[Frontiers in Bioscience 17, 1882-1890, January 1, 2012]

Effects of hypothermia on brain injury induced by status epilepticus

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1. ABSTRACT

We investigated the neuroprotective efficacy of hypothermia after the occurrence of status epilepticus (SE) in immature rats. In addition, the expression levels of NMDAR1 and c-Jun were measured to establish a possible signaling mechanism for hypothermia-induced neuroprotection. Pilocarpine-treated rats were randomly divided into 2 groups: group D (diazepam) and group DH (diazepam plus hypothermia). Pilocarpine-induced SE significantly enhanced the expression of NMDAR1 and c-Jun in the hippocampus compared to the control (group NS) rats. The numbers of necrotic and apoptotic pyramidal neurons were significantly higher in group D than in group NS rats. The DH group exhibited significantly fewer necrotic and apoptotic hippocampal pyramidal neurons and reduced NMDAR1 expression than group D. In contrast, early expression of c-Jun was significantly higher in the hippocampi of hypothermia-treated rats than in the hippocampi of group D, while late c-Jun expression was significantly lesser than group D. Our results show mild post-ictal hypothermia partially rescues/delays neuronal cell death in the hippocampus following SE. We further suggest that elevated NMDAR1 expression exacerbates SE-induced neuronal death in pilocarpine-treated rats, while early c-Jun overexpression, concomitant with hypothermia, suppresses subsequent neuronal death.

2. INTRODUCTION

Convulsive disorders are relatively common in children. The incidence of convulsions in children is 3–4 per 100 children per year; and 20% of these convulsions can be classified as status epilepticus (SE). Status epilepticus is defined as a single epileptic seizure lasting more than 30 min or repeated seizures occurring over a period of more than 30 min without recovery of consciousness. While many childhood seizures are benign, SE can cause severe and irreversible brain injury. The anticonvulsant efficacy of mild brain hypothermia following experimental SE has been confirmed (1). However, the possible neuroprotective effects and mechanisms of post-SE hypothermia have not been examined. To this end, we measured the neuronal necrosis and apoptosis in the hippocampus of pilocarpine-treated young rats and in rats exposed to mild hypothermia following pilocarpine-induced SE. To establish a possible signaling mechanism for hypothermia-mediated neuroprotection, we measured the post-SE expression of NMDAR1 and c-Jun.

3. MATERIALS AND METHODS

3.1. Animals

Three-week-old male Wistar rats (50–60 g) were obtained from the laboratory animal center (Shanghai, Chinese Academy of Science). During the experiments, the

rats were kept under controlled environmental conditions (24–25°C, 50–60% humidity, 12-h light/dark cycle, lights on at 6 a.m.). Our experimental procedures were approved by the local committee on ethics in animal research. All efforts were taken to minimize the number of animals used and their sufferings.

3.2. Pilocarpine-induced SE

Rats were injected intraperitoneally with either pilocarpine (380 mg/kg; Sigma-Aldrich Co Ltd, Ayrshire ,United Kingdom) dissolved in isotonic normal saline (pH 7.2) or normal saline alone (control or NS group). Only animals that reached stage 5 of the Racine's motor seizure scale and experienced a 30-min SE were selected for post-ictal treatments. These SE rats were randomly divided into 2 groups: the first group was injected with 5 mg/kg diazepam (Shanghai XuDong HaiPu Pharmaceutical Co Ltd, China) (D group) to stop acute seizure activity; and the second group was injected with 5 mg/kg diazepam at the same time as group D and then maintained under mild hypothermia for 2 h (DH group). The control group (NS) was injected with diazepam at an equivalent time after saline injection. Each group (NS, D, and DH) was divided into 4 subgroups that were examined at 3, 6, 24, or 72 h after SE onset (n = 5 rats in each subgroup).

3.3. Mild hypothermia

Mild hypothermia (33 ºC) was induced by placing the rat under a fan and covering the rat's torso with an icepack. Core temperature, as measured with a rectal thermometer, decreased to an average of 33°C within 6–10 min and was maintained for 2 h.

