Neonatal Hypoxia-Ischemia Differentially Upregulates MAGUKs and Associated Proteins in PSD-93–Deficient Mouse Brain

Xiangning Jiang, MD, PhD; Dezhi Mu, MD, PhD; R. Ann Sheldon, MA; David V. Glidden, PhD; Donna M. Ferriero, MD

Background and Purpose—Postsynaptic density (PSD)-93 and PSD-95 are the major membrane-associated guanylate kinases (MAGUKs) at excitatory synapses of the brain linking the N-methyl-d-aspartate receptor (NMDAR) with neuronal nitric oxide synthase (nNOS), which contributes to cell death after neonatal hypoxia-ischemia (HI). We investigated whether deletion of PSD-93 would dissociate the NMDAR from nNOS and be neuroprotective.

Methods—Postnatal day 7 wild-type (+/+), heterozygous (+/−), and homozygous (−/−) PSD-93 knockout mice were subjected to HI by permanent ligation of the right carotid artery, followed by exposure to 8% O2/92% N2 for 1 hour. Brains were scored 5 days later for damage with cresyl violet and iron stains. Western blot and communoprecipitation were used to determine the expression and association of the major PSD proteins.

Results—There was no significant difference between PSD-93 (−/−) and (+/+), (+/-) mice in mortality or degree of brain injury. In the absence of PSD-93, PSD-95 still interacted with NR2B and nNOS. Under physiological conditions, PSD-95, nNOS, NR2A, and NR2B were unaltered in the (−/−) pups. However, at 24 hours after HI, protein expression of PSD-95, nNOS, and NR2A but not NR2B was markedly higher in the (−/−) than in the (+/+) pups. In (+/+) pups, HI resulted in decreased expression of NR2A but not NR2B in cortex and decreased NR2A and NR2B expression in hippocampus, but this reduction was not observed in (−/−) pups.

Conclusions—PSD-93 is not essential for baseline synaptic function but may participate in regulation of NMDAR-associated signaling pathways after HI injury. Deletion of PSD-93 alone does not provide neuroprotection after neonatal HI, possibly a result, in part, of upregulation of PSD-95. MAGUKs may substitute for another, allowing normal NMDAR function in the postnatal period. (Stroke. 2003;34:2958-2963.)

Key Words: brain injuries growth and development mice receptors, N-methyl-d-aspartate

One of the major causes of hypoxic-ischemic (HI) injury to the newborn brain is neonatal stroke, which occurs in 1/4000 live births, an incidence equal to that of stroke in the elderly.1 Evidence from experimental models and clinical investigation indicates that excitotoxicity mediated by N-methyl-d-aspartate receptor (NMDA)-type glutamate receptors is a trigger for neonatal stroke. NMDA receptors (NMDARs) are heteromeric complexes of the NR1, NR2 (A through D), and NR3 subunits. The NR1 subunit is essential for functional NMDAR channels, whereas 4 members of the NR2 subunits potentiate channel activity and modulate functional properties. Dynamic changes in temporal and spatial expression patterns exist during development; NR1-NR2B receptors predominate in the neonatal brain, whereas NR1-NR2A receptors predominate at more mature synapses.

NMDARs are anchored in the postsynaptic densities (PSD) by interactions between their NR2 subunits and the PSD-95/

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cortical cultures. Recent pursuits of therapy aimed against NMDAR–PSD-95 protein interactions have led to promising treatment for stroke in adult animal models. Despite these exciting findings, evidence is lacking regarding mechanisms of HI-induced structural reorganization of immature synaptic networks and how this disruption might contribute to neuronal cell death.

