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Decreased levels of pNR1 S897 protein in the cortex of neonatal Sprague Dawley rats with hypoxic-ischemic or NMDA-induced brain damage

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Abstract

Our objective was to investigate the protein level of phosphorylated N-methyl-D-aspartate (NMDA) receptor-1 at serine 897 (pNR1 S897) in both NMDA-induced brain damage and hypoxic-ischemic brain damage (HIBD), and to obtain further evidence that HIBD in the cortex is related to NMDA toxicity due to a change of the pNR1 S897 protein level. At postnatal day 7, male and female Sprague-Dawley rats (13.12 ± 0.34 g) were randomly divided into normal control, phosphate-buffered saline (PBS) cerebral microinjection, HIBD, and NMDA cerebral microinjection groups. Immunofluorescence and Western blot (N = 10 rats per group) were used to examine the protein level of pNR1 S897. Immunofluorescence showed that control and PBS groups exhibited significant neuronal cytoplasmic staining for pNR1 S897 in the cortex. Both HIBD and NMDA-induced brain damage markedly decreased pNR1 S897 staining in the ipsilateral cortex, but not in the contralateral cortex. Western blot analysis showed that at 2 and 24 h after HIBD, the protein level of pNR1 S897 was not affected in the contralateral cortex ($P > 0.05$), whereas it was reduced in the ipsilateral cortex ($P < 0.05$). At 2 h after NMDA injection, the protein level of pNR1 S897 in the contralateral cortex was also not affected ($P > 0.05$). The levels in the ipsilateral cortex were decreased, but the change was not significant ($P > 0.05$). The similar reduction in the protein level of pNR1 S897 following both HIBD and NMDA-induced brain damage suggests that HIBD is to some extent related to NMDA toxicity possibly through NR1 phosphorylation of serine 897.

Key words: N-methyl-D-aspartate receptor 1; Phosphorylation; Neonate; Hypoxic-ischemic brain damage; Cerebral microinjection; Brain damage

Introduction

The neonatal brain is vulnerable to hypoxia-ischemia (HI), which may cause excitotoxic cell death mediated by N-methyl-D-aspartate (NMDA) receptors (1). Animal studies have demonstrated that overactivation of NMDA receptors (NR) is the primary step leading to neuronal injury after HI insults (2). There are seven NR subunits: one NR1, four NR2 (A to D), and two NR3 (A and B) (3). The functional NR channel must contain at least one NR1 subunit, which has several variants generated by alternate splicing of the C-terminus (4). NR phosphorylation is linked to oxidative stress (5) and plays a central role in the regulation of the function and cellular distribution of NRs (6). NR1 phosphorylation by cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) occurs at serine 897 (pNR1 S897), as shown by site-specific mutagenesis and phosphorylation site-specific antibodies. In addition, PKA can directly phosphorylate NR1 *in vitro* in transfected cells and in hippocampal slices (7).

During HI injury, NR overexpression in the developing brain of neonates may induce excitotoxicity and is a major trigger of neuronal death (8). In rats of different ages, HI influences pNR1 S897 expression differently, possibly explaining the age-related sensitivity to HI (9). It has been shown that abolishing NR1 S897 phosphorylation leads to glutamatergic hypofunction, which may underlie behavioral deficits in psychiatric disorders (10). Our preliminary experiments showed decreased protein levels of pNR897 in the cortex of Sprague-Dawley rat pups, a result that is also included in the present paper.

Since it is unclear whether the decreased expression

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of pNR1 after a neonatal HI insult is related to NMDA excitotoxicity, we decided to conduct further experiments looking at the change of pNR897 protein level in the cortex by establishing NMDA-induced brain damage. The objective was to investigate the protein level of pNR1 S897 in both NMDA-induced brain damage and hypoxic-ischemic brain damage (HIBD), in order to explore whether HIBD in the cortex is related to NMDA toxicity through NR1 S897 phosphorylation. We established a neonatal rat pup model of NMDA-induced brain damage using cerebral NMDA microinjection. The protein level of pNR1 S897 was compared in these two situations using immunofluorescence and Western blot.

Material and Methods

The present study was approved by the Medical Ethics Committee of the Third Xiangya Hospital and carried out according to the guidelines for animal experimentation at Central South University.

Animals

Postnatal day 7 (P7) Sprague Dawley rat pups (mean weight: 13.12 ± 0.34 g) were randomly divided into four groups: normal control group (N = 20), phosphate-buffered saline (PBS) cerebral microinjection group (N = 20), HIBD group (subdivided into 2- and 24-h subgroups, total N = 40), and NMDA cerebral microinjection group (N = 20). Half of the animals in each group were used for immunofluorescence analysis, and the other half for Western blotting.

