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PSD-93 Deficiency Protects Cultured Cortical Neurons from NMDA Receptor-triggered Neurotoxicity

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Abstract

It has been reported that N-methyl-D-aspartate receptor (NMDAR)-triggered neurotoxicity is related to excessive Ca^{2+} loading and an increase in nitric oxide (NO) concentration. However, the molecular mechanisms that underlie these events are not completely understood. NMDARs and neuronal NO synthase each binds to the scaffolding protein PSD-93 through its PDZ domains. In this study, we determined whether PSD-93 plays a critical role in NMDAR/ Ca^{2+} /NO-mediated neurotoxicity. We found that the targeted disruption of the PSD-93 gene attenuated the neurotoxicity triggered by NMDAR activation, but not by non-NMDAR activation, in cultured mouse cortical neurons. PSD-93 deficiency reduced the amount of NMDAR subunits NR2A and NR2B in synaptosomal fractions from the cortical neurons and significantly prevented NMDA-stimulated increases in cyclic guanosine 3',5'-monophosphate and Ca^{2+} loading in the cortical neurons. These findings indicate that PSD-93 deficiency could block NMDAR-triggered neurotoxicity by disrupting the NMDAR- Ca^{2+} -NO signaling pathway and reducing expression of synaptic NR2A and NR2B. Since NMDARs, Ca^{2+} , and NO play a critical role during the development of brain trauma, seizures, and ischemia, the present work suggests that PSD-93 might contribute to molecular mechanisms of neuronal damage in these brain disorders.

Keywords

N-methyl-D-aspartate receptors; PSD-93; Ca^{2+} loading; Neuronal nitric oxide syntheses

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Introduction

PSD (postsynaptic density)-93, also known as chapsyn (channel-associated protein of synapses)-110, is one of a growing superfamily of PDZ-domain-containing proteins. It physically links proteins together into macromolecular structures. PSD-93 was found to have a structural similarity with three other PDZ-domain-containing proteins: PSD-95/synapse associated protein 90 (SAP90), SAP102, and SAP97. These proteins are generically referred to as membrane-associated guanylate kinases (MAGUKs) and contain multiple protein-protein interaction domains, including three PDZ domains, an src homology domain 3, and a guanylate kinase homology domain (Brenman et al., 1996; Tao and Johns, 2006; Tao and Raja, 2004). PDZ domains of MAGUKs are motifs of ~90-amino acid repeats that have been recognized to mediate protein-protein interactions. For example, the first and second PDZ domains of PSD-93 and PSD-95 bind to the C-termini of NMDAR subunits NR2A and NR2B as well as to neuronal nitric oxide synthase (nNOS). PSD-95 enhances NMDAR clustering at synapses and inhibits NR2B-mediated internalization (Roche et al., 2001). Co-expression of PSD-95 with NMDARs increases surface expression of NMDARs and enhances synaptic NMDAR function (Carroll and Zukin, 2002). Consistently, the deletion of PDZ domains from PSD-93 disrupts interaction between PSD-93 and NR2A, NR2B, and nNOS, reduces NMDAR clustering at cellular membranes, and diminishes NOS activity (Brenman et al., 1996). These findings suggest that MAGUK proteins are required for synaptic expression of NMDARs and NMDAR-triggered activation of downstream signals. Thus, MAGUK proteins might also participate in NMDAR-mediated pathological processes.

The role of MAGUK proteins in NMDAR-mediated neurotoxicity has been examined. Using PSD-95 antisense oligonucleotides, previous studies showed that suppressing the expression of PSD-95 protected against excitotoxicity evoked by NMDA in cultured cortical neurons (Sattler et al., 1999) and increased the number of surviving pyramidal neurons in the postischemic hippocampus (Hou et al., 2005). However, using the same PSD-95 antisense oligonucleotides, Gardoni *et al* (Gardoni et al., 2002) revealed that lack of PSD-95 drove neuronal cell death in both primary hippocampal cultures and organotypic hippocampal slices. It appears that the functions of MAGUK proteins in neurotoxicity are still not completely understood. In the present study, using a genetic knockout strategy, we examined whether and how targeted disruption of the PSD-93 gene affects NMDAR/NO-dependent neurotoxicity in cultured cortical neurons.