We have previously shown that pharmacological elimination of neurons containing nNOS in the neonatal rat results in protection against HI and that neonatal mice with targeted disruption of the nNOS gene are less vulnerable to HI brain injury. Based on these findings, we used PSD-93 knockout mice in this study to test whether partially dissociating NMDAR from nNOS would provide neuroprotection to the neonatal brain after HI injury. Studies in PSD-93 knockout mice have revealed that neuronal MAGUKs are not required for development or function of parallel fiber synapses in cerebellum, but the effect of PSD-93 deficiency during pathological stimuli, such as ischemia, has not been investigated. In addition to examining the contribution of PSD-93 in the susceptibility of neonatal brain to HI damage, we also studied whether PSD-93 elimination affects protein expression of other major PSD components, including NR2A, NR2B, PSD-95, and nNOS, under physiological conditions and after HI.

Materials and Methods

Animals
All animal research was approved by the University of California San Francisco Committee on Animal Research and was performed with the highest standards of National Institutes of Health guidelines. C57/b6 heterozygous (+/−) PSD-93 knockout mice were derived from the founder stock described by McGee and colleagues. These mice were bred with each other for 5 generations to generate the wild-type (+/+), 31; (−/−), 26% (+/−), and (+/−) states.12 Homozygous (+/−) and PS-93 knockout mice. The genotypes of all mice were confirmed by polymerase chain reaction. The PSD-93 (−/−) mice have normal phenotype. PSD-93 protein levels in the (+/+) mice have normal phenotype. PSD-93 protein levels in the (−/−) mice are 50% of those in the (+/+) and are absent in the (−/−) state.2

Induction of HI
A total of 70 animals (+/+, 19; (+/−), 31; (−/−), 20) were used for the HI procedure. We adapted the Vannucci model for neonatal HI at postnatal day 7 (P7) as previously described. A duration of 60 minutes of hypoxia was chosen to produce the maximal degree of brain injury with the least amount of mortality. C57/b6 strain is immediately susceptible to brain damage between outbred (CD1) and inbred (129Sv) strains. Sham-operated controls received halothane anesthesia and exposure of the right common carotid artery without coagulation and hypoxia.

Mortality data include animals that died during the hypoxic period or that survived the hypoxia but died at some point before being euthanized 5 days later.

Brain Injury Score
Five days after HI, animals were anesthetized with pentobarbital (50 mg/kg) and perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer. Brains were removed, postfixed for 4 hours, and transferred to 30% sucrose in 0.1 mol/L phosphate buffer. Coronal sections were cut at 30-µm intervals with a Vibratome (Ted Pella, Inc). Alternate sections were stained with cresyl violet for morphology or with Perl’s stain to localize iron deposition.

Brains were scored in a blinded fashion by 2 observers for degree of damage with the stains as described. The score for each region was summarized for a final score ranging from 0 to 24.

Western Blot Analysis
In separate experiments, 3 animals per genotype were used for Western blot at 24 hours after HI. Because injury evolves over a long time period after HI, changes in protein expression can be multimodal. We chose this time point to determine changes in NMDAR signaling but not modifications like phosphorylation, which would be done earlier. Homogenates of the ipsilateral and contralateral cortex and hippocampus from both HI-injured and sham-operated animals were prepared in RIPA buffer (1% phosphate buffer solution, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing protease inhibitor cocktail solution (Complete Mini, Roche).

An equal amount of protein samples (10 µg) was separated with 4% to 12% sodium dodecyl sulfate-polycrylamide gel electrophoresis (Invitrogen) and blotted to polyvinylidene difluoride membranes (Bio-Rad) as described. The blots were probed with the following 5 antibodies or antisera overnight at 4°C: mouse monoclonal nNOS antibody (1:1000, BD Transduction Laboratory), rabbit polyclonal NR2B or NR2A antibody (1:1000, Chemicon), and sheep PSD-95 and goat PSD-93 antisera (1:200, gifts from Dr David Bredt, University of California San Francisco). Mouse β-actin antibody (Sigma) was used as loading control. An internal control sample was run on each gel for normalization. Appropriate secondary horseradish peroxidase–conjugated antibodies (1:2500, Santa Cruz) were used, and signal was visualized with ECL (Amersham). NIH Image was used to measure the optical densities and areas of protein signal on radiographic films after scanning.