3.4. Tissue processing

At 3, 6, 24, or 72 h post SE (or after vehicle treatment for the NS group), rats were anesthetized using chloral hydrate (2 mg/kg), sacrificed, and the brains were removed. Brains were bisected along the interparietal suture. Left hemi-brains were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.6) for 72 h and then embedded in paraffin. Transverse sections (6 μm) were cut from embedded brains through the dorsal hippocampus and subsequently processed for immunohistochemistry. Whole hippocampi dissected from the right hemi-brains were placed into 1 mL of TRIzol reagent (Invitrogen, Carlsbad,USA), frozen in liquid nitrogen, and stored at -80 °C until they were used for qRT-PCR (section 3.8).

3.5. Histological evaluation of the extent of neuronal damage

Hematoxylin-eosin (H&E) staining and terminal deoxynucleotidyl transferase (dUTP) nick-end labeling (TUNEL staining) were used to identify the necrotic and apoptotic neurons in the hippocampal CA1 and CA3 stratum pyramidale at 72 h post SE (n = 5). Necrotic (acidophilic) and non-necrotic neurons were counted in 5 grids—each of 1 mm^2—by using the 40× objective, and the percentage of necrotic neurons was determined by dividing the number of necrotic neurons by the total number of neurons counted. Necrotic neurons were identified using common morphological features, including shrunken cell bodies, karyolysis, and intensely eosinophilic cytoplasm. The number of TUNEL-positive neurons was also counted in five 1-mm^2 grids (using the 40× objective) per hippocampal CA1 and CA3. Neuronal counting was performed by investigators unaware of the treatment histories of the brain sections.

3.6. TUNEL staining

TUNEL staining was performed using the *in situ* cell death detection kit (POD, Roche,Indianapolis,USA). Paraffin-embedded tissue sections were first mounted on glass slides. Slides were heated to 60°C and subjected to xylene wash, a graded series of ethanol washes, and rinsed in double-distilled water. Subsequently, slides were treated with protease K for 20–30 min at 37°C, washed in PBS with 1% Triton X-100 for 2 min, rinsed again in PBS, and incubated for 60 min at 37°C with 500 μL of TUNEL reaction mixture. Finally, slides were incubated for 30 min at 37°C with 50 μL converter-POD (peroxidase), rinsed in PBS, and incubated for 10 min at 15–25 °C with 50 μL of DAB substrate solution.

3.7. Immunohistochemistry

Brain sections were immunostained for NMDAR1 at 72 h post SE (n = 5). Other sections were immunostained for c-Jun at 3, 6, 24, and 72 h post SE (n = 5). Briefly, the sections were first de-paraffinized using a graded series of xylene and alcohol washes, treated with 0.3% H_2O_2 in 100% methanol for 25 min, washed sequentially in 50% methanol and 0.1 M PBS (pH 7.6), and microwaved (700 W) for 25 min in 0.01 M citrate buffer (pH 6.0) to enhance antigen exposure. After cooling and washing in PBS, the sections were incubated overnight at 4°C with rabbit polyclonal anti-NMDAR1 antibody (1:100 in PBS; stock, 200μg/mL; Wuhan Boster Bio-Engineering Co Ltd,China). The sections were washed 3 times in PBS and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin at a dilution of 1:200 (EnVision, DAKO, Carpinteria, California,USA) in PBS for 120 min at 37°C. Stained sections were washed 3 times in PBS and immunoreactivity was visualized with 3,3′-diaminobenzidine (DAB; Chromogen- Kit, DAKO, Carpinteria, California,USA). As a negative control, the primary antibody was omitted for some brain sections. The protocol outlined above was also used for c-Jun immunohistochemistry (rabbit polyclonal anti-c-Jun, 1:100 of a 200 μg/mL stock; Santa Cruz Biotechnology,California,USA). Immunoreactivity for NMDAR1 was analyzed using a Leica imaging system (Leica, Germany) and Image-pro Plus analysis software. The mean optical density was calculated from the accumulated optical density values in five 1-mm^2 grids (using the 40× objective) per hippocampal CA1 and CA3. The number of c-Jun positive neurons was counted in five 1 mm^2 grids (using the 40× objective) per hippocampal CA1 and CA3. Optical density measurements and neuron counting were performed by investigators who were unaware of the treatment histories.

