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The distal carboxyl terminal of rat NR3B subunit regulates NR1-1a/NR3B and NR1-2a/NR3B surface trafficking

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ABSTRACT

N-Methyl-D-aspartate (NMDA) receptors are multi-subunit receptors formed from assembly of NR1 with NR2 and/or NR3 subunits. In this study, we investigated the role of a conserved RERLR motif present in a region within the distal carboxyl terminal of rat NR3B (between residues 952 and 984) in targeting NR1-1a/NR3B and NR1-2a/NR3B receptors to the cell surface. Surface biotinylation, confocal immunofluorescence microscopy and site-directed mutagenesis studies showed RERLR motif does not influence the surface expression of NR1-1a/NR3B NMDA receptor complex. Our bioinformatics analysis further showed this region can also exist as a coiled-coil domain. Truncation of this putative coiled-coil domain in NR3B affects surface expression of NR1-1a/NR3B and NR1-2a/NR3B receptors similarly suggesting that NR1 C1 cassette is not involved in the effect mediated by the distal carboxyl region of NR3B. This study represents the first attempt to evaluate a specific motif in regulating rat NR3B surface expression.

1. Introduction

N-Methyl-D-aspartate (NMDA) receptors are complexes assembled from NR1 subunits, NR2 subunits (NR2A-D) and/or NR3 subunits (NR3A-B) (Dingledine et al., 1999). Each subunit comprises an extracellular N-terminus, four membrane domains (M1–M4), and an intracellular C-terminus.

Endoplasmic reticulum (ER) retention signals mediate retention of improperly folded or assembled polypeptides. They are present in the C1 cassette of NR1 and M3 region of NR1 and NR2B subunits (Horak et al., 2008; Horak and Wenthold, 2009). Masking of these signals is required for the release of subunits from the ER (Standley et al., 2000; Hawkins et al., 2004). During co-assembly, inter-subunit interactions bring subunits into close proximity; thereby allowing the masking of ER retention signals (Zerangue et al., 1999; Standley et al., 2000; Hawkins et al., 2004). Although the amino-terminal domain (ATD) is sufficient for assembly and oligomerization (Meddows et al., 2001; Horak et al., 2008), more distal regions of the subunits may subsequently interact to result in physical masking of retention motifs in M3 and C-terminus of NR1 and NR2B (Schorge and Colquhoun, 2003; Horak et al., 2008).

Most studies to-date have focused on the trafficking signals present in NR1 and NR2B subunits (Okabe et al., 1999; Hawkins et al., 2004; Horak et al., 2008). There is a lack of knowledge pertaining to trafficking signals in NR3. The precise motif(s) responsible for the trafficking of NR3B-containing receptors from ER to the cell surface remain unclear although a previous study had identified the region between amino acid residues 952 and 985 in the C-terminus of mouse NR3B as a critical determinant for cell surface expression of NR1-1a/NR3B receptors (Matsuda et al., 2003).

In this study, we focused on the distal C-terminus of rat NR3B (rNR3B) and investigated the role of a conserved RERLR motif within this region. We also investigated a plausible role of NR3B C-terminus in masking ER retention signal(s) on NR1 subunit by co-expressing the full length and truncated rNR3B separately with rNR1 splice variants that possess or lack the C1 cassette.

2. Materials and methods

2.1. Molecular cloning

The rat NR3B clone (rNR3B) was kindly provided by Dr. Zhang, D. Burnham Institute, La Jolla, CA, USA. The rNR3B-HA construct was generated by adding a hemagglutinin (HA) tag to the distal end of the C-terminus as described elsewhere previously (Matsuda et al., 2003). rNR3B C-terminal truncation constructs were

Abbreviations: HEK, human embryonic kidney cells; ER, endoplasmic reticulum; ATD, amino-terminal domain; M4, transmembrane domain 4; HA, hemagglutinin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylene diamine tetraacetic acid; BSA, bovine serum albumin.

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created as described previously (Matsuda et al., 2003) and mutations were generated by overlap extension PCR (Wee et al., 2010). All mutations were confirmed by DNA sequencing.