EXPERIMENTAL PROCEDURES

Animals

The PSD-93 knockout (KO) mice (C57BL/6 genetic background) were generated as described previously (Xu et al., 2004). Female PSD-93 KO mice and wild type (WT) littermates were obtained by interbreeding PSD-93 heterozygous mice. All animal experiments were carried out in accordance with the National Regulations of Experimental Animal Administration and with the approval of the Committee of Experimental Animal Administration at Nanjing University and Johns Hopkins University. The experimenter was blind to the genotype of the mice in all studies.

Primary neuronal culture

Primary cortical neurons were prepared from day-17 to -19 embryos of WT and PSD-93 KO mice as described (Xu et al., 2004). Cortices were dissected from the hippocampus and striatum, treated with trypsin, and plated at 4×10^4 , 0.5×10^4 , and 8×10^4 cell/ml on poly-D-lysine-coated 24-well plates, glass cover slips, and 10-cm dishes, respectively. Cells were grown in

Neurobasal medium supplemented with B27 supplement (Invitrogen, Carlsbad, CA) and 25 μ M glutamine at 37°C in a humidified incubator with 5% CO₂.

Drug exposure

On day 10 after plating, cultured neurons were exposed to NMDA at different doses (0, 10, 20, 30 40, and 60 μ M) for 60 min at room temperature in the presence of 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, an AMPA/kainate antagonist) and 2 μ M nimodipine (a Ca²⁺ channel antagonist) to prevent both activation of AMPARs and voltage-gated Ca²⁺ channels, respectively, and then incubated in Neurobasal medium for 24 h at 37°C. To examine whether NMDA-induced neurotoxicity was dependent on the NMDA receptor/NO signaling pathway, the cultured neurons were pre-incubated for 60 min with 10 μ M MK-801 (an NMDA antagonist; Tocris, Ellisville, MI) or 30-60 μ M *N*-Nitro-L-arginine methyl ester hydrochloride (L-NAME, a NOS inhibitor; Sigma, St. Louis, MO) at 37°C. To determine whether PSD-93 deletion affected non-NMDAR-mediated neurotoxicity, the cultured neurons were exposed to kainate at doses of 0, 30, 60, 100, and 300 μ M in the presence of 10 μ M MK-801 and 2 μ M nimodipine for 60 min at room temperature.

Plasmids and transient transfection

Small RNA (siRNA) Target Finder software (from Genescript Corporation, <http://www.genscript.com>) was used to design siRNA and construct small hairpin RNA against PSD93 (shRNA). shRNAs were synthesized and subsequently cloned into pCMV-U6 vector using BbsI and BglII (Genescript, USA). Five PSD93 siRNAs sequences (siP1–siP5) were designed to target mouse PSD93 open reading frame-ORF (GenBank NM_011807). The plasmid expressing siRNA against luciferase (pSiLuc) was used as a negative control. shRNA sequences were as follows:

siP1:

1F:
tttgcGGATATTACTCACTCTTATTCTCGAGAATAAGAGTGAGTAATATCC
GTTTTT

1R:
gatcAAAAACGGATATTACTCACTCTTATTCTCGAGAATAAGAGTGAGTA
ATATCCG

siP 2:

2F:tttgcCCCATGAAGGATCGAATCAATCTCGAGATTGATTGATTCGATCCTTCAT
GGGTTTTT

2R:
gatcAAAAACCCATGAAGGATCGAATCAATCTCGAGATTGATTGATTCGATCCT
TCATGGG

siP 3:

3F:
tttgcCCTGGTGTGATTGATTCCAAACTCGAGTTTGGGAATCAATCACACCAG
GTTTTT

3R:
gatcAAAAACCTGGTGTGATTGATTCCAAACTCGAGTTTGGGAATCAATCA
CACCAGG

siP 4:

4F:
tttgcGGAATAACGTAAAGAAGTATCTCGAGATACTTCTTTACGTTAGTCC
GTTTTT

4R:
gatcAAAAACGGACTAACGTAAAGAAGTATCTCGAGATACTTCTTTACGT
TAGTCCG

siP 5:

5F:
tttgcCAGTCATTCTCAGCACAGTACTCGAGTACTGTGCTGAGAATGACTG
GTTTTT

5R:
gatcAAAAACCAGTCATTCTCAGCACAGTACTCGAGTACTGTGCTGAGAA
TGACTGG

The oligonucleotides were synthesized by Biocolor BioScience & Technology Company (Invitrogen, US), and control siRNA(pCMV-U6/siLuc) was purchased from Genscript Corporation. siRNAs were transfected into neurons respectively using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. At 24h post transfection, the cells were harvested for RT-PCR and western blotting.