3.8. Real-time qPCR

Quantitative real-time PCR (qRT-PCR) was used to estimate mRNA expression in the hippocampus at 3, 6, 24, and 72 h post SE (n = 5). Total RNA was extracted

Table 1. Percentage of necrotic neurons in the CA1 and CA3 stratum pyramidale (mean ± SD)

Group	CA1	CA3
NS	3.54±0.4	4.64±0.53
D	26.37±3.95*	27.66±3.3*
DH	14.77±3.35*#	17.15±2.80*#

*$P < 0.05$ vs NS, #$P < 0.05$ vs D, n=5

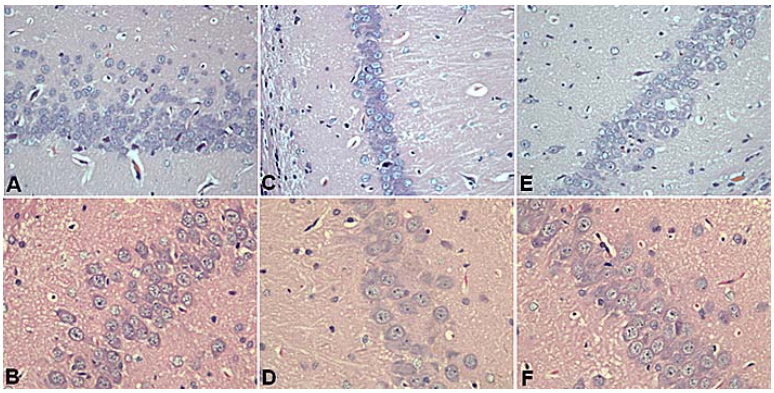

Figure 1. H&E staining of hippocampal sections isolated from control rat brains and rat brains following SE. Pyramidal neurons from CA1 (A) and CA3 (B) showed no change in H&E staining in the control NS group. In contrast, a fraction of pyramidal neurons exhibited the morphologic features of necrosis (including H&E staining) 72 h post SE in D group rats (C: CA1, D: CA3). Sections from rats treated with 2 h hypothermia after SE (DH group; E: CA1, F: CA3) had fewer necrotic pyramidal neurons than matched sections from D group rats.

using the RNeasy mini Kit (Qiagen, Hilden, Germany). The first cDNAs were synthesized from 2 μg of total RNA by using oligo-dT primers (1 μL), 1.25 μL of dNTP, 0.6 μL of RNase Inhibitor (Promega Co, Wisconsin,USA), 1 μL of M-MLV RTase (Promega Co, Wisconsin,USA), and 5 μL of l5× reaction buffer. Quantitative real-time PCR was performed according to the manufacturer's instructions by using the ABI7000 system (ABI Co,Los Angeles, USA) and QuantiTech SYBR Green PCR kit (Qiagen, Hilden, Germany). The gene-specific primers were designed and synthesized by Sangon Biotech Co Ltd (Shanghai, China). Sense and antisense primers were: 5′-GCAAGTGGGCATCTACAAT-3′ and 5′-GCTTGACATACACGAAGGGTT-3′ for NMDAR1, 5′-ACGACCTTCTACGACGATG-3′ and 5′-GTCGGTGTAGTGGTGATGTG-3′ for c-Jun, and 5′-AACCCTAAGGCCAACAGTGAAAAG-3′ and 5′-TCATGAGGTAGTCTGTGAGGT-3′ for β-actin (internal reference gene). Each primer pair was tested by a logarithmic dilution of the cDNA mix to generate a linear standard curve that was then used to calculate the primer pair efficiency. The PCR reactions were performed in 25-μL volumes by using a thermocycler protocol of 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. The generation of specific PCR products was confirmed by the melting curve analysis and agarose gel electrophoresis. The data were analyzed using the ABI Software, and all expression levels were normalized to β-actin.

3.9. Western immunoblot analysis

For western blot analysis, protein extracts were resolved on 12% SDS-polyacrylamide gels, and equal amounts of protein (20 μg) were loaded onto a 5% acrylamide stacking gel and separated by SDS-PAGE by using a 10% separating gel. Following the transfer of separated proteins, nitrocellulose membranes were blocked and probed overnight at 4°C with rabbit polyclonal antibodies to NMDAR1 (abcam,Massachusetts,USA) and rabbit polyclonal antibodies to c-Jun (abcam, Massachusetts,USA). The membrane was then probed for 1 h at room temperature with goat anti-rabbit peroxidase-conjugated IgG (Kirkegaard & Perry Laboratories,Maryland,USA), and the immunoreactivity was detected by chemiluminescence. Protein quantification was done by normalizing each band density to the GAPDH protein.

