Identification of N-Methyl-\(\alpha\)-aspartic Acid (NMDA) Receptor Subtype-specific Binding Sites That Mediate Direct Interactions with Scaffold Protein PSD-95*<sup></sup><br遭到更对的</br>
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**Background:** NMDA receptors are organized via scaffold proteins into macromolecular signaling complexes at synapses. 

**Results:** Novel binding sites that govern NMDA receptor/scaffold protein association have been delineated.

**Conclusion:** The scaffold, PSD-95, binds to two sites that differ between NMDA receptor NR2A and NR2B subunits.

**Significance:** The molecular basis of protein/protein interactions that contribute to organization of synaptic signaling complexes is extended and enhanced.

N-methyl-\(\alpha\)-aspartate (NMDA) neurotransmitter receptors and the postsynaptic density-95 (PSD-95) membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins are integral components of post-synaptic macromolecular signaling complexes that serve to propagate glutamate responses intracellularly. Classically, NMDA receptor NR2 subunits associate with PSD-95 MAGUKs via a conserved ES(E/D)V amino acid sequence located at their C termini. We previously challenged this dogma to demonstrate a second non-ES(E/D)V PSD-95-binding site in both NMDA receptor NR2A and NR2B subunits. Here, using a combination of co-immunoprecipitations from transfected mammalian cells, yeast two-hybrid interaction assays, and glutathione S-transferase (GST) pulldown assays, we show that NR2A subunits interact directly with PSD-95 via the C-terminal ESDV motif and additionally via an Src homology 3 domain-binding motif that associates with the Src homology 3 domain of PSD-95. Peptide inhibition of co-immunoprecipitations of NR2A and PSD-95 demonstrates that both the ESDV and non-ESDV sites are required for association in native brain tissue. Furthermore, we refine the non-ESDV site within NR2B to residues 1149–1157. These findings provide a molecular basis for the differential association of NMDA receptor subtypes with PSD-95 MAGUK scaffold proteins. These selective interactions may contribute to the organization, lateral mobility, and ultimately the function of NMDA receptor subtypes at synapses. Furthermore, they provide a more general molecular mechanism by which the scaffold, PSD-95, may discriminate between potential interacting partner proteins.

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Excitatory N-methyl-\(\alpha\)-aspartate (NMDA)<sup>2</sup> neurotransmitter receptors are important because of the central role they play in long term potentiation and long term depression, mechanisms of learning and memory, in synaptogenesis during the development of the central nervous system, and as a potential therapeutic target in neurodegenerative and psychiatric disorders that include stroke, neuropathic pain, epilepsy, schizophrenia, and Alzheimer disease (reviewed in Ref. 1).

NMDA receptors are tetramers formed by the co-assembly of two copies of the obligatory NR1 subunit together with either two copies of a single type of the four NR2 subunits (i.e. NR2A to NR2D), a single copy of two types of the NR2 subunit class, or a single copy of an NR2 subunit together with one of the NR3 class. The major NMDA receptor subtypes in mammalian brain are NR1/NR2A, NR1/NR2B, and NR1/NR2A/NR2B (reviewed in Ref. 2).

The localization of NMDA receptor subtypes at synapses in adult brain is highly organized. NR2A-containing receptors are found anchored at synaptic sites. In contrast, the distribution of NR2B-containing receptors is more diffuse. They are found predominantly at extra-synaptic sites but can be found within synapses due in part to their enhanced lateral mobility compared with NR2A receptors (3). This specialized distribution of the different NMDA receptor subclasses is thought to be mediated via their association with the post-synaptic density (PSD)-95 membrane-associated guanylate kinase (MAGUK) family of scaffold proteins (reviewed in Ref. 4). Indeed, NMDA receptors and PSD-95 MAGUKs are integral components of post-synaptic macromolecular signaling complexes that serve to propagate glutamate responses intracellularly. The different distribution of these complexes results in distinct functional properties and activation of separate downstream signaling pathways (reviewed in Ref. 5).

PSD-95 is the prototypic member of the PSD-95 MAGUK family. It contains three N-terminal PDZ domains of ~90 amino acids, PDZ1, PDZ2, and PDZ3, an SH3 domain, and a C-terminal guanylate kinase (GK)-like domain. Early studies found that PSD-95 associated with NMDA receptors via their PDZ1 and PDZ2 domains. These bind to an ES(E/D)V motif that is found at the distal intracellular C termini of all four NR2 subunits (6–9). Because each NR2 subunit has this C-terminal ES(E/D)V motif, this implies that all NR2 subunits should inter-

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* This work was supported by the Biotechnology and Biological Sciences Research Council (United Kingdom).