RT-PCR

Five siRNAs were transfected into neurons overnight respectively. Total RNA of the cells was extracted with a Trizol commercial kit (Invitrogen, USA). The cDNA was synthesized using a reverse transcriptase kit (Fermentas, USA). The oligonucleotide primer sequences were as follows: PSD-93: Forward: GCTGCACTCGGGTGCCTCTG Reverse: TTGCATCAGCACTGCCGGGG GAPDH: Forward: GCCAAGGCTGTGGGCAAGGT Reverse: TCTCCAGGCGGCACGTCAGA. Reaction cycle: 95°C for 5min, followed by 30 cycles of 95°C×40s, 58°C×40s, 72°C×40s and 72°C×10min. Reaction products were separated on an agarose gel with ethidium-bromide. The bands were visualized under UV illumination and photographed. GAPDH was as loading control.

Assessment of cell viability and cell death

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described (Xu and Tao, 2004). The cultured neurons were incubated in 0.5 mg/ml MTT at 37°C for 1.5 h and solubilized in a solution of anhydrous isopropanol, 0.1 N HCl, and 0.1% Triton X-100. The optical density was measured at 570 nm. To determine percentage of cell death, cells were stained with 6.25 µg/ml propidium iodide (PI) (a marker of cell death; Sigma) and 0.25 ng/ml calcein AM (a marker of living cells; Invitrogen Corporation, Carlsbad, CA) as described previously (McCollum et al., 2002; Xu and Tao, 2004; Xu et al., 2004). Fluorescent images were captured with a digital camera (TOSHIBA, Japan) on an Olympus microscope with Image Pro Plus v.3.0 software. PI- and calcein AM-positive cells were counted by an observer blinded to the genotype and experimental treatment. The percentage cell death was determined as: (number of PI-positive cells)/(total number of PI-positive + calcein AM-positive cells) × 100.

cGMP activity

After being treated with or without 30 µM NMDA for 1 h at room temperature, cultured neurons of WT and PSD-93 KO mice were incubated in Neurobasal medium for 24 h at 37°C. Neurons were then harvested, homogenized in 0.2 ml of cold 20% trichloroacetic acid, and centrifuged at 1,200 g for 5 min. The supernatant was collected, washed four times with water-saturated

diethyl ether, and neutralized with 1 N NaOH to pH 7.4 before cyclic guanosine 3',5'-monophosphate (cGMP) level was determined with a commercial radioimmunoassay (Amersham Biosciences, Arlington Heights, IL, USA) as described (Xu et al., 2004).

Measurement of Ca²⁺ loading

Neuronal cultures were challenged for 5 or 10 min with 30 μ M NMDA, 10 μ M CNQX, and 2 μ M nimodipine in a solution containing ⁴⁵CaCl₂ (0.85 μ Ci/well, 0.5 mM) (Czyz et al., 2002; Jiang et al., 2005). After being washed, the cultured neurons were lysed with 0.2% sodium dodecyl sulfate, and level of radioactivity was measured in a scintillation counter. In parallel cultures, neuronal morphology after Ca²⁺ loading was examined by labeling cultures on glass cover slips with the membrane tracer DiI (1,1'-dioctadecyl-3,3,3', 3'-tetramethylindocarbocyanine perchlorate).

Subcellular fractionation of proteins

Biochemical fractionation was carried out according to previous studies with minor modification (Liaw et al., 2008). Briefly, cultured neurons were homogenized in a homogenization buffer and centrifuged at 1,000 $\times$ g for 20 min at 4°C. The supernatant (total soluble fraction) was collected and centrifuged at 10,000 $\times$ g for 20 min at 4°C to produce a pellet. The pellet was lysed hypo-osmotically in water and centrifuged at 25,000 $\times$ g to produce the crude synaptosomal membrane fraction.