3.10. Statistical analysis

Data are presented as mean ± standard deviation (SD). For statistical comparison, one-way ANOVA followed by pairwise LSD-t post-hoc tests were employed. Values of $P < 0.05$ were considered to be statistically significant.

4. RESULTS

4.1. Hippocampal cell damage following SE

Representative photomicrographs of hematoxylin and eosin (HE)-stained sections are shown in Figure 1. Hippocampal neurons were intact and no obvious signs of necrosis were observed in the NS group (Figure 1A, B) at 72 h after saline injection. However, at 72 h post SE chromatorrhexis, karyolysis, and loosely packed neurons were observed in both CA1 and CA3 regions of the hippocampus in sections from D group rats (Figure 1C, D), and significantly more necrotic neurons were counted (NS CA1: 3.54 ± 0.4%; D CA1: 26.37 ± 3.95%, $P < 0.05$) (Table 1), while significantly fewer necrotic neurons were found in the hippocampal sections from DH group rats (DS

Table 2. TUNEL positive neurons in the CA1 and CA3 stratum pyramidale (mean ± SD)

Group	CA1	CA3
NS	0.52±0.51	0.71±0.46
D	11.5±2.18*	9±2.72*
DH	7.2±1.41*#	5.88±2.35*#

*P < 0.05 vs NS, #P<0.05 vs D, n=5

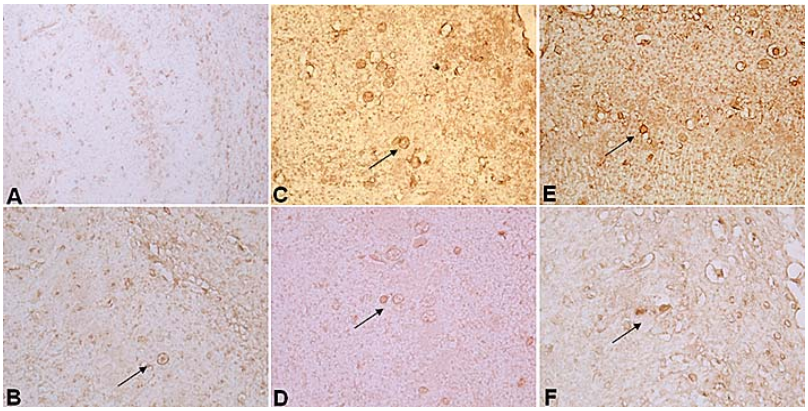

Figure 2. TUNEL staining of hippocampal sections showing variable apoptotic rates in pyramidal neurons in the different treatment groups. Some neurons were weakly stained in group NS (A: CA1, B: CA3, arrow). Neurons with the typical apoptotic morphology, including condensed and fragmented chromatin, were observed in group D sections 72 h post SE (C: CA1, D: CA3, arrows). Hypothermia reduced the number of apoptotic neurons (E: CA1, F: CA3, arrows).

CA1: 14.77 ± 3.35%; P < 0.05 compared to the CA1 from D group; Figure 1 E, F; Table 1).

4.2. TUNEL staining

Neurons that display morphological features of apoptosis can be visualized by the TUNEL staining technique. Some neurons were weakly stained by the TUNEL reaction in hippocampal CA1 and CA3 sections from control rats (Figure 2A, B; Table 2). At 72 h post SE, the number of TUNEL-positive CA1 and CA3 pyramidal neurons in group D rats were significantly higher than the control group, NS (Figure 2C, D; Table 2). However, this enhanced post-SE apoptosis was partially rescued by hypothermia (DH group rats; Figure 2 E, F; Table 2).

4.3. NMDAR1 protein and mRNA expression

Immunoreactivity of NMDAR1 in group NS rats was detected within neuronal perikarya and dendrites (Figure 3A,B; Table 3). At 72 h post SE, enhanced NMDAR1 immunostaining was observed in the hippocampal CA1 and CA3 of group D rats (Figure 3C, D; Table 3). This enhanced post-SE expression was significantly reduced in the hypothermia-treated (group DH) rats compared to group D rats (Figure 3E, F; Table 3). Similarly, NMDAR1 mRNA expression at 72 h post SE was reduced by hypothermia (Figure 6; Table 4).