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<sup>2</sup> The abbreviations used are: NMDA, N-methyl-\(\alpha\)-aspartic acid; GK, guanylate kinase; PDZ, post-synaptic density protein-95, *Drosophila* disc large tumor suppressor (DlgA), and Zo-1 protein; SH3, Src homology 3; YPAd, yeast extract peptone dextrose; MAGUK, membrane-associated guanylate kinase.
act similarly with each PSD-95 MAGUK. There is evidence, however, to suggest that NR2A receptors associate preferentially with PSD-95, whereas NR2B-containing receptors complex with SAP102 (10), although this has been more recently disputed (11). The mapping of the PSD-95/NR2 protein-protein-binding sites was carried out initially using yeast two-hybrid interaction assays. Although the distal C-terminal ES(E/D)V motif was identified as the main site of association, there was some evidence that more N-terminal upstream NR2 peptide sequences could contribute to their association with PSD-95 (6, 9). We extended these findings to investigate NR2A–PSD-95 and NR2B/PSD-95 interactions using a mutagenesis strategy in conjunction with immunoprecipitations to show that deletion of the ESDV domain of either NR2A or NR2B subunits by truncation did not prevent the co-immunoprecipitation of assembled NR1/NR2A or NR1/NR2B receptors with PSD-95 suggesting that there may be additional sites of interaction (12). Indeed, additional PSD-95-binding sites that differed between NR2A and NR2B subunits and mapped to NR2A(1382–1420) and NR2B(1086–1157) were found (12). The experimental approach that we employed for these studies could not, however, distinguish between direct and indirect association between NR2 and PSD-95. Therefore, the possibility that PSD-95 immunoprecipitates with assembled NR1/NR2 subunits via an intermediary protein that binds to e.g. NR2A(1382–1420) could not be eliminated.

Here, we have extended these studies to identify an SH3 domain-binding motif within the NR2A subunit that binds to PSD-95. We refine the upstream NR2B PSD-95 binding domain. We demonstrate definitively that the interactions of NMDA receptors with these second PSD-95 binding domains are direct.

EXPERIMENTAL PROCEDURES

Constructs and Antibodies

Mammalian Expression Constructs—For all NMDA receptor subunit constructs, amino acid numbering begins at the start of methionine in the signal peptide. pCISNR1-1a and pCISNR2A were as in Ref. 13; pCISNR2BFLAG was as in Ref. 14; pCISNR2A1460 and pCISNR2BFLAG/1478 were as in Ref. 15; and pCISNR2A1441, pCISNR2A1420, pCISNR2A1382, pCISNR2B1137, pCISNR2BFLAG/1458, pCISNR2BFLAG/1157, and pCISNR2BFLAG/1086 were as in Ref. 12. pCISNR2AEDAV, pCISNR2ASDA, pCISNR2A1460-ASDA, pCISNR2A1420-ASDA, pCISNR2BFLAG/EDAV, pCISNR2BFLAG/1157-ARSA, and pCISNR2BFLAG/1157-ADA were generated using the QuikChangeTM mutagenesis kit (Stratagene, La Jolla, CA). pCISNR2A1389, pCISNR2BFLAG/1120, and pCISNR2BFLAG/1149 were generated by PCR amplification and insertion into the EcoRI/Xbal (pCISNR2A1389) and EcoRI/BamHI (pCISNR2BFLAG/1120 and pCISNR2BFLAG/1149) sites of pCIS. pGW1PSD-95c-Myc was a kind gift from Dr. M. Sheng (Genentech Inc.).

Yeast Two-hybrid Expression Constructs—The DNAs encoding the C-terminal constructs (NR1-1a(834–938); NR2A(838–1464); NR2B(838–1149)) sites of pCISNR1 and pCISNR2B were generated by PCR amplification and inserted in-frame into the EcoRI/XbaI (pCISNR2A1389) and EcoRI/BamHI sites of pGADT7 yeast bait vector to generate the following: pGBK7NRT1-1a, pGBK7NR2A, pGBK7NR2AEDAV, pGBK7NR2ASDA, pGBK7NR2A1460, pGBK7NR2A1460-ASDA, pGBK7NR2A1441, pGBK7NR2A1420, pGBK7NR2A1420-ASDA, pGBK7NR2A1389, pGBK7NR2A1382, and pGBK7NR2A1157; pGBK7NR2B, pGBK7NR2BEDAV, pGBK7NR2B1478, pGBK7NR2B1458, pGBK7NR2B1157, pGBK7NR2B1149, pGBK7NR2B1120, and pGBK7NR2B1086. The DNAs encoding PSD-95c-Myc, PSD-95c-Myc(1–393), PSD-95c-Myc(1–430), PSD-95c-Myc(431–724), PSD-95c-Myc(431–495), and PSD-95c-Myc were generated by PCR from pGWI1PSD-95c-Myc and cloned into the EcoRI/XhoI sites of pGADT7 to generate pGADT7PSD-95c-Myc, pGADT7PSD-95c-Myc(1–393), pGADT7PSD-95c-Myc(1–430), pGADT7PSD-95c-Myc(431–724), and pGADT7PSD-95c-Myc(431–495). PSD-95c-Myc(W470L) was generated from pGADT7PSD-95c-Myc using the QuikChangeTM mutagenesis kit. Expression of all constructs was verified by immunoblotting (results not shown).

Bacterial Expression Vectors—The DNAs encoding the C-terminal constructs NR2A(838–1464), NR2A(838–1464EDAV), NR2A(838–1464), NR2A(838–1420), and NR2A(838–1382) were generated by PCR from the appropriate mammalian expression construct and subcloned in-frame into the EcoRI/XhoI sites of pGEX-4t to generate pGEX-4t-NR2A, pGEX-4t-NR2AEADV, pGEX-4t-NR2A1458, and pGEX-4t-NR2A1389.

Mammalian expression constructs were verified by DNA sequencing (Wolfson Centre, University College, London, UK).

Antibodies—Affinity-purified anti-NR1 C2(911–920), anti-NR2A(44–58 Cys), and anti-NR2A(1381–1394) antibodies were raised and characterized as reported previously (3, 16, 17). Anti-NR2A(1252–1391) was from Millipore (Temecula, CA). Mouse monoclonal anti-FLAG M2 antibodies were from Sigma; anti-c-Myc clone 4A6 mouse monoclonal antibodies were from Upstate (Charlottesville, VA), and anti-PSD-95 antibodies were from Abcam (Cambridge, UK).