Western blot analysis

Equal amounts of protein from total soluble and synaptosomal membrane fractions were separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. Membranes were blocked with 3% non-fat dry milk for 1 h and incubated overnight at 4°C with rabbit anti-PSD-93 (1:1,000; Alomone Labs Ltd, Jerusalem, Israel), rabbit anti-NR2A (1:200, Upstate/Chemicon, Temecula, CA), rabbit anti-NR2B (1:200, Upstate/Chemicon), mouse anti-PSD-95 (1:1,000; Upstate/Chemicon), rabbit anti-N-cadherin (1:1,000; BD Biosciences, Palo Alto, CA), or monoclonal mouse anti- β -actin (1:10,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). N-cadherin was used as a loading control and marker for the crude synaptosomal fraction, and β -actin was used as a loading control for the total soluble fraction. The proteins were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies and visualized with chemiluminescence reagents provided with the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to film. The intensity of the blots was quantified with densitometry. The blot density from naïve animals was set as 100%.

Statistical analysis

Data were statistically assessed as mean $\pm$ SEM by analysis of variance. Intergroup differences were analyzed by the Newman-Keuls test. $P < 0.05$ was considered statistically significant. The statistical software package SigmaStat (Systat, Port Richard, CA) was used to perform all statistical analyses.

RESULTS

siRNA knockdown the mRNA level of PSD93 in neurons

To test the efficacy of siRNAs, five siRNAs (siP1 to siP5) were transfected into neurons. And mRNA and protein expression level of PSD93 were measured by RT-PCR and western blot. The results indicated that siP3 significantly decreased the mRNA of PSD93 by 80.2% of control (Fig.1A) and the protein of PSD93 by 79.5% compared to control (Fig.1B-C). We then used this siRNA for the further experiments.

Effect of PSD-93 deficiency or knockdown on NMDA-induced neurotoxicity

Consistent with previous reports (Aarts et al., 2002; Sattler et al., 1999), NMDA produced significant and dose-dependent neurotoxicity in cultured cortical neurons from WT mice. The MTT assay showed that NMDA at doses of 10, 20, 30, 40, and 60 μ M decreased the percentage of neuronal viability to 77.31% ($P = 0.15$), 46.81% ($P < 0.05$), 36.22% ($P < 0.05$), 31.66% ($P < 0.05$), and 37.22% ($P < 0.01$), of control levels, respectively (Fig. 2A). PI/calcein staining also showed that NMDA at these doses increased the percentage of neuronal death by 2.42-fold ($P = 0.07$), 3.19-fold ($P < 0.01$), 5.21-fold ($P < 0.01$), 5.37-fold ($P < 0.01$), and 6.11-fold ($P < 0.01$), respectively, compared to the control (Fig. 2B). Cultured neurons from PSD-93 KO mice were used to define the role of PSD-93 in NMDA-induced neuronal death. The Basal level of neuronal viability and neuronal death were similar in both PSD-93 KO and the WT neurons (Fig. 2). However, the neurons from PSD-93 KO mice showed increased viability caused by NMDA at doses of 20, 30, and 40 μ M by 1.60-fold ($P < 0.05$), 1.71-fold ($P < 0.05$), and 1.55-fold ($P < 0.05$), respectively, compared to the corresponding WT neurons, although neuronal viability in NMDA-treated PSD-93 KO neurons was lower than the basal levels of WT and PSD-93 KO neurons (Fig. 2A). The Percentage of neuronal death in PSD-93 KO neurons treated with 10, 20, 30, 40, and 60 μ M NMDA was reduced by 26.00% ($P < 0.05$), 32.84% ($P < 0.01$), 30.15% ($P < 0.05$), 26.03% ($P = 0.06$), and 5.07% ($P = 0.12$) of the corresponding WT levels, respectively, although the level remained higher compared with the basal levels of WT or PSD-93 KO neurons (Fig. 2B and 2C). To further determine the role of PSD-93 in neurotoxicity in mature cortical neurons, siRNA was used to knockdown PSD-93 expression in cultured neurons and the cells, then were treated with 30 μ M NMDA. Cell viability was measured by MTT. The results showed that the cell viability reduced by 44.12% when exposed to NMDA ($p < 0.01$). However, siP3 can alleviate this toxicity, the cell viability increase 17% ($P < 0.05$ vs siLuc) (Fig. 2D), which was similar with that of PSD93 KO.

The NMDAR antagonist MK-801 significantly reduced the NMDA-induced neurotoxicity, although MK-801 treatment alone did not affect the basal level of neuronal viability or neuronal death, in cultured neurons from WT mice (Fig. 4). However, MK-801 treatment did not significantly change the levels of NMDA-induced neurotoxicity in cultured neurons from PSD-93 KO mice (Fig. 3).