4.4. c-Jun protein and mRNA expression

In hippocampal sections from control rats, only the occasional, weakly positive c-Jun immunostained neuron was observed in the hippocampal CA1 and CA3 stratum pyramidale (Figure 5A-D; Table 5). At 24 h and 72 h post SE, the number of c-Jun-positive neurons in group D was significantly higher than that in the control group NS rats (Figure 5E-H; Table 5). Hypothermia increased the number of c-Jun-positive neurons observed at 3 and 6 h post SE but significantly reduced the number of c-Jun-positive neurons at 24 h and 72 h post SE compared to group D (Figure 5I-L; Figure 7; Tables 5 and 6). Thus, hypothermia increases the early post-SE expression of c-Jun, which subsequently decreases the delayed expression of c-Jun.

5. DISCUSSION

Following the determination of a precise definition of SE in the 1990s by the International League against Epilepsy (IEAE), many studies have examined the relationship between SE and brain damage. It is well established that SE triggers neuronal loss in the immature brain, and there is a direct correlation between neuronal death and the duration of SE (2-7). We also observed a morphological sequence of necrosis and apoptosis in the hippocampal CA1 and CA3 stratum pyramidale at 72 h after SE. Uncontrolled seizures resulted in substantial damage to the hippocampal pyramidal neurons, since approximately 25% were necrotic and 10% apoptotic at 72 h post SE (suggesting a lower limit of 25% cell death under these conditions). Indeed, neuronal damage in the hippocampus appeared to be inevitable, highlighting the importance of safe and effective neuroprotective measures against SE-induced brain damage (in addition to novel treatments that prevent seizures). Neuroprotection against ischemic brain injury by mild hypothermia has been demonstrated in numerous animal models and clinical trials (8-10). In recent years, hypothermia has also shown to suppress spontaneous epileptiform activity and to have anticonvulsant properties (11). This study indicated that 2 h of mild hypothermia following SE partially rescued

Table 3. NMDAR1 immunoreactivity in the hippocampus as measured by optical density (mean ± SD)

Group	CA1	CA3
NS	0.07±0.005	0.08±0.015
D	0.35±0.038*	0.47±0.044*
DH	0.23±0.036*#	0.35±0.035*#

*P < 0.05 vs NS, #P<0.05 vs SE 30min D, n=5

Table 4. NMDAR1 mRNA expression in the hippocampus (mean ± SD)

Group	3h	6h	24h	72h
NS	3.41±0.11	3.51±0.10	3.49±0.19	3.40±0.14
D	5.18±0.44*	4.97±0.63*	4.72±0.26*	4.56±0.33*
DH	4.96±0.18*	4.44±0.33*	4.21±0.53*	4.03±0.71*#

*P < 0.05 vs NS, #P < 0.05 vs SE 30min D, n=5

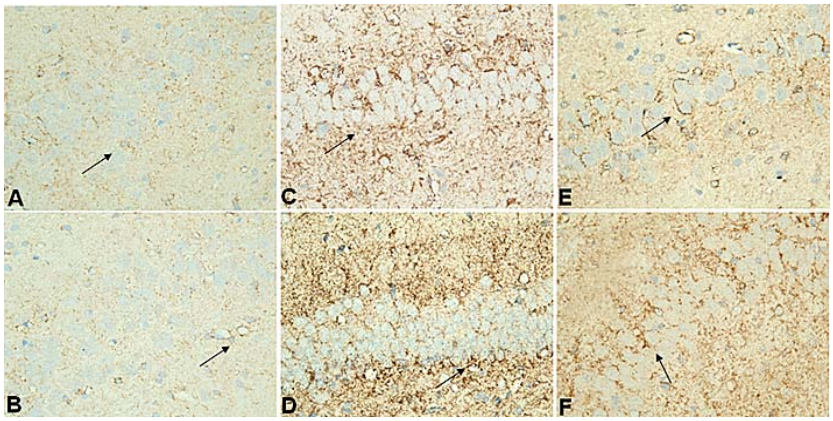

Figure 3. NMDAR1 immunohistochemistry in hippocampus sections from control, SE and SE plus hypothermia rats. Immunoreactivity of NMDAR1 in control group NS was detected within neuronal perikarya and dendrites (A: CA1, B: CA3, arrows). At 72 h post SE, enhancement of NMDAR1 immunostaining was observed in group D (C: CA1, D: CA3, arrows) while NMDAR1 immunostaining was reduced in hypothermia-treated group DH rats compared to group D rats (E: CA1, F: CA3, arrows).