Neonatal rat pup model of HIBD

The P7 neonatal rat pup model of HIBD was established as previously described (11). Briefly, the right common carotid artery was permanently ligated under isoflurane inhalation anesthesia. After resting with its dam for 2 h at room temperature, the pup was subjected to humidified 8% O_2/92% N_2 hypoxia in an air-tight chamber at 34°C for 2 h (10 pups/time). The end of hypoxia was considered to be 0 h after HI. Animals were euthanized 2 or 24 h after HI. This technique has been successfully used in our laboratory for years in a series of HIBD studies (12,13). The time points of 2 and 24 h after HI and 2 h after cerebral NMDA microinjection were chosen based on preliminary experiments when the immunohistochemical staining changes showed the most significant changes.

Cerebral microinjection

P7 neonatal rat pups were anesthetized by hypothermia. In the NMDA group, NMDA was prepared at a final concentration of 10 nM in 0.5 µL PBS, pH 7.4, and microinjected into the cortex using Hamilton syringes at a rate of 0.1 µL/20 s. A Cunningham mouse and neonatal rat stereotaxic adaptor (Stoelting Co., USA) was used to keep the animal's head in the correct position. The injection site was 3 mm rostral to the bregma, 3 mm right lateral to the sagittal fissure, and 1 mm below the skull surface. For the PBS group, the same volume of PBS was injected by the same method. After injection, the rats were kept warm and allowed to recover and euthanized 2 h after injection. The cortex was chosen as the target brain region mainly because, based on our preliminary experiments, the immunohistochemical staining of pNR897 in the cortex after HI was the most homogenous and the cerebral microinjection of NMDA into the cortex was technically easier and mechanically less traumatic for the rat pups than other brain regions.

Brain section preparation

P7 neonatal rat pups were sacrificed by transcardial perfusion with 4% paraformaldehyde in PBS. After cryoprotection in 30% sucrose in PBS, the brains were cryosectioned into 10-µm thick coronal sections and collected on chrome-album-coated slides and air-dried.

Immunofluorescence

The brain sections were first blocked with 3% BSA, 0.1% Triton-X, and 15% normal goat serum in PBS for 1 h at room temperature, stained with rabbit anti-pNR1 S897 primary antibody (1:500; Upstate Biotechnology, USA) at 4°C overnight, and then stained with fluorescein isothyocyanate-labeled goat anti-rabbit IgG (1:200, Pharmingen, USA) for 1 h at room temperature. Images were analyzed using an image analyzer (Microbrightfield, USA) connected to a fluorescence microscope (Leica DMRB, Germany), loaded into the Image Processing System, and examined using the Stereo investigator 3.0 software.

Western blot

Brain tissues in the right (ipsilateral) and left cortex were removed separately, placed immediately in liquid nitrogen, and homogenized in 50 mM Tris buffer, pH 7.4. The homogenate was centrifuged twice at 10,000 *g* for 10 min at 4°C. The supernatant was discarded and the protein concentration of the homogenate was measured using a bicinchoninic acid kit (Pierce, USA). Equal amounts of protein (60 µg) were fractionated by 5% (w/v) SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, first stained with rabbit anti-pNR1 S897 primary antibody (1:500) overnight at 4°C, and then stained with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, USA). The blots were developed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, USA) and band intensity was quantified using densitometric scanning analyses. β-actin was used as an internal control.

Statistical analysis

Data were analyzed using the SPSS13.0 software. Groups were compared by one-way analysis of variance. The *post hoc* test was used to keep the experiment error

rate at acceptable levels. Data was analyzed at the Department of Medical Statistics, Public Health Institute of Central South University.

Results

Protein level of pNR1 S897 determined by immunofluorescence staining

We first examined how HIBD or NMDA-induced brain damage affected the protein level of pNR1 S897 by using immunofluorescence staining. Both the control group (Figure 1a) and PBS group (Figure 1b) showed significant cytoplasmic staining of pNR1 S897 in the cortex. No staining was observed when the primary antibody was not added (Figure 1c). After HIBD, pNR1 S897 staining in the contralateral cortex remained the same (Figure 1d: 2 h, and 1e: 24 h), whereas the staining in the ipsilateral cortex was markedly decreased (Figure 1g: 2 h, and 1h: 24 h). Interestingly, the staining of pNR1 S897 under NMDA-induced brain damage showed a similar pattern. Specifically, at 2 h after 10 nM NMDA injection, pNR1 S897 staining was unchanged in the contralateral cortex (Figure 1f), but was significantly decreased in the cortex of the ipsilateral hemisphere (Figure 1i).