2.2. Cell culture and transfection

Human embryonic kidney 293FT (HEK293FT) cells (Invitrogen) were cultured according to manufacturer's recommendation. All tissue culture media and supplements were obtained from Invitrogen. Rat cDNAs encoding rNR1-1a (NM_017010), NR1-2a (U08262) and rNR3B (NM_133308) were co-transfected using Lipofectamine™ and Plus Reagent™ (Invitrogen) at 1:2 (NR1:NR3B) ratio according to the manufacturer's protocol.

2.3. Surface biotinylation

16–24 hours post-transfection, transfected HEK293FT were washed twice with PBS, pH 8.0 followed by incubating for 15 min at 4 °C with 0.5 mg/mL of EZ link Sulfo NHS-SS-biotin (Pierce) in PBS, pH 8.0. Reaction was terminated by adding 50 mM Tris–HCl, pH 7.5 and washed 4 times with wash buffer (50 mM Tris–HCl pH 7.5, 115 mM NaCl) (Matsuda et al., 2003). Cells were lyzed using 400 μl of TNE buffer (50 mM Tris–HCl pH 8.0, 50 mM NaF, 1% NP-40 Igepal, 20 mM EDTA, 0.1% SDS). For NeutrAvidin precipitation of biotinylated proteins, 200–300 μg cell lysate was incubated with 50–100 μl of immobilized NeutrAvidin (Pierce) for 1 h at room temperature with inversion. NeutrAvidin beads were washed 5–7 times with TNE buffer before eluting the bound proteins. Elution was carried out by heating the NeutrAvidin precipitates at 95 °C for 5 min in SDS-PAGE sample buffer. The eluates and total cell lysates were subjected to Western blot analysis. 1 μg of total lysate was loaded for analysis.

PVDF membranes were blocked with 5% milk in PBS, pH 7.4 containing 0.1% (v/v) Tween-20. Primary antibodies used were mouse anti-NR1 (BD Pharmingen; 1:100), goat anti-NR3B (Santa Cruz, sc-55727; 1:100) and rat anti-HA Clone 3F10 (Roche, Cat. No. 11867423007; 1:500). Membrane was incubated with secondary HRP-tagged antibodies. All incubation times were kept to 1 h at room temperature. Chemiluminescence detection was performed using ECL or ECL Plus (Amersham Corp.).

2.4. Immunocytochemistry

Transfected cells were fixed 24 h post-transfection using ice-cold 4% paraformaldehyde (15 min, 4 °C). For surface labelling, incubations were conducted without Triton X-100. Cells were blocked using 3% BSA (w/v) in PBS (1 h, room temperature), and incubated with an anti-NR3B targeting the extracellular region (Santa Cruz; 1:100) (4 °C, overnight). After 3 times wash with PBS, cells were incubated with secondary antibody in the dark (1 h, room temperature). Subsequently, blocking and permeabilization were performed using 3% BSA (w/v) in PBS containing 0.1% (v/v) Triton X-100 (PBS-0.1% TX). Mouse anti-hemaglutinin (HA) (Roche; 1:500) was added and cells were washed 3 times using PBS-0.1% TX. Cells were incubated with secondary antibodies, washed and mounted as described previously (Wee et al., 2008). Secondary antibodies used were Alexa Fluor 555 donkey anti-goat and Alexa Fluor 647 donkey anti mouse (Molecular Probes; 1:800).

2.5. Densitometric analysis

Scanned images were digitized using UN-SCAN-IT gel™ Version 6.1 (Silk Scientific Corp.). To compare the effect of NR3B RERLR mutants and NR1 splice variants on surface expression, normalization was carried out for each NR3B wild type and mutant construct using the following formula:

$$\frac{\text{NR3B band intensity for surface fraction/NR3B band intensity for total fraction}}{\text{NR1 band intensity for surface fraction/NR1 band intensity for total fraction}}$$

Normalization takes into account two factors that may confound the analysis: total NR3B protein expression and NR1 expression. The normalized value for each mutant was then compared to the control NR1-1a/NR3B and NR1-2a/NR3B values to obtain the percentage surface expression of each mutant relative to control. Statistical analysis was performed using GraphPad Prism™ software, version 4.02 by using one-way ANOVA and paired *t*-tests.