We further determined whether the protection against NMDA-induced neurotoxicity in PSD-93-deficient neurons was due to its role on NMDARs. Non-NMDAR toxicity was induced by kainic acid at different doses (30 to 300 μ M) in the presence of 10 μ M MK-801 and 2 μ M nimodipine. Consistent with previous studies (Kanada et al., 2005; Sattler et al., 1999), kainic acid led to a dose-dependent decrease in neuronal viability or a dose-dependent increase in the percentage of neuronal death in the cultured neurons from WT mice (Fig. 4). This kainate toxicity was unchanged in PSD-93-deficient neurons (Fig. 4).

Effect of PSD-93 deficiency on synaptic NR2A and NR2B expression

Western blot analysis showed that PSD-93 deficiency did not change the total protein levels of NR2A, NR2B, PSD-95 (Fig. 5), or nNOS (data not shown) in cultured cortical neurons, a finding consistent with those in previous studies (Liaw et al., 2008; McGee et al., 2001; Tao et al., 2003; Xu et al., 2004). However, the levels of NR2A and NR2B, but not of PSD-95, in the synaptosomal membrane fraction were markedly decreased in cultured cortical neurons of PSD-93 KO mice (Fig. 5). The amounts of NR2A and NR2B were reduced by 57.32% ($P < 0.01$) and by 49.19% ($P < 0.01$), respectively, compared to those in WT mice.

Effect of PSD-93 deficiency on NMDA-induced increase in Ca^{2+} loading

Excessive Ca^{2+} loading through NMDARs underlies the mechanisms of NMDAR-mediated neurotoxicity (Aarts and Tymianski, 2004; Arundine and Tymianski, 2003). Therefore, we

further examined whether PSD-93 deficiency affects NMDA-induced increases in Ca^{2+} loading. Ca^{2+} influx results from activation of NMDARs, AMPA/kainate and Ca^{2+} channel respectively. In order to address Ca^{2+} influx attributing to the activation of NMDARs by NMDA, 10 μM CNQX, an AMPA/kainate antagonist and 2 μM nimodipine, a Ca^{2+} channel antagonist are used to block AMPA and Ca^{2+} channel pathways (Sattler et al., 1999). The basal level of Ca^{2+} loading in cultured neurons from PSD-93 KO mice was similar to that from WT mice (Fig. 6). Consistent with previous studies (Lu et al., 1996), NMDA significantly increased Ca^{2+} loading by 2.24-fold ($P < 0.01$) of the corresponding control levels at 5 min and 1.49-fold ($P < 0.05$) at 10 min in cultured neurons from WT mice (Fig. 6). However, NMDA did not significantly increase the Ca^{2+} loading in cultured neurons from PSD-93 KO mice (Fig. 6), indicating that PSD-93 deficiency produces a resistance against Ca^{2+} loading induced by NMDAR activation.

Effect of PSD-93 deficiency on NMDA-induced increase in cGMP production

Consistent with previous studies (Dawson et al., 1996; Girouard et al., 2009; Liu et al., 2007), we found that NMDA-induced neurotoxicity was dependent on NOS activity. Pretreatment with L-NAME (a non-selective NOS inhibitor) at the doses of 30 and 60 μM significantly attenuated the NMDA-induced reduction in neuronal viability and the increase in percentage of neuronal death in the cultured neurons from WT mice (Fig. 7A). This result indicates that activated nNOS participates in NMDAR-triggered neurotoxicity.

PSD-93 presents nNOS to the NMDAR complex through its binding to NR2A, NR2B, and nNOS to allow prompt activation of nNOS by Ca^{2+} that enters through NMDARs in the cortical neurons (Brenman et al., 1996; Xu et al., 2004). This suggests that PSD-93 deficiency might affect the NMDAR-triggered increase in NOS activity as determined by NO-stimulated cGMP formation in the cortical neurons. The basal level of cGMP was not significantly different between WT and PSD-93 KO neurons (Fig. 7B). NMDA treatment of WT neurons increased the cGMP concentration to a level 3.45-fold greater than that in the untreated control group ($P < 0.01$; Fig. 7B). In contrast, cGMP concentrations in NMDA-treated neurons from PSD-93 KO mice were similar to the control levels from WT and PSD-93 KO mice ($P > 0.05$; Fig. 7B). The cGMP concentrations in NMDA-treated neurons from PSD-93 KO mice were 42.74% less than those in NMDA-treated neurons from WT mice ($P < 0.01$; Fig. 7B), indicating that NOS is not activated by NMDA stimulation in the absence of PSD-93.

