2a in NR1/NR2 transfections (Fig. 1, B and C). These results imply that the insertion of the c-Myc epitope does not affect the integrity of the NMDA receptor ion channel.

[3H]MDL105,519 is a competitive glycine site antagonist and binds with high affinity to NR1 subunits expressed alone. Fig. 2D shows representative [3H]MDL105,519 saturation curves for binding to NR1-2a and NR1-2a-c-Myc. The binding parameters are as follows: \( K_D = 1.9 \pm 0.6 \) nM, \( B_{max} = 1.4 \pm 0.4 \) pmol/mg protein (n = 3; NR1-2a), \( K_D = 1.2 \pm 0.2 \) nM, and \( B_{max} = 0.8 \pm 0.2 \) pmol/mg protein (n = 3; NR1-2a-c-Myc). There was thus no significant difference in the affinity and the \( B_{max} \) values between wild-type and mutant receptors. These [3H]MDL105,519 binding studies are in agreement with the comparison of expression levels by immunoblotting, and furthermore, they imply that the introduction of the c-Myc epitope at amino acid NR1-2a 81 does not perturb the NR1 glycine binding domain.

**Fig. 3.** Subcellular fractionation of NR1-2a-c-Myc/NR2A NMDA receptors and demonstration of the association of calnexin with the NR1-2a subunit. A, HEK 293 cells were transfected with NR1-2a/NR2A or NR1-2a-c-Myc/NR2A receptors; post-nuclear supernatants were prepared, loaded on a continuous 5–20% (v/v) Optiprep gradient, and centrifuged at 135,000 \( \times g \) for 3 h at 4°C. Fractions were collected and subjected to immunoblotting with anti-NR1 C2 and NR2A-(1381–1394), calnexin, and LAMP-II as shown. Arrows denote the positions of NR1 115 and 226 kDa, NR2A 180 kDa, calnexin 95 kDa, and LAMP-II 125-kDa immunoreactive species. B, HEK 293 cells were co-transfected with either NR1-2a/NR2A or NR1-2a-c-Myc/NR2A clones; cell homogenates were collected, solubilized, and immunoprecipitated with anti-NR1 C2 antibodies, and immune pellets were analyzed by immunoblotting using anti-calnexin antibodies. Lanes 1–3 indicate cells transfected with NR1-2a/NR2A; lanes 4–6 indicate cells transfected with NR1-2a-c-Myc/NR2A. Lanes 1 and 4 indicate solubilized, transfected cells; lanes 2 and 5 indicate cells immunoprecipitated with anti-NR1 C2 antibodies; lanes 3 and 6 indicate immunoprecipitated with non-immune Ig.
that implied that NMDA receptor/PDZ-containing proteins associate early in the secretory pathway rather than at the plasma membrane.

**NR1-2a/NR2A and NR1-2a-cMyc/NR2A Heteromers, Comparative Analysis by SDS-PAGE Under Reducing and Non-reducing Conditions**—In immunoblots of HEK 293 cells homogenates expressing NR1-2a/NR2A and NR1-2a-cMyc/NR2A receptors probed with anti-NR1-C2 antibodies, in addition to the 115 ± 2-kDa (n = 7) immunoreactive species, i.e. the mature, glycosylated NR1 subunit, a second immunoreactive band with 226 ± 5 kDa (n = 8) was observed for wild-type but not for the mutant NR1-2a (Figs. 1B and 5A). This molecular weight is compatible with an NR1-NR1 dimer. It was also detected for the NR1-1a splice variant (data not shown). The c-Myc epitope tag is inserted at NR1-2a-(81). There is a cysteine residue at the NR1-1a splice variant (data not shown). The c-Myc epitope tag is inserted at NR1-2a-(81). There is a cysteine residue at the NR1-1a splice variant (data not shown). The c-Myc epitope tag is inserted at NR1-2a-(81). There is a cysteine residue at the NR1-1a splice variant (data not shown). The c-Myc epitope tag is inserted at NR1-2a-(81).
the tag may prevent disulfide bond formation involving residue Cys-79. The 226-kDa immunoreactive band seen for wild-type NR1-2a may thus be SDS-resistant, non-reduced NR1-NR1 dimers. To investigate this further, HEK 293 cell homogenates of NR1-2a/NR2A and NR1-2ac-Myc/NR2A transfections and brain membranes were all analyzed by immunoblotting following SDS-PAGE under both reducing and non-reducing conditions. The results are shown in Fig. 5A.