Coimmunoprecipitation
Cortical homogenates of sham-operated PSD-93 (+/+, +/−), and (−/−) mice (n=3 per genotype) were used for coimmunoprecipitation (Co-IP) experiments. Co-IP was performed with the Catch and Release Immunoprecipitation System (Upstate). An equal amount of protein (500 µg) was incubated with 4 µg rabbit polyclonal nNOS antibody (BD Transduction Laboratory) or 1.5 µg rabbit polyclonal NR2B antibody and 1 µg Antibody Capture Affinity Ligand for 15 minutes at room temperature and then transferred onto spin columns. After centrifugation for 6 minutes at 1500g and washing, 35 µL of 2% IP elution buffer was added onto the spin columns to elute the immune complexes. An equal volume of eluate was loaded onto 4% to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and blotted to polyvinylidene difluoride membrane, and blots were reacted with the same 5 primary antibodies or antisera used in Western blot analysis. Immunoreactive proteins were detected with ECL.

Statistical Analysis
Data are presented as median and interquartile range for brain injury score using Statview 5.0 nonparametric tests for analysis of variance (ANOVA; Kruskal-Wallis). Contingency tables were used to determine mortality differences. Data of optical densities of immunoblots are presented as mean±SD and were evaluated statistically using ANOVA with Bonferroni/Dunn posthoc tests. Differences were considered significant at P<0.05.

Results
Deficiency in PSD-93 Does Not Alter Mortality or Severity of HI Injury in the Immature Brain
To investigate the contribution of PSD-93 in the susceptibility of neonatal brain to HI damage, we initially examined the effect of PSD-93 deficiency on mortality and degree of brain injury after HI. Mortality was 32% (+/+), 26% (+/−), and...
15% (−/−), with no significant difference between (+/+), (+/−), or (−/−) mice (P=0.1406; the Table).

There was no difference in degree of brain injury between (+/+), (+/−), or (−/−) mice (P=0.598; the Table and Figure 1) as a composite score or when evaluated by regions (cortex, hippocampus, and striatum).

Deficiency in PSD-93 Does Not Alter the Expression of PSD-95 and Associated Proteins Under Physiological Conditions

We next determined the effect of deficiency in PSD-93 on the expression of PSD-95, another major component of MAGUK family and its associated proteins. In sham-operated animals, Western blot revealed that PSD-95 expression was unaltered in both cortex and hippocampus of the (−/−) mice (Figure 2). This result was consistent with the previous report that levels of other neuronal MAGUKs, PSD-95, SAP-97, and SAP-102, all occur at normal levels in the brains of the PSD-93 (−/−) mice. Likewise, the expression of other components of the NMDAR–PSD-95/93–nNOS signaling pathway; NR2A, NR2B, and nNOS, was also unchanged in the PSD-93 (−/−) mice under physiological conditions (Figures 2 and 3).

Figure 1. Effect of PSD-93 elimination on HI injury in the immature brain. Wild-type (+/+); heterozygous (+/−), and homozygous (−/−) PSD-93 knockout mice were used for the HI procedure at P7. Data are represented by box and whisker plot of composite brain injury scores (Kruskal-Wallis test). For each column, horizontal line represents the median score; space within the box, scores in the 25th to 75th percentiles; and vertical lines extending from the box, range of scores.

Table: Effect of PSD-93 Deficiency on Mortality and Degree of Brain Injury After HI

<table>
<thead>
<tr>
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<th>No.</th>
<th>Mortality, %</th>
<th>Injury Score</th>
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<tbody>
<tr>
<td>+/+</td>
<td>19</td>
<td>32</td>
<td>9</td>
</tr>
<tr>
<td>+/−</td>
<td>31</td>
<td>26</td>
<td>9</td>
</tr>
<tr>
<td>−/−</td>
<td>20</td>
<td>15</td>
<td>7</td>
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−/− vs +/+: P=0.1406 (mortality); P=0.598 (injury score).

