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DELINEATION OF ADDITIONAL PSD-95 BINDING DOMAINS WITHIN NMDA RECEPTOR NR2 SUBUNITS REVEALS DIFFERENCES BETWEEN NR2A/PSD-95 AND NR2B/PSD-95 ASSOCIATION

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Abstract—*N*-methyl-D-aspartate (NMDA) receptors are clustered at synapses via their association with the PSD-95 (post-synaptic density-95) membrane associated guanylate kinase (MAGUK) family of scaffolding proteins. PSD-95 is the best characterized of this family. It is known to associate with NMDA receptor NR2 subunits via a conserved ES(E/D)V amino acid sequence located at their C-termini and thus to promote the clustering, regulation and the trafficking of assembled NR1/NR2 NMDA receptors at synapses. Here we have investigated in more detail NMDA receptor NR2/PSD-95 protein–protein association. Wild-type NR1 and PSD-95α were co-expressed with a series of rodent C-terminal truncated constructs of either NR2A or NR2B subunits in human embryonic kidney (HEK) 293 cells and the association of PSD-95α with assembled receptors determined by immunoprecipitation. Additional PSD-95 binding domains that differed between NR2A and NR2B subunits were identified. These domains mapped to the amino acid sequences NR2A (1382–1420) and NR2B (1086–1157). These results suggest that NR2A and NR2B may associate with PSD-95 but with different affinities. This may be important in the determination of the lateral mobility of NMDA receptor subtypes in post-synaptic membranes. © **2009 IBRO. Published by Elsevier Ltd. All rights reserved.**

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N-methyl-D-aspartate (NMDA) receptors are one of the major mediators of fast excitatory neurotransmission in the CNS. They are a subclass of ionotropic glutamate neurotransmitter receptors that are activated by the binding of co-agonists, L-glutamate and glycine, together with the alleviation of a voltage-dependent blockade by magnesium ions that is achieved at synapses by the activation of adjacent non-NMDA glutamate receptors (reviewed in Dingledine et al., 1999). NMDA receptors are important because of the central role they play in long-term potentiation (LTP) and long-term depression (LTD), mechanisms of learning and memory; in synaptogenesis during the development of the CNS and as a potential therapeutic target in neurological and psychiatric disorders that include stroke, neuropathic pain, epilepsy, schizophrenia and Alzheimer's disease. Seven genes encode the seven NMDA receptor subunits, the NR1, NR2A–NR2D, NR3A–NR3B subunits. The NR1 subunit undergoes extensive splicing to yield eight variants NR1-1a, 1b to NR1-4a, 4b. Functional NMDA receptors are formed from the co-assembly of the obligatory NR1 glycine binding subunit with the L-glutamate binding NR2 subunit and/or NR3 subunits. The subunits probably assemble as tetramers with stoichiometry $(NR1)_2(NR2)_2$ (Furukawa et al., 2005; Ulbrich and Isacoff, 2007).

At synapses, NMDA receptors are associated with a network of proteins that mediate their clustering, their activity and downstream signaling events. Pivotal to all these activities are the post-synaptic density (PSD)-95 membrane-associated guanylate kinase (MAGUK) family of scaffold proteins. PSD-95 and the other members of this family, chapsyn-110 (also termed PSD-93), synapse-associated protein 102 (SAP102) and SAP97 are post-synaptic density protein-95, *Drosophila* disc large tumor suppressor (DlgA), and Zo-1 protein (PDZ) domain-containing scaffolding proteins (Kim and Sheng, 2004). PSD-95 is the prototypic member of this family. It is known to be localized at post-synaptic membranes in adult brain. It is a 95 kDa protein with three PDZ domains, of ~90 amino acids in the N-terminal region, PDZ1, PDZ2 and PDZ3, followed by an SH3 domain and a C-terminal guanylate kinase-like domain. PSD-95 and the other PSD-95 MAGUKs associate with each of the putative major subtypes of NMDA receptors via an ES(E/D)V domain found at the distal, intracellular C-termini of all NR2 subunits (Kornau et al., 1995; Niethammer et al., 1996; Cousins et al., 2007). PSD-95 has been shown to enhance the expression of NR2A and NR2B subunits. This enhancement is ESDV-dependent i.e. removing the motif results in the loss of the PSD-95 mediated increase in NR2A and NR2B expression (Rutter and Stephenson, 2000).