Probing with anti-NR1-C2 antibodies, as observed previously under reducing conditions for transfected cells, two immunoreactive bands with 115 and 226 kDa were observed for wild-type with one 116 ± 2 kDa (n = 4) band seen for NR1-2ac-Myc. Under non-reducing conditions for NR1-2a, specific immunoreactive bands with 105 ± 2 kDa (n = 7), 226 ± 5 kDa (n = 6), and 244 ± 4 kDa (n = 6) were observed as well as significant immunoreactivity that did not enter the gel (Fig. 5A). If NR1-NR1 subunits are disulfide-linked as suggested above, one would expect the disappearance of the 115-kDa monomer. However, it has been shown that in heterologous expression systems and indeed in the brain, there exists a pool of unassembled NR1 subunits (18, 30); under non-reducing conditions, the 105-kDa species is presumably unassembled NR1. For native NMDA receptors, only the 115-kDa NR1 immunoreactive species was detected under reducing conditions. Under non-reducing conditions, no immunoreactive species were detected. This may be explained by both the relatively low expression of NR1 homomers in brain compared with transfected cells, and the molecular weight of assembled NMDA receptors, i.e. >600,000, means that they would not enter the running gel.

For NR1-2ac-Myc under non-reducing conditions, interestingly the major immunoreactive band migrated with a significantly different molecular weight to that found for wild-type NR1-2a. Values are as follows: $M_w = 113 ± 2$ (n = 4; NR1-2ac-Myc) versus $M_w = 105 ± 2$ (n = 7; NR1-2a). The 8-kDa difference in molecular mass between wild-type NR1-2a and NR1-2ac-Myc is attributed to differences in folding perhaps due to intra-disulfide as opposed to inter-disulfide bond formation in NR1-NR1 homodimers and hence mobility in SDS-PAGE of the NR1-2a subunits (see below for further discussion). No differences were found in the migration properties of monomeric NR2A subunits under reducing and non-reducing conditions (results not shown).

To substantiate that the 226-kDa species in NR1-2a/NR2A receptors contained NR1 disulfide-linked subunits, two-dimensional SDS-PAGE was carried out. The first dimension SDS-PAGE was carried out under non-reducing conditions to max-
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**A**

![NR1-2a Cys → Ala mutant subunits all co-associate with NR2A and calnexin. HEK 293 cells were co-transfected with different NR1-2a cysteine to alanine mutants and NR2A clones; cell homogenates were collected and solubilized; and equal volumes were immunoprecipitated with anti-NR1 C2 antibodies and immune pellets analyzed by immunoblotting using either anti-NR1 C2 (A) or anti-calnexin antibodies (B) all as described under “Experimental Procedures.” The gel layout is the same in both A and B where lane 1 is wild-type NR1-2a/NR2A; lane 2 is NR1-2a C79A/NR2A; lane 3 is NR1-2a C308A/NR2A; lane 4 is NR1-2a C79A C308A/NR2A, and in lane 5, HEK 293 cells were transfected with NR1-2a/NR2A, and immunoprecipitation was with non-immune Ig. The positions of molecular weight standards (×10^3 kDa) are shown on the right.

**B**

**DISCUSSION**

In this paper we have described the properties of an NR1-2a NMDA receptor subunit tagged with the c-Myc epitope in the N-terminal domain. This NR1-2a-c-Myc subunit when co-expressed with NR2A was not targeted to the plasma membrane. Studies aimed at understanding why cell surface expression was not attained led to an investigation into the cysteine residues located within the LIVBP domain. It was concluded that cysteine residues Cys-79 and Cys-308 are requisite for efficient insertion into the plasma membrane. Thus, efficient trafficking of functional NMDA receptors to the plasma membrane requires co-synthesis of NR1 and NR2 subunits in the endoplasmic reticulum (12), the formation of NR1-NR1 disulfide-linked homodimers initiated, mediated, and dictated by the maturation of the NR1 LIVBP domain (13, 14), and assembly of NR1-NR1 dimers with NR2 subunits possibly via NR1-NR1 disulfide linkage. There may possibly favor disulfide bridging between other cysteines, e.g. those located within the lysine-arginine-ornithine and glutamine-binding protein and/or S2 domains.