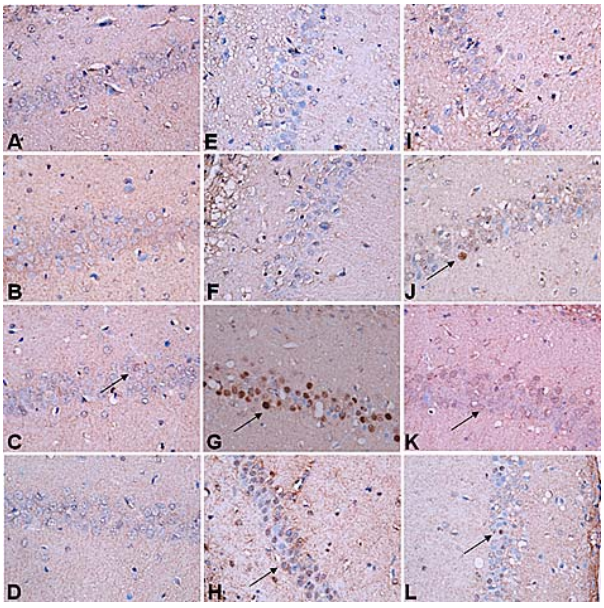

Figure 4. c-Jun immunohistochemistry in hippocampal CA1 pyramidal neurons in control, SE, and SE plus hypothermia rats. Some neurons were weakly immunoreactive for c-Jun in control group NS (A-D, arrows). At 24 h and 72 h post SE, strong staining and increased numbers of c-Jun positive neurons were found in group D rats (E-H, arrows). Hypothermia increased the number of c-Jun-positive neurons at 6 h post SE relative to the D group, but reduced the number of c-Jun-positive neurons at 24 and 72 h post SE (I-L, arrows).

Table 5. c-JUN positive neurons in hippocampal CA1 and CA3 stratum pyramidale (mean ± SD)

	CA1				CA3			
Group	3h	6h	24h	72h	3h	6h	24h	72h
NS	3.36±1.38	3.52±1.42	3.56±1.50	2.84±1.21	2.80±0.96	2.4±1.04	2.48±1.05	2.16±0.58
D	3.96±1.21	5.4±1.41	26.8±3.87*	16.44±5.48*	2.64±1.11	2.92±1.26	18.76±4.48*	12.04±4.48*
DH	4.64±1.25	9.44±2.16*#	16.0±3.11*#	11.24±3.97*#	3±1.22	4.52±1.53*#	12.24±3.46*#	7.56±2.97*#

*$P < 0.05$ vs NS, #$P < 0.05$ vs SE 30min D, n=5

Table 6. c-JUN gene expression as measured by qRT-PCR (mean ± SD)

Group	3h	6h	24h	72h
NS	3.70±0.08	3.77±0.12	3.73±0.11	3.75±0.12
D	5.14±0.20*	4.91±0.48*	5.19±0.32*	5.00±0.36*
DH	5.54±0.29*#	5.01±0.30*	4.43±0.50*#	4.38±0.36*#