In 2002, an electrophysiological and pharmacological study showed that NMDA receptors are mobile and move between synaptic and extra-synaptic compartments (Tovar and Westbrook, 2002). This was substantiated by Groc et al. (2004) who demonstrated that indeed NMDA receptors do have lateral mobility in post-synaptic membranes, at least in hippocampal neurones in primary culture, using single particle and single-molecule approaches. Further, in a later study Groc et al. (2006) reported differences be-

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Abbreviations: HEK, human embryonic kidney; MAGUK, membrane-associated guanylate kinase; NMDA, *N*-methyl-D-aspartate; NR1, NR2A etc., *N*-methyl-D-aspartate receptor NR1 subunit, *N*-methyl-D-aspartate receptor NR2A subunit etc.; PDZ, post-synaptic density protein-95, *Drosophila* disc large tumor suppressor (DlgA), and Zo-1 protein; PSD, post-synaptic density; SAP, synapse-associated protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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tween the lateral mobilities of NR2A- and NR2B-subunit-containing NMDA receptors in post-synaptic membranes. NR2A subunit-containing receptors were relatively immobile and localized to synaptic sites whereas NR2B subunit-containing receptors were observed to move rapidly between extra-synaptic and synaptic regions (Groc et al., 2006). The scaffold protein, PSD-95, is thought to be the key molecule immobilizing NMDA receptors at synaptic sites yet both NR2A and NR2B subunits contain the distal C-terminal ESDV PSD-95 binding motif. The mapping of the PSD-95 binding region of NR2 subunits was carried out initially using yeast two-hybrid interaction assays and, although the distal C-terminal ESDV domain was identified as the main site of association, there was some evidence that more upstream NR2 peptide sequences could contribute to their association with PSD-95 (Kornau et al., 1995; Bassand et al., 1999). Here, we have extended these studies and carried out a comparative investigation of NR2A/PSD-95 and NR2B/PSD-95 interaction regions using a mutagenesis, immunoprecipitation strategy. Additional but different NR2A and NR2B PSD-95 binding domains were identified. These may contribute to differences in lateral mobilities of NMDA receptor subtypes at post-synaptic membranes.

EXPERIMENTAL PROCEDURES

Mammalian cell transfection and preparation of cell homogenates

Human embryonic kidney (HEK) 293 cells were transfected with either wild-type pCISNR1/pCISNR2A+PSD-95$\alpha^{c\text{-}Myc}$, pCISNR1/pCISNR2B^{FLAG}+PSD-95$\alpha^{c\text{-}Myc}$ or with NR1+PSD-95$\alpha^{c\text{-}Myc}$ and NR2A or NR2B^{FLAG} C-terminal constructs, i.e. NR1/NR2A^{1157}, NR2A^{1382}, NR2A^{1420}, NR2A^{1441}, NR2A^{1460}, NR2B$^{FLAG/1086}$, NR2B$^{FLAG/1157}$, NR2B$^{FLAG/1458}$ and NR2B$^{FLAG/1478}$ using the calcium phosphate method with a total of 20 μg DNA and cultured post-transfection in the presence of 1 mM ketamine to prevent NMDA receptor–mediated cytotoxicity (Cik et al., 1993). NMDA receptor and PSD-95$\alpha^{c\text{-}Myc}$ MAGUK clones were co-transfected using a 1:1 ratio with NR1:NR2 subunits being in a ratio of 1:3 thus for 20 μg DNA, 2.5 μg pCISNR1:7.5 μg pCISNR2:10 μg pGW1PSD-95$\alpha^{c\text{-}Myc}$ clones were used. All experiments used the NR1-1a splice variant. The NR2B^{FLAG} clone was used because other available anti-NR2B antibodies do not recognize NR2B subunits in immunoblots. The FLAG tag is introduced between amino acids 53 and 54 of the mature NR2B subunit and it was shown to behave as wild-type NR2B with respect to radioligand binding and functional properties (Hawkins et al., 1999; Prybylowski et al., 2002). Cells were harvested 24–36 h post-transfection, homogenates prepared, adjusted to 0.5 mg protein/ml and analyzed by quantitative immunoblotting.