Peptides

Peptides with >98% purity were synthesized by GenScript (Piscataway, NJ). They were NR2A(1381–1390), RCCPSD-PYKHS, termed SH3, and corresponded to the amino acid sequence around the SH3 binding domain; SH3 scrambled, DSYSRPHCKP; NR2A(1450–1464), NRRVYKMPSESVDV, termed PDZ, and corresponded to the amino acid sequence at the C-terminal ESDV domain and the same as used previously (18), and PDZ scrambled, KRSMNIVEKDPVYRS.
**Mammalian Cell Transfections**

Human embryonic kidney (HEK) 293 cells were cultured and transfected using the calcium phosphate method as described previously (13). Cells were incubated post-transfection in the presence of 1 mM ketamine to prevent NMDA receptor-mediated cytotoxicity (13). HEK 293 cells were transfected in parallel with either NR1-1a + PSD-95αC-Myc and either wild-type or mutant/truncated NR2A or NR2BFLAG clones. The ratio of DNAs for the transfections were NR1/NR2/PSD-95αC-Myc, 1:3:4, which corresponded to 2.5, 7.5, and 10 μg for a 20-μg total DNA for transfection of a 1 × 250-ml flask.

**Immunoprecipitation Assays**

HEK 293 cells were harvested 24 h post-transfection. Cell homogenates were prepared and solubilized for 1 h at 4°C at a concentration of 1.5 mg of protein/ml with solubilization buffer (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 phenylmethylsulfonyl fluoride (1 mM)). Samples were diluted to 1 mg of protein/ml with the above solubilization buffer, and solubilized material was collected by centrifugation at 100,000 × g for 40 min at 4°C. The detergent extracts were incubated with affinity-purified rabbit anti-NR1 C2 antibodies (5 μg) or protein A-purified nonimmune rabbit IgG (5 μg) as control overnight at 4°C. Protein A-Sepharose (25 μl) was added, and samples were incubated for 1 h at 4°C. Immune pellets were collected by centrifugation for 15 s at 600 × g, washed three times with 1 ml of solubilization buffer, and then analyzed by immunoblotting.

For immunoprecipitations from adult rat brain, 1% (w/v) sodium deoxycholate detergent-solubilized 100,000 × g extracts were prepared as described (19). Triplicate samples were incubated with 100 μl test and control peptides and anti-NR2A(44–58 Cys) or nonimmune rabbit Ig primary antibodies for 16 h at 4°C. Immunoprecipitations were completed as above.

**Immunoblotting**

Immunoblotting was performed as described previously using 25–50 μg of protein/sample precipitated using the chloroform/methanol method and SDS-PAGE under reducing conditions in 7.5% (w/v) polyacrylamide slab minigels all as described previously (20). Primary antibodies used were as follows: anti-NR1 C2(911–920); anti-NR2A(44–58 Cys), anti-NR2A(1381–1394), anti-FLAG M2; anti-c-Myc clone 4A6, and anti-PSD-95. Rabbit or mouse horseradish-linked secondary antibodies (GE Healthcare) were used at a final dilution of 1:2000, and immunoreactivities were detected using the ECL Western blotting system.

**Yeast Two-hybrid Interaction Assays**

Competent yeast cells were prepared according to the method of Schiestl and Gietz (21). A 16–18-h culture was prepared by inoculating 5 ml of yeast extract peptone dextrose with adenine (YPAD) media with a single colony of AH109 or Y189 Saccharomyces cerevisiae cells. A starting A<sub>s</sub> = 600 nm = 0.15 was prepared in 50 ml of YPAD media. The culture was incubated at 30°C at 250 rpm for ~4 h until an A<sub>s</sub> = 600 nm = 0.4–0.5 was obtained. The cells were pelleted by centrifugation at 2500 × g at room temperature. The supernatant was discarded, and the pellet was resuspended in 25 ml of double distilled H<sub>2</sub>O, and cells were pelleted by centrifugation as above. The cell pellet was resuspended in 12.5 ml of lithium sorbitol solution (100 mM lithium acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 1 m sorbitol) followed by centrifugation as above. The cells were resuspended in 300 μl of lithium sorbitol solution, 80 μl of salmon sperm DNA (2 μg/ml, freshly boiled for 5 min and then rapidly cooled in ice for 5 min), and 1 ml of lithium/polyethylene glycol solution (100 μl lithium acetate, 10 μl Tris-HCl, pH 8.0, 1 mM EDTA, and 40% (w/v) polyethylene glycol, M<sub>w</sub> 3500), was added. Y189 S. cerevisiae cells were transformed with 1 μg of either C-terminal NR1-1a, C-terminal wild-type NR2A, C-terminal wild-type NR2B, C-terminal NR2A and NR2B truncations, or point mutated pGBK7 bait constructs. AH109 S. cerevisiae cells were subsequently transformed with 1 μg of fish constructs, which were wild-type pGADT7-PSD-95αC-Myc or pGADT7-PSD-95α<sup>C-Myc</sup> truncations or the point mutation pGADT7-PSD-95<sup>W470M</sup>. For both transformations, 100-μl aliquots were gently vortexed for ~2 s and incubated for 20 min at room temperature. Dimethyl sulfoxide (DMSO, 12 μl) was added and mixed by gentle vortexing for ~2 s. The yeast cells were heat shocked for 10 min at 42°C, and cells were pelleted by centrifugation at 12,000 × g for 30 s at room temperature. The transformed yeast cells were resuspended in 100 μl of double distilled H<sub>2</sub>O and plated onto the appropriate 2% (w/v) agar plate, i.e. a ~ Trp dropout medium for the pGBK7– bait constructs and ~Leu dropout medium for the pGADT7– fish constructs. Yeast cells were incubated at 30°C for ~5 days until colonies appeared. Single colonies were picked for each transformed yeast strain and added to 1 ml of YPAD media, and cells were dispersed by vortexing for ~10 s. AH109 or Y189 S. cerevisiae yeast mating assays were carried out in 24-well tissue culture plates. To each well, YPAD media (500 μl) were added followed by 45 μl of the fish and bait constructs. The mating yeast mix was incubated at 30°C for 22 h at 220 rpm. Aliquots (70 μl) of the diploid cells were plated onto 2% (w/v) agar plates containing either ~Leu/~Trp (to select for diploid formation) or ~Leu/~Trp/~His/~Ade (to activate reporter gene activity). Plates were inverted and incubated for 7 days at 30°C and the colonies counted. Positive and negative controls were always carried out in parallel. The positive control was AH109 S. cerevisiae cells pre-transformed with pTD1-1 and Y187 S. cerevisiae cells pre-transformed with pVA3-1. The negative controls were either empty pGBK7– and pGADT7– vectors or empty fish or bait, respectively, to check for auto-activation of either the bait or fish constructs.