Protein level of pNR1 S897 determined by Western blot

We further confirmed our observation of the protein level of pNR1 S897 by using Western blot analysis (Figure 2). Our results showed that at 2 and 24 h after HIBD the protein level of pNR1 S897 in the contralateral cortex was not affected ($P > 0.05$), whereas the level in the ipsilateral cortex was reduced ($P < 0.05$). There was no significant difference between the 2- and 24-h subgroups ($P > 0.05$). At 2 h after 10 nM NMDA injection, the protein level of pNR1 S897 in the contralateral cortex was not affected ($P > 0.05$).

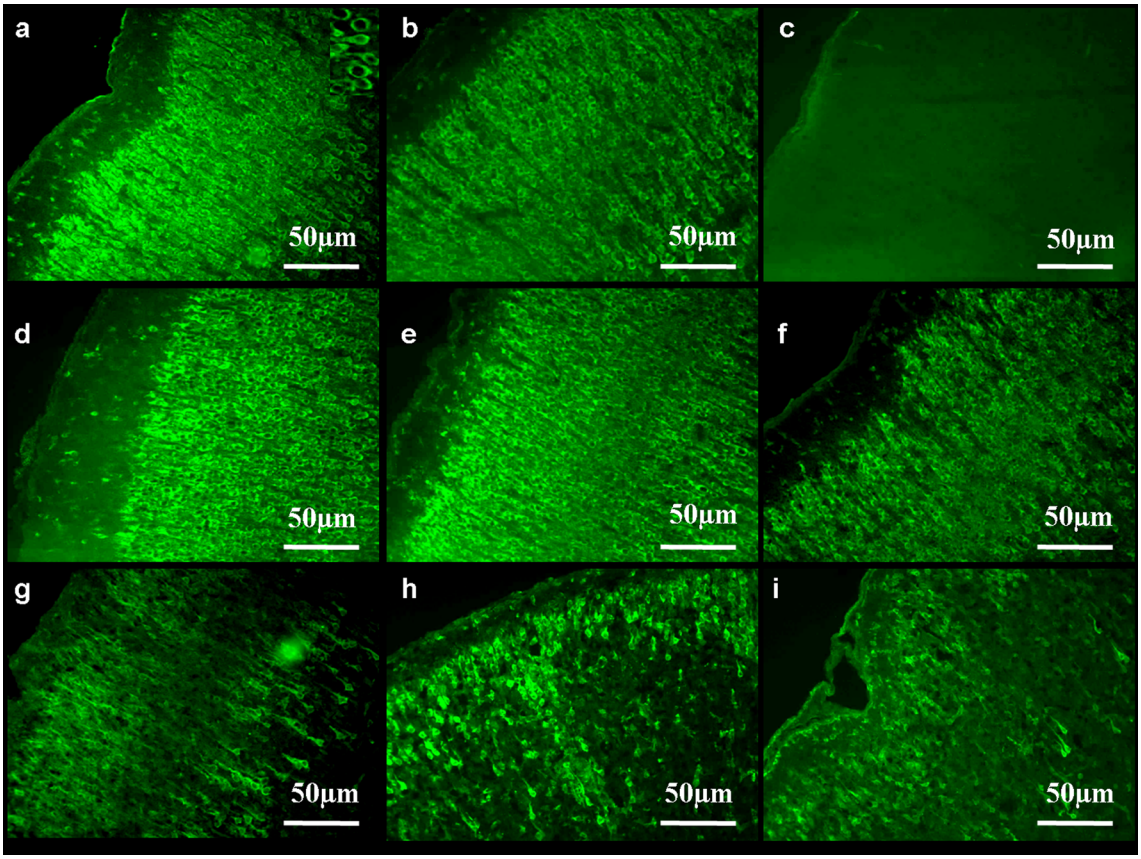

Figure 1. Immunofluorescence staining of pNR1 S897 in the cortex following hypoxic-ischemic brain damage (HIBD) or NMDA-induced brain damage (200X). Representative figures of pNR1 S897 immunofluorescence staining from the control group (*a*), PBS group (*b*), no primary antibody group (*c*), contralateral cortex 2 h after HIBD (*d*), contralateral cortex 24 h after HIBD (*e*), contralateral cortex 2 h after 10 nM NMDA injection (*f*), ipsilateral cortex 2 h after HIBD (*g*), ipsilateral cortex 24 h after HIBD (*h*), and ipsilateral cortex 2 h after 10 nM NMDA injection (*i*). Both HIBD and NMDA-induced brain damage reduced pNR1 S897 staining in the ipsilateral cortex (*g*, *h*, and *i*), but not in the contralateral cortex (*d*, *e*, and *f*).