Despite impaired trafficking to the cell surface, NR1-2aC79A, NR1-2aC308A, and NR1-2aC79A C308A were all shown to associate with NR2A subunits by immunoprecipitation (Fig. 7A). (Note again the absence of the 226-kDa species in NR1-2a subunits lacking Cys-79.) Furthermore, calnexin was found to co-immunoprecipitate with NR1-2aC79A, NR1-2aC308A, and NR1-2aC79A C308A all co-expressed with NR2A (Fig. 7B). The amount of calnexin associating with NR1-2a increased with increased cysteine mutations. Thus overall, mutation of Cys-79 in the LIVBP domain yields NR1-2a subunits that possess similar properties to the epitope-tagged NR1-2a.
deduced that in the NR1 subunit two pairs of cysteine residues were important for the redox sensitivity of NMDA receptors. One pair in the S2 extracellular loop, i.e. Cys-744 and Cys-798, is responsible for the slow persistent component of the redox modulation of NR1/NR2B, NR1/NR2C, and NR1/NR2D receptors (32). A second pair, Cys-79 and Cys-308, predicted but not proven to form an intra-disulfide bond within NR1, was reported to be responsible for an intermediate component of redox modulation (33). Although the studies reported here agree that these two cysteines are important, they differ in that we predict that NR1 subunits are linked via disulfide bridging involving Cys-79. No studies involving mutation of Cys-22 were reported, but the maximum current response for NR1-1a/NR2A was −60% of wild-type NR1-1a/NR2A receptors. This correlates with the decrease in cell surface expression of NR1-2a_c79S/NR2A receptors (Fig. 6D). It was notable that despite this significant decrease in cell surface expression of the NR1-2a cysteine mutants, there was no change in the percentage cell cytotoxicity post-transfection (Table I). This may be explained by the fact that the cytotoxicity assay was saturated. Alternatively, it may be possible that introduction of the cysteines 79 and 308 affects ligand binding or channel gating leading to enhanced Ca²⁺ entry and an apparent increased cytotoxicity post-transfection. Choi et al. (33), however, reported that the NMDA and glycine EC₅₀ values for activation of NMDAR-1a (C79S,C308S)/NR2A were −60% of wild-type NR1-1a/NR2A receptors. This correlates with the decrease in cell surface expression of NR1-2a_c79S/NR2A receptors reported here (Fig. 6D). It was notable that despite this significant decrease in cell surface expression of the NR1-2a cysteine mutants, there was no change in the percentage cell cytotoxicity post-transfection (Table I). This may be explained by the fact that the cytotoxicity assay was saturated. Alternatively, it may be possible that introduction of the cysteines 79 and 308 affects ligand binding or channel gating leading to enhanced Ca²⁺ entry and an apparent increased cytotoxicity post-transfection. Choi et al. (33), however, reported that the NMDA and glycine EC₅₀ values for activation of NMDAR-1a (C79S,C308S)/NR2A receptors were either not significantly changed (glycine EC₅₀) or were actually increased but only −1.25-fold (NMDA EC₅₀). Thus, although the point mutation was to serine rather than alanine as described here, changes in ligand binding or channel activation are unlikely. The lack of correlation between cell surface expression and cell cytotoxicity may also be explained by an apparent decrease in cell surface expression due to a reduced reactivity of anti-NR2B antibodies, used to measure cell surface expression of NR1-2a/NR2B receptors, and due to possible changes in conformation introduced by cysteine → alanine mutations.

AMPA receptors, ionotropic glutamate receptors, belong to the same superfamily as the NMDA receptors. Most interesting, when analyzed under non-reducing conditions, it was reported that multiple high molecular weight species were observed that were absent in reducing conditions. These subunits could correspond to AMPA receptor subunit disulfide-linked dimers (34). Within the N-terminal domain of non-NMDA, AMPA-type receptors, there are four cysteines, Cys-73, Cys-88, Cys-194, and Cys-308. The crystal structure of the ligand binding domain of the GluR2 AMPA receptor subunit has been determined (9, 35). This corresponds, however, only to residues GluR2(404–796); thus structural information regarding the N-terminal region of GluR2 and related sequences is currently not available. Similarly, the crystal structure of the NMDA receptor NR1 S1S2 domain was recently reported (36), but again, this excluded the LIVBP domain.

Fidelity of disulfide bond formation is important in that it both stabilizes the native conformation of proteins, and it maintains the integrity of secreted soluble proteins and cell surface receptors. Indeed, intradisulfide bonds have been shown to fulfill these functions in, for example, the acetylcholine-binding protein (37) and the Kir2.1 inwardly rectifying potassium channel (38). More recently, the integrity of disulfide bond formation was shown to be important for the trafficking to the cell surface of human P2X, ATP-gated ion channels (39). The results described here reveal another example whereby appropriate NR1-NR1 intersubunit disulfide bond formation involving cysteine 79 is requisite for the efficient trafficking of NR1/NR2 NMDA receptors to the cell surface. It is as yet unclear whether this appropriate disulfide bridging is a necessary step in the formation of NR1-NR1 dimers, whether it initiates the NR1-NR1 dimer formation, whether it occurs subsequently in order to stabilize NR1-NR1 prior to assembly with NR2-NR2 dimers as proposed by Schorge and Colquhoun (14), or indeed whether appropriate disulfide bridge formation merely serves as a quality control checkpoint in the exit of assembled, functional NMDA receptors from the endoplasmic reticulum.

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Appropriate NR1-NR1 Disulfide-linked Homodimer Formation Is Requisite for Efficient Expression of Functional, Cell Surface N-Methyl-D-aspartate NR1/NR2 Receptors

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