**Glutathione S-Transferase Pulldown Assays**

Glutathione S-transferase (GST) in vitro pulldown GST protein/protein interaction kit (Pierce). pGEX-4t-NR2A, pGEX-4t-NR2A<sup>1382</sup>, pGEX-4t-NR2B<sup>1382</sup>, pGEX-4t-NR2A<sup>1420</sup>, pGEX-4t-NR2A<sup>1460</sup>, and pTrcHisB-PSD-95α<sup>C-Myc</sup> were each
transformed into BL21 Escherichia coli cells. Cultures (100 ml) were grown to an \( A_{600} = 0.6 \), and protein expression was induced with 1 mM of isopropyl \( \beta \)-d-1-thiogalactopyranoside for 4 h. E. coli cells were centrifuged at 3000 \( \times g \) for 15 min at 4 °C. The proteins were extracted by resuspending the bacterial pellets in 8 ml of 50 mM Na\( \text{H}_2\text{PO}_4 \), pH 8.0, 500 mM NaCl followed by the addition of lysozyme (8 mg) and incubated on ice for 30 min. Cell extracts were lysed by sonication on ice, with six 10-s bursts with 10-s cooling rests. Soluble extracts were collected by centrifugation at 3000 \( \times g \) for 15 min at 4 °C. His-PSD-95αc-Myc was purified using a ProBond\textsuperscript{TM} Ni\textsuperscript{2+} affinity column following the manufacturer’s instructions (Invitrogen). Each NR2A C-terminal truncation construct was bound to glutathione-agarose, and the agarose was washed five times with wash solution and then incubated for 2 h at 4 °C with purified His-PSD-95αc-Myc. Glutathione-agarose was washed five times with 400 \( \mu \)l of wash solution, and proteins were eluted using 100 mM glutathione. Eluted samples were analyzed by immunoblotting.

Analysis of Results

All results are the means \( \pm \) S.E. and were analyzed by the Student’s two tailed paired \( t \) test.

RESULTS AND DISCUSSION

Refinement of the Second PSD-95-binding Site within the NMDA Receptor NR2A C-terminal Domain—We reported previously that the second non-ESDV PSD-95-binding site within the NMDA receptor NR2A C-terminal domain mapped to NR2A(1382–1420) (Fig. 1A). This sequence contains a putative SH3 domain-binding site, PSDPYK, at amino acids 1383–1388. To refine the site further, a new truncation was made at NR2A\textsuperscript{1389}. This incorporated the SH3 domain-binding site with an additional amino acid, His. Concurrently, the point mutated NR2A subunit, NR2A\textsuperscript{EADV}, was generated. This was because Przybylowski et al. (22) removed the NR2B subunit ESDV PSD-95-binding site by point mutation (\( i.e. \) NR2B\textsuperscript{EADV}) rather than by truncation as described in our previously reported findings (12). NR2B\textsuperscript{EADV} did not co-immunoprecipitate with PSD-95 (22). This observation is in contrast to NR2A\textsuperscript{1460} and NR2B\textsuperscript{1478} (\( i.e. \) NR2A and NR2B subunits lacking the ESDV site following truncation) where association was found for both with PSD-95 (12). Thus, it is important to reconcile the differences between the two reports.