DISCUSSION

The major new findings in the present study are: 1) NMDAR-mediated neurotoxicity is suppressed in cortical neurons lacking or knockdown of PSD-93; 2) PSD-93 deficiency decreases Ca^{2+} influx through NMDARs in the cortical neurons; 3) PSD-93 deletion prevents NMDA-stimulated increase in cGMP; and 4) PSD-93 deficiency reduces the expression of synaptic NR2A and NR2B. These findings suggest that PSD-93 deficiency could block NMDAR-triggered neurotoxicity by altering the NMDAR- Ca^{2+} -NO signaling pathway and the expression of synaptic NR2A and NR2B.

NMDAR-mediated excitotoxicity in neuronal damage after stroke has been well established, but the underlying mechanisms are still elusive (Liu et al., 2007; von Engelhardt et al., 2007). The best model to address this issue may be an *in vitro* neuronal culture that is challenged with NMDA, a specific NMDAR agonist. Consistent with previous studies (Aarts et al., 2002; Cui et al., 2007), we showed that NMDA caused neuronal death in a dose-dependent manner in cultured cortical neurons. This response was NMDAR-mediated, as MK-801, a selective NMDAR antagonist, significantly attenuated the NMDA-induced decrease in neuronal viability and the increase in percentage of neuronal death. In previous studies, use of a PSD-95 antisense oligonucleotide knockdown approach has led to mixed findings regarding the role

of PSD-95 in neurotoxicity (Gardoni et al., 2002; Hou et al., 2005; Sattler et al., 1999). We showed that both targeted disruption and knockdown of PSD-93 could weaken NMDA-induced neurotoxicity. Moreover, this protective effect was specific to NMDARs, as PSD-93 deficiency did not affect non-NMDAR-mediated neurotoxicity. Our findings support the functional roles of MAGUK proteins in the mechanisms of neurotoxicity triggered by NMDAR activation.

PSD-93 is a scaffolding protein that is required for expression and function of synaptic NMDARs. Deletion of PDZ domains of PSD-93 not only disrupted the interaction between PSD-93 and NR2A or NR2B but also reduced NMDAR clustering at cell membranes *in vitro* (Kim et al., 1996). Our quantitative Western blot analysis showed a significant decrease in the amounts of NR2A and NR2B proteins in the synaptosomal membrane fractions of PSD-93 KO neurons. This finding is consistent with our previous *in vivo* study, which revealed that targeted disruption of the PSD-93 gene reduced synaptic expression of NR2A and NR2B proteins in the adult forebrain cortex and spinal cord dorsal horn (Liaw et al., 2008; Tao et al., 2003). Since PSD-93 deficiency did not affect total amounts of NR2A and NR2B proteins in the cultured cortical neurons, it is very likely that NR2A and NR2B are distributed predominantly in cytoplasm and/or extrasynaptic membranes of cortical neurons in PSD-93 KO mice. PSD-93 might be important in synaptic NMDAR targeting and function. This conclusion is supported by our previous observation that targeted disruption of the PSD-93 gene resulted in impaired NMDAR-mediated excitatory postsynaptic functions in cortical neurons (Tao et al., 2003). Given that synaptic NMDAR expression is required for the induction of neurotoxicity (Hazell, 2007; Watanabe et al., 2003), the present results suggest that a reduction in synaptic expression of NR2A and NR2B is one possible molecular mechanism by which NMDAR-triggered neurotoxicity is prevented in the cortical neurons of PSD-93 KO mice.