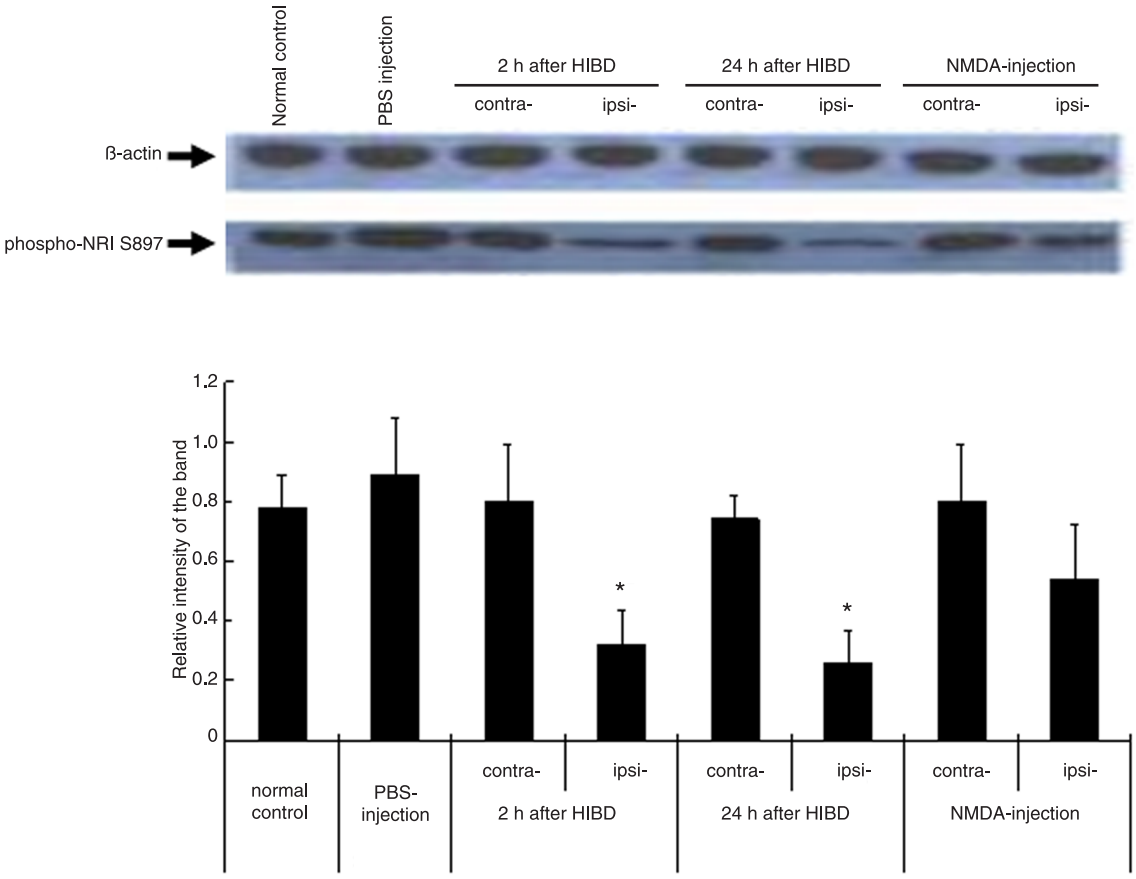

Figure 2. Western blot of pNR1 S897 in the cortex following hypoxic-ischemic brain damage (HIBD) or NMDA-induced brain damage. At 2 and 24 h after HIBD, the pNR1 S897 protein levels in the contralateral cortex were not affected ($P > 0.05$), whereas those in the ipsilateral cortex were reduced ($P < 0.05$). There was no significant difference between the 2- and 24-h subgroups ($P > 0.05$). At 2 h after 10 nM NMDA injection, the pNR1 S897 protein levels in the contralateral cortex were also not decreased ($P > 0.05$). The levels in the ipsilateral cortex were decreased, but the change was not significant ($P > 0.05$). In the "Normal control" and "PBS (phosphate-buffered saline) injection" groups the cortex used was from the right hemisphere, which was consistent with the ipsilateral hemisphere in both the HIBD model and NMDA-induced brain damage model. The band density of pNR1 S897 was normalized to that of β-actin. *$P < 0.05$ compared to control (two-tailed unpaired Student *t*-test).