Each new NR2A construct was co-expressed with NR1-1a and PSD-95αc-Myc in HEK 293 cells; cell homogenates were detergent-solubilized and immunoprecipitated with anti-NR1C2 or control nonimmune antibodies, and the pellets were analyzed by immunoblotting. Anti-NR1 C2 antibodies were selected for immunoprecipitation because they would pellet NR1-NR2A-P95 complexes. Positive and negative control immunoprecipitations were always carried out in parallel. The negative controls included the longest NR2A subunit that did not co-immunoprecipitate PSD-95, \( i.e. \) NR2A\textsuperscript{1382} (12) and an immunoprecipitation from HEK 293 cells expressing only NR1-1a and PSD-95αc-Myc. \( \text{Note that the NR1-3 and NR1-4 splice forms possess a PDZ-binding site motif, STVV, at their respective C termini, but this is not the case for NR1-1a, which does not associate with PSD-95 (6).} \) The positive controls included full-length NR2A and NR2A\textsuperscript{1460}, the shortest NR2A subunit, NR2A\textsuperscript{1420}, that was known to associate with PSD-95. The results are shown in Fig. 1A. NR2A\textsuperscript{EADV}, like the ESDV-truncated subunit, NR2A\textsuperscript{1460}, retained the ability to associate with PSD-95αc-Myc because anti-c-Myc immunoreactivity was detected in test but not in control pellets. NR2A\textsuperscript{1389} was also found to retain the ability to co-immunoprecipitate with PSD-95αc-Myc because anti-c-Myc immunoreactivity was found in test but not control immune pellets. Thus, the second PSD-95-binding site within the NMDA receptor NR2A C-terminal domain was further refined to NR2A(1382–1389). This contains the class II SH3 domain binding consensus sequence, PSDPYK, previously postulated to be the second site in NR2A for the binding of PSD-95 (12).

Association between PSD-95 and the Non-ESDV PSD-95 Binding Domain of the NMDA Receptor NR2A C-terminal Tail Is Direct—The method that was employed initially to study the molecular interactions between PSD-95 and NR2A C termini was via co-immunoprecipitation after expression in mammalian cells. Co-immunoprecipitation of proteins, however, does not demonstrate that proteins associate directly, \( i.e. \) it is possible two proteins may co-precipitate by virtue of each binding to an intermediary protein. Thus, to establish if there is a direct association between PSD-95 and the non-ESDV PSD-95 binding domain sites, two strategies were employed.

The first method was a sensitive yeast two-hybrid interaction assay that utilized two independent reporter gene activities. Various truncated C-terminal NR2A peptides, NR1-1a and NR2A\textsuperscript{EADV}, were used as the bait with full-length PSD-95αc-Myc as the fish in yeast two-hybrid mating assays. NR2A, NR2A\textsuperscript{EADV}, NR2A\textsuperscript{1460}, NR2A\textsuperscript{1441}, NR2A\textsuperscript{1420}, and NR2A\textsuperscript{1389} all showed diploid activity on mating with PSD-95αc-Myc indicating association of the respective fish and bait proteins (Fig. 1, C and D). In contrast, NR2A\textsuperscript{1382} and NR2A\textsuperscript{1157} yielded no diploid activity (Fig. 1C). NR1-1a also showed no diploid activity on mating with PSD-95αc-Myc (Fig. 1C). The number of diploid colonies for the full-length NR2A C-terminal peptide with PSD-95αc-Myc was 912 \( \pm \) 68 (\( n = 21 \)). Expression of constructs was verified by immunoblotting (supplemental Table 1 and supplemental Fig. 1). The yeast mating assays were semi-quantified by counting the number of diploid colonies and expressing these as a percentage of the number of colonies observed for the full-length NR2A C-terminal peptide. Removal of ESDV and all the other constructs that yielded diploid activity gave \( \sim50% \) of the number of colonies compared with the full-length control (Fig. 1D).

The second approach was to determine the ability of selected and immobilized GST-NR2A C-terminal and GST-NR2A\textsuperscript{EADV} fusion proteins to bind in vitro to purified His-tagged PSD-95αc-Myc (His-PSD-95αc-Myc) by GST pulldown assays. His-PSD-95αc-Myc was found to bind GST-NR2A, GST-NR2A\textsuperscript{EADV}, and GST-NR2A\textsuperscript{1420} but not GST-NR2A\textsuperscript{1382} (Fig. 1E).

Thus the results for these two sets of experiments correlate with each other and also with the findings of the co-immunoprecipitation assays reported previously (12) and extended in Fig. 1. Therefore, for the yeast mating assays, constructs NR2A, NR2A\textsuperscript{EADV}, NR2A\textsuperscript{1460}, NR2A\textsuperscript{1441}, NR2A\textsuperscript{1420}, and NR2A\textsuperscript{1389}
but not NR2A\textsuperscript{1382} and NR2A\textsuperscript{1157} show diploid activity on mating with PSD-95\textsuperscript{c-Myc}. For the\textit{ in vitro} GST pulldown assays, GST-NR2AEADV and GST-NR2A\textsuperscript{1420} but not GST-NR2A\textsuperscript{1382} bind His-PSD-95\textsuperscript{c-Myc}. It could be argued that in the co-immunoprecipitations, truncation of NR2A permitted association of PSD-95\textsuperscript{c-Myc} via a non-ESDV site with NR1-1a or possibly an intermediary protein that binds both PSD-95 and NR1-1a. Because NR1-1a assembles with all the NR2A truncations, NR2A would still have been present in immune pellets. However, importantly, NR1-1a did not show diploid activity on mating with PSD-95\textsuperscript{c-Myc}. This possibility is therefore excluded, and it can be concluded that the association between PSD-95\textalpha and the NR2A C-terminal non-ESDV site is direct.
Within the NR2A C-terminal Domain, Demonstration by Coincuboprecipitation and Yeast Two-hybrid Interaction Assays—To investigate the contribution of the NR2A (1382–1389), PSDPYK SH3 domain-binding motif to the binding of PSD-95 to NMDA NR2A subunits, the site was mutated. The mutation PSDP → ASDA was introduced into the following subunits for mammalian expression studies and into the C-terminal constructs for yeast two-hybrid interaction assays:

- Full-length NR2A, the ESDV truncation, i.e. NR2A1460, and NR2A1420, the shortest construct that remained the ability to co-immunoprecipitate with PSD-95.