Figure 2. Effect of neonatal HI on expression of PSD-95 and nNOS in PSD-93 (−/−) mice at 24 hours after HI by Western blot. Expression for the contralateral side is not shown because levels were similar to those in sham-operated (sham) animals. Primary antibodies used are indicated on the left of the blots. Relative optical densities (OD) of the blots normalized to an internal control is shown on the right. Data are mean±SD from 3 independent experiments. *P<0.05 vs (+/+). C indicates cortex; H, hippocampus.

PSD-95 Interacts With NR2B and nNOS in PSD-93 (−/−) Mice

To investigate whether, in the absence of PSD-93, PSD-95 could still associate with NMDAR and nNOS, we performed Co-IP experiments from cortex of sham-operated PSD-93 (+/+), (+/−), and (−/−) mice. Solubilized brain extracts were immunoprecipitated with an antibody specific for nNOS or NR2B, and immunoblots were probed with antibodies for NR2A, NR2B, PSD-95, and nNOS (Figure 4). As expected, deletion of PSD-93 successfully eliminated the association of NR2B with PSD-93 and nNOS with PSD-93. However, PSD-95 and nNOS specifically coimmunoprecipitated with NR2B; PSD-95, NR2A, and NR2B specifically coimmunoprecipitated with nNOS in PSD-93 (−/−) mice.
Different Responses of PSD-93 (−/−) Mice to HI in the Immature Brain

To explore the mechanisms responsible for the unaltered susceptibility of PSD-93 (−/−) mice to HI damage, we examined the protein expression of PSD-95 and other major PSD proteins at 24 hours after HI. Unlike the unchanged levels of PSD-95 and its associated proteins in (−/−) mice under physiological conditions, PSD-93 (−/−) mice showed different responses to HI than the (+/+) mice with significantly higher expression of PSD-95 and nNOS in both cortex and hippocampus in (−/−) than that in (+/+) (P<0.05), implying an upregulation of PSD-95 after HI in (−/−) mice (Figure 2). Additionally, posts ischemic responses of NR2A and NR2B differed in PSD-93 (−/−) and (+/+) mice; the expression of NR2A but not NR2B was significantly higher in (−/−) than in (+/+) mice in both ipsilateral cortex and hippocampus. In (+/+) mice, there was a 46% decrease in NR2A (P<0.05) with no change in NR2B in cortex and a 73% decrease in NR2A (P<0.05) and a 44% decrease in NR2B (P<0.05) in hippocampus compared with sham-operated animals by 24 hours of reperfusion. In contrast, these reductions were not observed in (−/−) brains (Figure 3).

Discussion

This is the first report of the expression of MAGUK proteins under physiological conditions and after HI in neonatal murine brain. Our data show that deletion of PSD-93 does not alter the mortality or attenuate brain damage after HI. The possible mechanisms could be that another MAGUK protein, PSD-95, interacts with the NR2B and nNOS in PSD-93–mutant mice and that upregulated PSD-95 after HI may have compensated for the deficient PSD-93, allowing normal NMDAR function in the postnatal period.

The PSD-93 gene was first identified in 1996 as a member of MAGUKs that binds directly to NMDAR and Shaker K+ channel subunits, as well as to nNOS. The PSD-93 protein is 71% identical to PSD-95 at the amino acid level and shows an identical domain organization, with 3 N-terminal PDZ domains, an SH3 domain, and a C-terminal region homologous to guanylate kinases. The temporal patterns of developmental change of PSD-93 and PSD-95 at rat hippocampal synapses are similar, with low levels of expression beginning at P10 and increasing throughout development. Overlapping postsynaptic distribution in forebrain and function allows PSD-93 to heteromultimerize with PSD-95. Both MAGUKs are recruited into the same NMDAR clusters for clustering of receptors and associated signaling proteins. In the absence of PSD-93, multiple channel binding sites per PSD-95 monomer are required for ion channel clustering, and only homomeric PSD-95 is recruited into NMDAR-associated complexes. For either K+ channel or NMDAR subunits, there appears to be no significant difference in the
relative clustering efficiencies of the homomeric PSD-93 or PSD-95 versus the heteromeric PSD-95/93 configurations. This might explain our observation that under physiological conditions the expression of PSD-95, NR2A, NR2B, and nNOS occurs at normal levels in PSD-93 (−/−) mice. This fact is generally consistent with previous studies using the same PSD-93 (−/−) mice or mice with mutant PSD-95 protein and other in vitro studies.6,8,12