2.6. Bioinformatics analysis

Multiple sequence alignment was performed on the following: rNR3B (NM_133308), mNR3B (NM_13055) and hNR3B (NM_138690) using the ClustalW2 software on the EMBL-EBI server. Rat NR3B sequence (Swiss-Prot ID: Q8VHN2) was analyzed for coiled-coil domain on Network Protein Sequence Analysis (NPS). The algorithm derives the probability that a residue in a protein is part of a coiled-coil structure from the comparison of its flanking sequences with sequences of known coiled-coil proteins. Residues with probabilities >50% are assumed to be part of a coiled-coil segment (Lupas et al., 1991). Probability values obtained using a 21 residue scanning window was used to determine the coiled-coil domain (Lupas et al., 1991).

3. Results

3.1. Mutations of the RERLR motif has no significant effect on the surface expression of rNR3B alone or NR1-1a/NR3B receptors

NR3B carboxyl-terminal contains a RERLR motif that is fully conserved between rat, mouse and human (Fig. 1A). This motif resembles the RXR (Arginine-X-Arginine) ER retention motif. Mutual masking of ER retention signals has been proven to be a mechanism for releasing NMDA receptor complexes from the ER (Horak et al., 2008). We investigated whether RERLR motif is the molecular determinant responsible for lack of surface trafficking of NR3B alone by expressing NR3B-containing single arginine mutation (R966A, R968A or R970A) or double mutations (R966A/R968A) in HEK293FT cells and compared against wild-type NR3B in the presence of NR1-1a. Our surface biotinylation data showed all mutants lack surface expression similar to wild-type NR3B when expressed alone (Fig. 1B).

When these NR3B mutants were co-expressed with NR1-1a, there was no significant difference ($p = 0.81$, one-way ANOVA) in all mutants' surface expression compared to wild-type NR3B as analyzed by surface biotinylation (Fig. 1C, $n = 3$) and confocal immunofluoresence microscopy (Fig. 1D, $n = 3$).

3.2. Distal C-terminus regulates rNR3B surface expression

We progressively truncated the distal 18 (amino acids 985–1002; hereafter named NR3B-T1) and 51 residues (amino acids 952–1002; hereafter named NR3B-T2) from the C-terminal end of rNR3B and showed co-expression of NR1-1a/NR3B and NR1-1a/NR3B-T1 mutually supported transport of the NR3B and NR3B-T1 proteins to the cell surface (Fig. 1E). In contrast, there was only approximately 10% NR3B-T2 protein detected on cell surface ($p < 0.05$, $n = 3$; Fig. 1E). Hence, an important determinant of surface expression must exist in the T2 region (residues 952–984), which is the region spanning the distal end of NR3B-T1 and NR3B-T2 (Fig. 1A).

3.3. The T2 region of NR3B is predicted to contain a coiled-coil domain

We questioned the possibility that the effect of T2 region on surface expression of NR1-1a/NR3B receptors might be due to a three-dimensional structural motif rather than a short molecular motif. Our bioinformatics analysis revealed a single region from residues 947–981 of rNR3B protein with a very high probability ($0.7 < x < 1.0$) of existing in the coiled-coil conformation (Fig. 1F). This predicted coiled-coil domain overlaps extensively with T2 region of rNR3B. Additionally, the length of this domain is 34 residues long, which falls within the expected length of 30–35 residues for coiled-coil domains (Lupas et al., 1991).

3.4. The C-terminus of NR3B exerts its effect on surface expression via a region other than the NR1 C1 cassette

We next investigated whether the C1 cassette of NR1-1a, which contains two separate ER retention signals (RRR and KKK) (Horak et al., 2008; Fig. 2A), could be masked by NR3B-T2 region within NR3B subunit. Pulldown of surface biotinylated NR3B-T2 when co-expressed with NR1-1a or NR1-2a, NR3B-T2 surface expression decreased in a similar fashion ($p < 0.05$, $n = 3$; Fig. 2B and C).

4. Discussion

Here, we show that NR3B distal carboxyl-terminal region regulates surface expression of NR1-1a/NR3B and NR1-2a/NR3B receptor complex which is not mediated by a well conserved