The results are shown in Fig. 2A. For the co-immunoprecipitations, all NR2A constructs were shown to immunoprecipitate with NR1 subunits. Introduction of the ASDA mutation in the full-length NR2A had no effect on the association of NR2A ASDA with PSD-95α-c-Myc because PSD-95 was found in immune pellets at similar levels to the control, i.e., full-length NR2A. However, when NR2A constructs lacking both the ESDV and the PSDP motifs were co-expressed with NR1-1a and PSD-95α-c-Myc, no PSD-95α-c-Myc was detected in immune pellets. This suggests that PSD-95α was now unable to bind to the mutated/truncated construct, NR2A1460-ASDA.

Similar findings were made for the yeast two-hybrid interaction assays, i.e., only when both sites were deleted were no diploid colonies found (Fig. 2B). These results are consistent with the NR2A PSDPYK, SH3 domain-binding site, NR2A(1382–1389), being the non-ESDV region mediating association with PSD-95α. Interestingly, the adjacent serine, Ser-1390, has been shown to be phosphorylated in vivo (23). This suggests that phosphorylation at Ser-1390 may regulate NR2A/PSD-95 association.

Peptide Inhibition of NR2A-PSD-95 Complexes, Immunoprecipitation from Detergent Extracts of Adult Rat Brain—To determine the importance of the ESDV and non-ESDV PSD-95α-binding site for the association of PSD-95 with native NMDA receptors, peptide inhibition of NR2A/PSD-95 co-immunoprecipitations from detergent extracts of adult rat brain were carried out. triplicate samples were co-incubated with anti-NR2A(44–58 Cys) and peptides encompassing the NR2A SH3 binding domain motif, the ESDV site, or the respective scrambled control peptides overnight at 4°C. Protein A-Sepharose was added for precipitation of antigen-antibody complexes, and immune pellets were analyzed by immunoblotting. The results are shown in Fig. 3. Incubation with any of the four peptides had no significant effect on the amount of NR2A immunoprecipitated. The anti-PSD-95 antibody recognized two molecular mass species, 113 ± 1 and 97 ± 1 kDa (n = 30) in detergent extracts and in immunoprecipitates. Characterization of this commercial antibody showed that it recognized all four PSD-95 MAGUKs (supplemental Fig. 2). The molecular
Determination of Localization of NR2A PSDPYK-binding Site within PSD-95—To determine where the PSDPYK amino acid sequence of NR2A binds to PSD-95α, a yeast two-hybrid approach was taken. In the first instance, a series of PSD-95α constructs were generated (Fig. 4A). These were used as the fish with NR2A full-length and ESDV-truncated NR2A, i.e. NR2A1460 C-terminal constructs as the baits in yeast two-hybrid mating assays. The results shown in Fig. 4B demonstrate that both the NR2A constructs yielded diploid activity with full-length PSD-95 in accord with earlier findings (Figs. 1 and 2). When PSD-95 was genetically engineered to contain the PDZ domains only or the PDZ domains plus the C-terminal 37 amino acids of the linker region, full-length NR2A yielded diploid activity in mating assays. This was not the case for NR2A1460 (Fig. 4B). This is in agreement with earlier findings that identified the PDZ domains I and II of PSD-95 as the binding site for the NR2 ESDV sequences (7). Because the second PSD-95α-binding site in NR2A, PSDPYK, is a class II consensus sequence for the binding of SH3 domains (24), it may be expected that it should bind to the SH3 domain of PSD-95α. Surprisingly, however, no diploid activity was evident for yeast co-transformations with the PSD-95α SH3 domain, i.e. PSD-95α(431–495), and full-length NR2A C terminus or with NR2A1460. When the SH3 domain construct was extended to include both the SH3 and GK-like domains, diploid activity was found for full-length NR2A but not the ESDV-truncated NR2A C-terminal domain, NR2A1460 (Fig. 4B). The SH3 domain of PSD-95 is known to form an intramolecular interaction with the GK-like domain (25). The x-ray crystal structure of the SH3-GK-like domain revealed that the two regions form an intertwined unit with the polypeptides of both domains being integrated together (26, 27). Moreover, Pan et al. (28) recently showed that for the ZO-1 MAGUK, the PDZ3 domain interacts extensively with its SH3 domain. They suggested, based on structure-based sequence analysis, that this would also be the case for other MAGUKs (28). Thus, it is highly likely that the folding of the SH3 domain in the absence of the PDZ3 and the GK-like domain may not resemble that found in native
PSD-95. Consequently, NR2A constructs containing the PSDP-binding site will not bind to the PSD-95 SH3 domain in isolation. Alternatively, it may be that the SH3 domain is not the binding partner for NR2A PSDP. It is unclear why wild-type NR2A binds to the tandem construct SH3-GK-like domain.

To investigate this further, a mutagenesis strategy was employed. It has been reported that a highly conserved tryptophan residue found in the hydrophobic binding surface of prototypical SH3 domains mediates interaction with proline-containing peptides such as those found in SH3 binding domains.