However, at 24 hours after HI injury, there are significantly higher levels of PSD-95, nNOS, and NR2A expression in PSD-93 (−/−) than in (+/+ ) mice. It is unclear why (−/−) mice respond differently to HI insult than the (+/+ ) mice and why this difference does not confer different susceptibility to HI in the immature brain. Evidence from adult animal studies has shown that transient global ischemia followed by reperfusion induces modifications of the network of PSD proteins that are linked to PSD-95. Therefore, PSD-95 may have an important role in regulating receptor function and signal transduction contributing to ischemic neuronal cell death.7 The reported beneficial effect of PSD-95 suppression in stroke treatment6,9 suggests that PSD-95 might be an important mediator of neuronal cell death after ischemic brain injury in the mature brain. However, contradictory evidence from studies in heterologous cells indicates that PSD-95 may play a protective role against neuronal excitotoxicity by decreasing glutamate sensitivity of NMDAR.18 Whether PSD-93 and PSD-95 have different functions in vivo remains uncertain. If distinct channel binding specificities exist in vivo, heteromerization would increase the variety of ion channels in a cluster and would regulate the heterogeneity and composition of PSD protein clusters.15

In our study, we found differential effects of HI on subunit expression of the NMDAR. In (+/+ ) mice, there was a 46% decrease in NR2A (P<0.05) with no change in NR2B in cortex and a 73% decrease in NR2A (P<0.05) and a 44% decrease in NR2B (P<0.05) in hippocampus compared with sham-operated controls by 24 hours of reperfusion. This finding is in accord with previous work with the same HI model, showing a 75% to 80% decrease in NR2A but not NR2B expression in P7 rat forebrains after 24 hours of recovery.10 Decreased expression and functionality of both NR2A and NR2B were also observed in adult rat hippocampus after transient cerebral ischemia.20 In PSD-93 (−/−) mice, the expression of NR2A but not NR2B was significantly higher than in (+/+ ) mice in both ipsilateral cortex and hippocampus. It is possible that PSD-93/95 prefers association with NR2A rather than NR2B, with expression of NR2A more commonly regulated by PSD-93/95. This hypothesis is supported by the coincident expression of NR2A during development with that of PSD-93 and PSD-95.16 In addition, recent studies demonstrate that PSD-95 overexpression in developing rat cerebellar granule neurons allows synaptic insertion of NR2A and depressing NR2B expression.21 After HI, the preferential and dramatic decrease in NR2A in (+/+ ) mice would result in the NMDAR composition in favor of NR2B-containing receptors. In contrast, in (−/−) mice, NMDAR is composed primarily of NR2A or has no change in 2A/2B proportions. NMDAR containing the NR2A subunit display faster kinetics and greater peak open probability and might be preferentially targeted to synaptic sites, whereas NR2B-containing receptors display slower kinetics and less peak open probability and are preferentially inserted at extrasynaptic sites.22 The different effects of HI on NMDAR subunit composition in PSD-93 (−/−) mice might play an important role in modulating their response to ischemic challenge during development. It remains to be investigated whether this difference could be related to the sensitivity to excitotoxic cell damage and the pathogenic events leading to neuronal death after an HI challenge.

In summary, this is the first demonstration of the contribution of the MAGUK family to neonatal HI brain injury and neuronal cell death. Deletion of PSD-93 does not affect normal NMDAR expression; instead, PSD-93 may regulate the NMDAR-associated signaling network after HI in the neonatal brain.

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References


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