An unexpected observation in this study was that PSD-93 deficiency reduced NMDAR-mediated Ca^{2+} loading in the cultured cortical neurons. NMDARs are slow gating channels that are highly permeable to Ca^{2+} . This permeability is governed by an asparagine residue (N598) in the channel-forming segment of the second transmembrane domain of NR1; however, substituting an asparagines at this site in NR2 subunits barely affects Ca^{2+} permeability (Burnashev et al., 1992). Since PSD-93 interacts directly with NR2A and NR2B but not with NR1, the mechanisms by which PSD-93 deletion alters NMDA-stimulated Ca^{2+} loading are still unclear. NR1 and NR2 are highly expressed in the cortical neurons and determine the cortical NMDAR currents (Lu et al., 1996; von Engelhardt et al., 2007). As discussed above, PSD-93 deficiency was shown to reduce NMDAR-mediated excitatory postsynaptic currents in cortical neurons (Tao et al., 2003). This suggests that PSD-93 deficiency might also affect synaptic expression of NR1, but this remains to be confirmed. It is well known that excessive influx of extracellular Ca^{2+} by NMDA stimulation leads to neurotoxicity (Mehta et al., 2007; Soriano et al., 2008). Thus, the reduction in NMDA-stimulated Ca^{2+} loading produced by PSD-93 deficiency might also contribute to the mechanism by which NMDAR-mediated neurotoxicity is attenuated in the cortical neurons of PSD-93 KO mice.

PSD-93 also assembles intracellular signaling proteins (e.g., nNOS) around NMDARs through PDZ domain and regulates NMDAR-dependent downstream signaling. We previously showed that PSD-93 co-localizes with NR2A and nNOS in the processes of cultured cortical neurons (Xu et al., 2004). PSD-93 also interacts with NR2A and nNOS, as PSD-93 antibody was able to immunoprecipitate both proteins in cultured cortical neurons (Xu et al., 2004). These findings indicate the formation of a ternary complex assembled by PSD-93 with NMDARs and nNOS in cortical neurons. This physical assembling may be important for NMDAR-

triggered nNOS activation because PSD-93 deficiency attenuated NMDA-stimulated increases in nNOS activity without affecting the total amount of nNOS protein in cortical neurons.

The use of siRNA to knockdown PSD93 further supports our conclusion that loss of PSD93 can be neuroprotective. Moreover, we found that synaptic expression of NR2A and NR2B, Ca²⁺ uptake through NMDARs and cGMP production were significantly reduced in PSD93 KO neurons. Indicating that nNOS activation is Ca²⁺ dependent, so less Ca²⁺ uptake would result in less nNOS activation. It's possible that PSD93 KO alters NMDAR localization and function in terms of Ca²⁺ handling.

It bears noting that PSD-93 deficiency did not completely abolish the NMDAR-mediated neurotoxicity. The cultured neurons from PSD-93 KO mice still displayed an NMDA-induced decrease in neuronal viability and an NMDA-stimulated increase in the percentage of neuronal death compared to the corresponding control groups from either WT or PSD-93 KO mice. It is known that in addition to PSD-93, two other MAGUK proteins, PSD-95 and SAP102, bind to NR2A and NR2B and are required for synaptic NMDAR expression (Elias et al., 2008; Sans et al., 2005). PSD-95 also interacts with nNOS (Cao et al., 2005; de Bartolomeis and Fiore, 2004). It is very likely that PSD-95 and SAP102 maintain partial synaptic NR2A and NR2B expression and some NMDAR/NO mice. In addition, the cortical culture might contain a few glial cells (< 10%) despite the fact that it is treated with cytosine- β -arabino-furanoside (a cell division inhibitor) (Xu et al., 2004). Because PSD-93 expression is undetectable in glial cells (Tao et al., 2003; Xu et al., 2004), PSD-93 deficiency might not affect NMDA-induced glial cell death. Thus, cell death in the culture from PSD-93 KO mice after NMDA exposure might include some glial cells.

In conclusion, this study demonstrates that targeted disruption of the PSD-93 gene reduces NMDAR-triggered neurotoxicity. PSD-93 deficiency also reduced synaptic expression of NR2A and NR2B and inhibited NMDA-stimulated nNOS activation and Ca²⁺ loading in cultured neurons. Since NMDARs, nNOS, and Ca²⁺ play critical roles in the development of brain injury, seizures, and ischemia (Aarts and Tymianski, 2004; Arundine and Tymianski, 2003), the present work suggests that PSD-93 might be required for the pathological mechanisms of neuronal damage during these brain disorders.

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Abbreviations

NMDAR	N-methyl-D-aspartate receptor
NO	nitric oxide
PSD	postsynaptic density
cGMP	cyclic guanosine 3',5'-monophosphate
MAGUKs	membrane-associated guanylate kinases
nNOS	nitric oxide synthase
KO	knockout

WT	wide-type
chapsyn	channel-associated protein of synapses
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
L-NAME	<i>N</i> -Nitro-L-arginine methyl ester hydrochloride

Reference List

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Fig. 1.