The levels in the ipsilateral cortex were decreased, but there was no significant difference between the ipsilateral and contralateral hemispheres ($P > 0.05$).

Discussion

The present study showed a similar reduction of protein level in NR1 S897 phosphorylation following both HIBD and NMDA-induced brain damage in the ipsilateral cortex, based on immunofluorescence and Western blot. The protein levels of pNR1 S897 in the ipsilateral cortex were decreased at both 2 and 24 h after HI insult, as well as at 2 h after NMDA microinjection into the cortex. This observation might indicate that HIBD is to some extent related to NMDA toxicity through NR1 S897 phosphorylation. But this needs to be further proven by using an NMDA antagonist in the same animal model to determine whether the reduction of the protein level of pNR897 can be reversed by the antagonist.

NMDA receptors are tetramers consisting of NR1 subunits and at least one type of NR2 subunits. Different NR subunits confer distinct electrophysiological and pharmacological properties to the receptors and couple them to different signaling mechanisms (14,15). NMDA

receptor phosphorylation regulates short- and long-term channel function, receptor integrity, and cellular location (16). NR1 is essential for the formation of functional NR ion channels and NR2 (or NR3) subunits determine specific pharmacological and biophysical properties of NR channels (17). The C1 domain of NR1 has three serines (890, 896, and 897), with S890 and S896 phosphorylated by PKC and S897 phosphorylated by PKA (7,18). Previous studies have shown that the simultaneous phosphorylation of S896 and S897 to glutamate increased NR1 surface expression in both heterologous systems and hippocampal neurons, whereas phosphorylation of S890 to glutamate had no effect (19), suggesting a role for the phosphorylation of S896 and S897, but not of S890, in NMDA receptor trafficking. The up-regulation of pNR1 S897 observed in this study has also been reported in a unilaterally lesioned model of Parkinson's disease, in which pNR1 S897 was increased in both non-denervated and denervated striatum of lesioned monkeys, but not in striatal cells from control monkeys (20). The fact that the protein level of pNR1 S897 is increased in the presence of different neurological injuries in several animal species suggests that the serine 897 site of NR1 may be a common target in neurological diseases.

In this study, we employed 120 min of hypoxic exposure to induce cortical damage in P7 rats. A previous study has used similar procedures inducing HI using 120 min (for P7 rats) or 90 min (for P21 rats) of hypoxic exposure (9). The brain sensitivity to HI damage is affected by age-related differences (21). The abundant pNR1 S897 positively stained cells in the cortex of normal P7 rat pups indicates that the protein level of pNR1 S897 may play an important role in maintaining normal neuronal function at this age. The P7 rat brain is histologically similar to that of a 32- to 34-week gestation of human fetus or newborn infant (11,22) in terms of cell proliferation, cortical organization, synapse number, neurotransmitter synthetic enzymes, and electrophysiological parameters (23). The vulnerability of gray matter, especially the cortex, to HI damage increases towards term gestation into the early postnatal period (24). Both the present immunofluorescence and Western blot analyses led us to conclude that the protein level of pNR1 S897 is high in normal P7 rat cortex, and is reduced by both HIBD and NMDA-induced brain damage.

In previous studies, NMDA-induced brain damage has been investigated by either microinjecting NMDA into the cerebrum *in vivo* (25) or adding NMDA to the cell culture medium *in vitro* (15,26). The concentrations of NMDA ranged from 10 to 100 nM. Since exposure of cortical neurons to high NMDA concentrations results in severe neuronal necrosis, rather than reversible apoptosis (27), we chose a low NMDA concentration (10 nM) for *in vivo* examination. Future studies are required to establish how different doses of NMDA affect the protein level of pNR1 S897 in the cortex of P7 Sprague-Dawley rats.

In addition, NRs are highly permeable to Ca^{2+} ions, which may activate many downstream signal transduction pathways to induce NMDA receptor-dependent synaptic plasticity. Electrophysiological studies of medium-sized striatal neurons showed that phosphorylation of NR1 at the serine 897 site by PKA also promoted Ca^{2+} influx (28). Excessive Ca^{2+} influx via NR ion channels leads to excitotoxic cell death (29). Therefore, further research may also target whether Ca^{2+} influx in NMDA-induced brain damage is positively proportional to the phosphorylation of NR1 at serine 897.

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