FIGURE 4. Mapping the NMDA receptor NR2A binding domains within PSD-95α by yeast two-hybrid interaction assays. A, schematic diagram showing the PSD-95 constructs highlighting the protein domains of PSD-95. B–E, Y189 S. cerevisiae yeast cells were transformed with pGBK7 NR2A C-terminal bait constructs, and AH109 S. cerevisiae cells were transformed with pGADT7-PSD-95α constructs as shown. Cells were mated and grown on −Leu/−Trp/−His/−Ade agar plates, and the number of diploid colonies counted after 7 days incubation at 30 °C all as described under “Experimental Procedures.” The number of diploid colonies were semi-quantified as a percentage of the number of colonies obtained for the NR2A full-length C-terminal domain, and thus 100%; 80–90%; 40–79%; < 10%, and 0 colonies. C and E are histogram summaries, where the values shown are the means ± S.E. for n = 3 (C) and n = 4 (E).
(29–31). This tryptophan is conserved in all the PSD-95 MAGUKs. Moreover, when mutated it abrogated the recruitment of phosphatidylinositol 3-kinase-C2α to the SH3-binding site of PSD-95 (32). Thus, the W470L point mutation was introduced into full-length PSD-95, and the interactions between wild-type and PSD-95W470L and wild-type and NR2A truncated and mutated C-terminal tails were investigated in parallel by yeast two-hybrid interaction assays. The results are summarized in Fig. 4C. As before (Figs. 1 and 2) NR2A, NR2A1460, NR2A ASDA but not NR2A1460-ASDA nor NR2A1382 yielded diploid activity with wild-type PSD-95. As may be predicted, wild-type NR2A associates with PSD-95W470L because it contains the ESDV motif, but surprisingly, NR2A ASDA, which retained the ESDV PDZ-binding motif, did not interact with PSD-95W470L. In contrast, despite not containing ESDV, NR2A1460 did yield diploid activity with PSD-95W470L. NR2A1460/ASDA and the negative control, NR2A1382 construct did not result in diploid activity (Fig. 4C).

These findings support the hypothesis that the second NR2A binding domain, PSDPYK, does indeed associate with PSD-95 via the SH3 domain. Furthermore, they imply that because NR2A ASDA does not interact with PSD-95W470L, the PSDPYK motif is essential and the first point of recognition in a two-step mechanism for the binding between the NR2A C terminus and PSD-95. The first step is the association between the SH3 domain and the proline-rich PSDPYK, the SH3 domain binding region followed by docking of NR2A to PSD-95 via the ESDV site. This mechanism would also fit with the peptide inhibition studies in rat brain. The hypothesis may explain the findings of Pryblyoski et al. (22). They showed that the synaptic localization of NR2A-containing NMDA receptors is not controlled by the PDZ binding domain. However, if the first step in the association of NR2A with PSD-95 is via the PSDPYK motif, this would still allow synaptic localization of NR2A. But, an unanswered question is why is PSD-95 targeted to synapses? The two N-terminal cysteines, PDZ I and PDZ II domains and a C-terminal sequence (residues 13–25 of the distal C-terminal domain) are all purported to play a role (33).

Refinement of Second PSD-95-binding Site within the NMDA Receptor NR2B C-terminal Domain and Demonstration That Association between PSD-95 and the Non-ESDV PSD-95 Binding Domain of the NMDA Receptor NR2B C-terminal Tail Is Direct—We reported previously that the second PSD-95-binding site within the NMDA receptor NR2B C-terminal domain mapped to NR2B(1086–1157) (Fig. 5) (12). This sequence is distinct from that of NR2A. Similarly to NR2A, this preliminary mapping of the second non-ESDV site identified an SH3 domain-binding motif (class I) within this region at (1111RRRPRSP1117; see Ref. 12) and a potential internal PDZ binding domain with the sequence TDI(1152–1154) (34). Thus, to refine the site further, two new truncations were made. These were NR2B1120 and NR2B1149. In addition, similarly to NR2A because Pryblyoski et al. (22) removed the NR2B subunit C-terminal ESDV site by point mutation, i.e. ESDV→EADV, to generate NR2B subunits that reportedly lacked PSD-95 binding capability, a new NR2B construct was generated, i.e. NR2B EADV. This mutation is particularly important for NR2B because truncation of the ESDV C-terminal sequence generates a new C-terminal sequence, SSI (35, 36). This sequence has characteristics of a PDZ binding domain raising concerns that the original NR2B truncation experiments may be an artifact resulting from the creation of a new PDZ domain.

Initially, NR1, PSD-95αC-Myc, and wild-type and mutated or truncated NR2B subunits were co-expressed in parallel in HEK 293 cells, and immunoprecipitations carried out using anti-NR1 C2 antibodies and associating proteins were identified by immunoblotting (Fig. 5). The results revealed that NR2B EADV did co-immunoprecipitate with PSD-95αC-Myc thus discounting the possible creation of an artificial PDZ site by truncation of the C terminus. Both the new constructs did not co-precipitate with PSD95αC-Myc and thus the non-ESDV PSD-95 binding domain must map to NR2B(1149–1157). These findings were verified by yeast two-hybrid interaction assays (Fig. 5C). Thus, as concluded for NR2A, the interaction between the non-ESDV site of NR2B and PSD-95 must be direct.