Efficacy of pCMV-U6-siRNA constructs. (A) RT-PCR analysis showed that a 495 bp product from PSD-93 was inhibited significantly by siP3, GAPDH mRNA was used as a loading Control. siP3 significantly decreased the mRNA of PSD93 by 80.2% of control. (B) Confirmation by western blot analysis of silencing PSD93 expression, The blots were reprobed with anti-GAPDH antibody to verify protein loading. (C) Relative protein level of PSD-93 after siRNA transfection, PSD93 decreased by 79.5% compared to control group when transfected with siP3.

Fig. 2.

PSD-93 deficiency or knockdown protects against NMDA-stimulated neurotoxicity. Cortical neurons were cultured from WT and PSD-93 KO mice and treated with NMDA (0, 10, 20, 30, 40, and 60 μM) in the presence of 10 μM CNQX and 2 μM nimodipine. (A) Neuronal viability was assessed by MTT assay. (B) Percentage of neuronal death was determined by propidium iodide and calcein AM staining. * $P < 0.05$, ** $P < 0.01$ vs corresponding WT. $n = 6$ repeats. (C) Representative photographs showing propidium iodide and calcein AM staining of cortical neurons cultured from WT and PSD-93 KO mice treated with or without 30 μM NMDA or 10 μM MK-801 + 30 μM NMDA. Scale bar: 60 μm. (D) acute knock down PSD-93 by siP3 can inhibit the toxicity induced by NMDA at the dose of 30μM. MTT assay shows the cell viability reduced by 44.12% ** $P < 0.01$ vs normal group. However, siP3 can alleviate this toxicity, the cell viability increase 17%, # $P < 0.05$ vs group transfected by siLuc.

Fig. 3. MK-801 attenuates NMDA-induced neurotoxicity in cultured cortical neurons from WT but not from PSD-93 KO mice. Percentage of neuronal death was determined by propidium iodide and calcein AM staining (top) and neuronal viability by MTT assay (bottom). * $P < 0.05$ vs the corresponding NMDA concentration alone. $n = 6$ repeats.

Fig. 4. PSD-93 deficiency has no effect on non-NMDA receptor-triggered neurotoxicity in cultured cortical neurons. Cultured neurons were treated with kainate at the doses shown in the presence of 10 μM MK-801 and 2 μM nimodipine. Percentage of neuronal death was determined by propidium iodide and calcein AM staining (top) and neuronal viability by MTT assay (bottom). $n = 6$ repeats.

Fig. 5. PSD-93 deficiency significantly reduces synaptic expression of NR2A and NR2B in cultured cortical neurons. (A) Representative Western blots showing the levels of PSD-93, PSD-95, NR2A, and NR2B in the total soluble and synaptosomal fractions. (B) Statistical summary of the densitometric analysis expressed relative to WT mice after normalization to corresponding β -actin or N-cadherin. ** $P < 0.01$ vs WT mice. $n = 3$ repeats.

Fig. 6. PSD-93 deficiency attenuates NMDA-stimulated Ca^{2+} loading in cultured cortical neurons. Cultures were challenged for 5 or 10 min with 30 μM NMDA, 10 μM CNQX, and 2 μM nimodipine in medium containing $^{45}\text{CaCl}_2$. CMP: counts per minute. * $P < 0.05$ vs corresponding control. $n = 6$ repeats.

Fig. 7.

PSD-93 deficiency decreases NMDA-stimulated increase in cGMP in cultured cortical neurons. (A) NMDA-induced neurotoxicity was NOS-dependent in cultured cortical neurons. Cultured neurons were treated with NMDA (30 μ M or 60 μ M) with or without L-NAME (10 μ M or 30 μ M). Neuronal viability was determined by MTT assay (top) and percentage of neuronal death by propidium iodide and calcein AM staining (bottom). * $P < 0.05$ vs NMDA alone. $n = 6$ repeats. (B) Cultured neurons from WT and PSD-93 KO mice were exposed to 30 μ M NMDA. PSD-93 deficiency decreased the NMDA-stimulated increase in cGMP level in cultured cortical neurons. * $P < 0.05$ vs the corresponding NMDA-treated group from WT mice. $n = 6$ repeats.