*$P < 0.05$ vs NS, #$P < 0.05$ vs SE 30min D, n=5

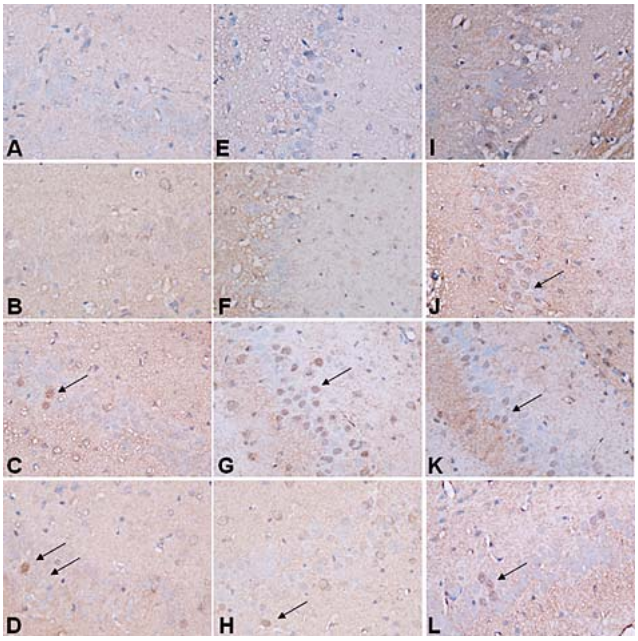

Figure 5. c-Jun immunohistochemistry in hippocampal CA3 pyramidal neurons. Some weakly c-Jun immunopositive neurons were observed in group NS (A-D arrows). At 24 h and 72 h post SE, strong staining and increased numbers of immunoreactive CA3 neurons were observed in group D (E-H arrows). Hypothermia increased the number of positive neurons at 6 h post SE but reduced staining and the number of positive neurons at 24 h and 72 h post SE (I-L, arrows).

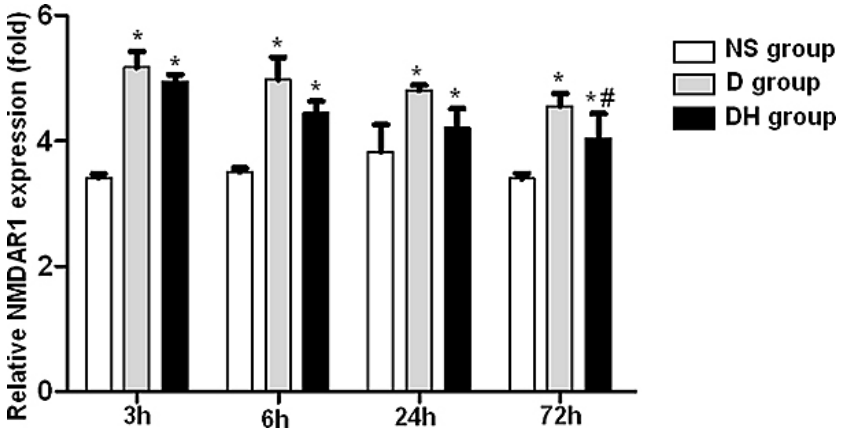

Figure 6. NMDAR1 mRNA expression in the hippocampus. Quantitative real-time PCR was used to estimate NMDAR1 mRNA expression in the hippocampus at 3, 6, 24, and 72 h post SE (n=5). Results are representative of three independent experiments. *$P < 0.05$ vs NS, #$P < 0.05$ vs SE.

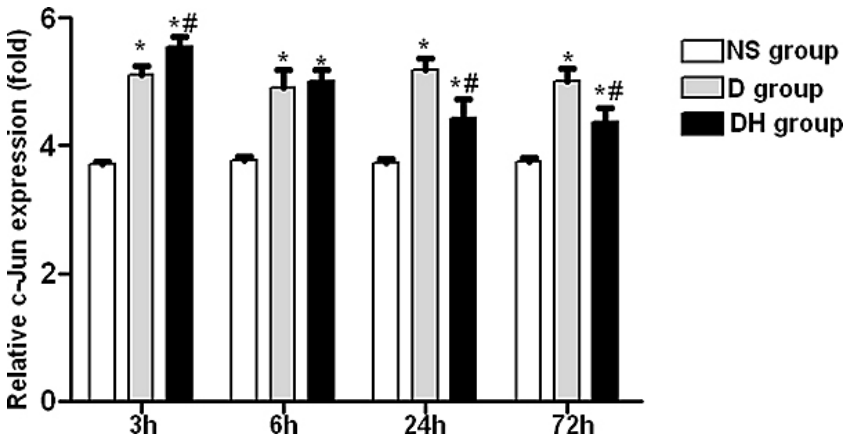

Figure 7. c-Jun mRNA expression in the hippocampus. Quantitative real-time PCR was used to estimate c-Jun mRNA expression in the hippocampus at 3, 6, 24, and 72 h post SE (n=5). Results are representative of three independent experiments. *$P < 0.05$ vs NS, #$P < 0.05$ vs SE.