Immunoblotting

Immunoblotting was performed as described previously using 25–50 μg of protein/sample precipitated using the chloroform/methanol method and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions in 7.5% polyacrylamide slab minigels (Papadakis et al., 2004). Affinity-purified anti-NMDA receptor, i.e. anti-NR1 C2 (Chazot et al., 1994), anti-NR2A 44–58 (Groc et al., 2006), anti-FLAG antibodies and anti-c-Myc Clone 4A6 mouse monoclonal antibodies (Upstate USA Inc., Charlottesville, VA, USA) were used at final concentrations of 1–5 μg/ml. Rabbit and mouse horseradish-linked secondary antibodies (Amersham Biosciences Ltd., Little Chalfont, Bucks, UK) were used at a final dilution of 1:2000 and immunoreactivities were detected using the ECL Western blotting system. Immunoreactive bands were quantified using the GeneGnome Chemiluminescence Capture and Analysis System (Syngene, Cambridge, Cambs., UK).

Immunoprecipitations

HEK 293 cells were transfected with either wild-type pCISNR1/pCISNR2A+PSD-95$\alpha^{c\text{-}Myc}$, pCISNR1/pCISNR2B^{FLAG}+PSD-9595$\alpha^{c\text{-}Myc}$ or with NR1/NR2A^{1157}, NR1/NR2A^{1382}, NR1/NR2A^{1420}, NR1/NR2A^{1441}, NR1/NR2A^{1460}, NR1/NR2B$^{FLAG/1086}$ NR1/NR2B$^{FLAG/1157}$, NR1/NR2B$^{FLAG/1458}$, NR1/NR2B$^{FLAG/1478}$+PSD-9595$\alpha^{c\text{-}Myc}$. Cells were harvested 24 h post-transfection, cell homogenates prepared and solubilized for 1 h at 4 °C with 50 mM Tris–citrate, pH 7.4, 240 mM NaCl, 5 mM EDTA, 5 mM EGTA, 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 phenylmethylsulphonyl fluoride (1 mM). Samples were diluted to 1 mg protein/ml and the solubilized material collected by centrifugation at 100,000×g for 40 min at 4 °C. Aliquots (800 μl) were incubated with affinity-purified anti-NR1 C2 antibodies or protein A purified non-immune Ig (5 μg) overnight at 4 °C. Protein A-Sepharose (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 as described by Papadakis et al. (2004).

Generation of NMDA receptor NR2A and NR2B C-terminal truncated subunits

C-terminal truncations of NR2A and NR2B subunits were generated by the introduction of a stop codon after the requisite amino acid using the Quikchange™ mutagenesis kit (Stratagene, La Jolla, CA, USA) with the complementary oligonucleotide primers listed in Table 1 or as in Rutter and Stephenson (2000) for the NR2A^{1460} and NR2B$^{FLAG/1478}$. All constructs were verified by DNA sequencing (MWG-Biotech AG, Ebersberg, Germany).

RESULTS

Rationale for the selection of NR2A and NR2B C-terminal truncations

An alignment of the intracellular, C-terminal amino acid sequences of the NR2A and NR2B subunits is shown in Fig. 1 together with a schematic diagram depicting the truncated NR2A and NR2B subunits generated. Overall, NR2A and NR2B C-terminal domains share 31% amino acid identity and 48% similarity. This rises to 58% identity and 67% similarity for the 24 C-terminal amino acids. The extreme C-terminal NR2A^{1460} and NR2B^{1478} truncation mutants were created since this deleted the PSD-95 ESDV binding motif at the C-termini of the respective NR2 subunits. Since, from the literature, ESDV was identified as the PSD-95 binding motif, this truncation should in theory result in the loss of NMDA receptor/PSD-95 protein–protein association. In initial experiments however (see below), it was found that the deletion of this domain did not result in the loss of PSD-95 in NMDA receptor/PSD-95 co-immunoprecipitation experiments suggesting that additional NR2 upstream amino acid sequences may contribute to the formation of this complex. Truncations were subsequently generated at NR2A^{1441} and NR2B^{1458} which removed the