To refine the NR2B non-ESDV-binding site further, two NR2B mutations were generated. These were pCIgNSR2B FLAG/1157-ARSA and pCIgNSR2B FLAG/1157-ADA. NR2B FLAG/1157-ARSA mutated the class I SH3 domain-binding motif, which is outside the site mapped by the truncation experiments (NR2B(1114–1117)) and therefore serves as a control. NR2B FLAG/1157-ADA mutated the putative internal PDZ binding domain (NR2B(1152–1154)) within the identified NR2B non-ESDV-binding sequence, NR2B(1149–1157). Mutation of the SH3 domain-binding motif did not affect NR2B EADV/1157-ARSA/PD95αC-Myc association because NR2B, PSD-95αC, and NR1-1a were all found in immune pellets (Fig. 5B). This was also the case for NR2B EADV/1157-ADA thus excluding the TDI sequence as an internal PDZ binding domain. The NR2B non-ESDV site thus maps to the sequence DLTDIYKE. This may be a novel PSD-95αC protein-binding sequence that associates with non-PDZ domains of PSD-95. There is a precedent for this. Kalia et al. (37) showed that Src binds directly via its SH2 domain to a 12-amino acid sequence in the N-terminal region of PSD-95. It should be noted that Chen et al. (38) recently reported that deletion of the PDZ ligand, ESDV, from NR2B resulted in a loss of interaction with PSD-95. This was shown by yeast two-hybrid interaction assays. The methodology used however was different from that employed here. Chen et al. (38) measured reporter gene activity after yeast co-transformations utilizing a single reporter gene, HIS3. Here, two reporter genes, HIS3 and ADE, were used to increase the stringency of any observed protein/protein associations. These were utilized in conjunction with nutritional markers in yeast mating assays with the resultant diploid formation reflecting specific protein/protein interactions. We have found that using this additional yeast mating step gives an approximate 5-fold increase in sensitivity, which may explain the differences between the two groups.

Concluding Remarks—In this paper, we have determined the PSD-95 non-ESDV-binding site within the NMDA receptor NR2A subunit. We have shown that this second site is important for the association between NR2A and PSD-95.
in vivo. Furthermore, we have refined the non-ESDV site within the NR2B subunit. These findings provide a molecular basis for the differential association of NMDA receptor subtypes with PSD-95 MAGUK scaffold proteins that may contribute to the organization, lateral mobility, and ultimately the function of these receptors at synapses.

Fig. 6 summarizes possible modes of interaction between NR2A subunits and PSD-95 within assembled NR1/NR2A receptor complexes. These models include the binding of a single PSD-95 molecule to two sites within one NR2A subunit, the binding of 1× PSD-95 molecule to 2× NR2A subunits, and the binding of 2× PSD-95 molecules to 1× NR2A subunit. These possibilities would result in a ratio of PSD-95/NMDA receptor ranging from 1 to 8 given that PSD-95 is known to dimerize. The number of PSD-95 molecules per post-synaptic density (estimated 200–300) exceeds the number of NMDA receptors by 20–30 (for review see 39). However, PSD-95 does bind other ESDV-containing synaptic proteins (4). A recent quantitative proteomic study yielded a ratio of 12 PSD-95/1 NR2 subunit (40). This is sufficient to accommodate all the models proposed here together with binding to other ESDV-containing proteins. Further studies are necessary to resolve this issue.

More generally, given the relative ubiquity of ESDV motifs in synaptic proteins, we have here provided a molecular mechanism by which the scaffold, PSD-95, may discriminate between potential interacting partner proteins. It would be interesting to determine whether other ESDV-containing proteins, such as voltage-gated and inward-rectifying K channels (4), also contain non-ESDV, PSD-95 binding domains.

FIGURE 5. Refinement of a novel binding domain that contributes to the association of the NR2B C-terminal tail with PSD-95, demonstration by co-immunoprecipitation and yeast two-hybrid interaction assays. A, schematic diagram showing the NR2B C-terminal region highlighting the ESDV PDZ binding domain, the amino acid sequence of the second PSD-95 binding domain, and the arrow denoting the position of the new truncation. Also highlighted are the potential protein binding domain sites that are mutated, i.e. PRSP → ARSA and TDI → ADA. B and C, HEK 293 cells were co-transfected in parallel with pCISNR1-1a/pCISNR2BFLAG, pCISNR1-1a/pCISNR2BFLAG/ESDV, pCISNR1-1a/pCISNR2BFLAG/ARSA, and pCISNR1-1a/pCISNR2BFLAG/ADA. Transfected cells were harvested 24 h post-transfection; cell homogenates were prepared and detergent-solubilized; immunoprecipitations carried out using anti-NR1 C2 or anti-nonimmune IgG antibodies, and immune pellets were analyzed by immunoblotting using anti-NR1 C2, anti-FLAG, or anti-c-Myc antibodies to detect NR1, NR2B, and PSD-95 as shown. The number of diploid colonies were semi-quantified as a percentage of the number of colonies obtained for the NR2B full-length C-terminal peptide is 100% (422 ± 54 colonies, n = 18); *, p < 0.05; **, p < 0.025; ****, p < 0.001.
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FIGURE 6. Proposed mechanisms for the interaction between NMDA receptor NR2A C-terminal tails and PSD-95. The figure shows possible models for the association between PSD-95 and NR2A subunits within an assembled tetrameric NMDA receptor. A, PSD-95 binds to one NR2A subunit via both the ESDV and non-ESDV site leading to a ratio of NR2A/PSD-95, 2:1. B, PSD-95 binds to one NR2A via the ESDV site and to the second NR2A via the non-ESDV site leading to a ratio of NR2A/PSD-95, 2:1. C, NR2A/PSD-95 ratio is 1:2 because two molecules of PSD-95 bind to one NR2A, one via the ESDV site and the other via the non-ESDV site.

Acknowledgments—We thank Dr. Kieran Brickley and Tom Randall (School of Pharmacy, University of London) for help with antibody production and affinity purification and assistance with the yeast two-hybrid interaction assays, respectively. We also thank Professor Morgan Sheng (Genentech Inc.) for the gifts of the pGWIPSD-95c-Myc and pCMVneoSAP102c-Myc.

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