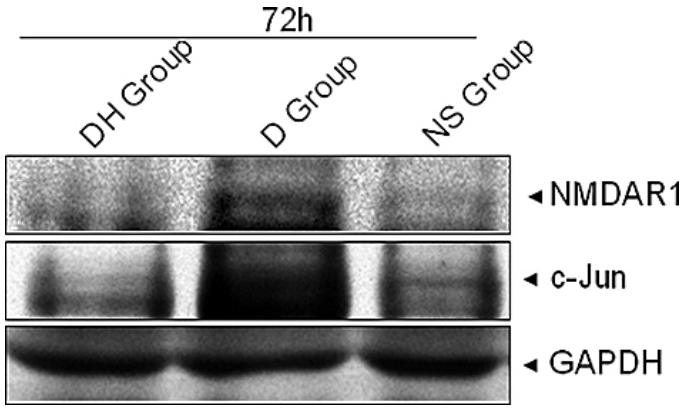

Figure 8. c-Jun and NMDAR1 protein expression in the hippocampus. Western blot was explored using rabbit polyclonal to NMDAR1 and rabbit polyclonal to c-Jun, with the expression of GAPDH as a normalized control.

neurons from post-ictal necrosis and apoptosis in immature rats.

Seizures cause prolonged periods of neuron depolarization (paroxysmal depolarizing shifts); they are associated with the generation of free radicals due to hypermetabolic exhaustion and enhance excitatory amino acid release from synapses. The concurrence of depolarization and glutamate release results in the overactivation of NMDARs, NMDAR-mediated Ca^{2+} influx, and in the activation of a myriad of downstream Ca^{2+}- and free radical-dependent neurodegenerative pathways (12). The mammalian brain has 5 types of glutamate receptors: NMDA receptors, kainic acid receptors, QA receptors, L-2-amino-phosphono butyric acid receptors, and metabotropic receptors. As the main calcium channel mediated by glutamate receptor, the level of NMDA expression is closely related to the susceptibility to convulsions and post-ictal brain injury. Excessive Ca^{2+} influx, mediated by hyperactivation of NMDARs, induces intracellular Ca^{2+} overload and concomitant activation of Ca^{2+}-dependent endonucleases, phospholipases (like PLC and A2), neuronal NO synthase (nNOS), and oxidative stress from oxygen free radicals. These toxic factors all synergize to produce the acute necrosis of neurons (13,14). In addition, elevated intracellular calcium concentrations can also phosphoactivate the transcription factor CREB and alter the expression of several immediate-early genes (IEGs).

Immediate-early gene is the general name for genes that are rapidly induced (and short-lived) in response to many forms of extracellular stimulation. They encode transcription factors, including c-Jun, c-Fos, and c-Myc. In humans, the c-Jun gene is localized to chromosome 1p32–31, and its expression product, c-Jun protein, is composed of 308 amino acid residues. Brain injury due to various causes can activate c-Jun transcription. The c-Jun mRNA is translated in the cytoplasm and translocates rapidly to the nucleus. Through activation by c-Jun N-terminal kinase (JNK), c-Jun and c-Fos proteins form the Fos-Jun heterodimer AP-1 transcription factor. Under some conditions, AP-1 activation participates in the regulation of apoptotic gene expression. Activation of apoptotic proteases (caspases) then initiates neuronal apoptosis (15). In addition, c-Jun also participates in the induction of long-

term functional alterations in neurons during development (proliferation and differentiation) and in response to neuron damage (16-18). The first peak of IEG mRNA expression following pathogenic stimulation has been associated with the neuronal capacity for self-repair and the rapid re-establishment of basal signal transduction (19, 20). In contrast, the second delayed peak of IEG mRNA expression may trigger neurodegeneration. Indeed, this second wave of expression after pilocarpine-induced SE only occurs in 3-week-old rats and adult mice, but curiously, not in 10-day-old neonatal rats. In 10-day-old rats, only small numbers of apoptotic neurons were found in the cortex, while apoptosis was widespread in the cortices of 3-week-old rats and adult mice (21). Thus, while the first peak is associated with an endogenous neuroprotective capacity, the second is linked to the activation of neuronal apoptosis.

This study examined the neuroprotective effects of mild hypothermia on NMDAR1 and c-Jun expression in hippocampal pyramidal neurons. Both NMDAR1 protein levels and mRNA expression were significantly reduced in diazepam- and hypothermia-treated (DH group) rats compared to those receiving diazepam alone (D group rats) at 72 h post SE. This suggests that group DH rats may experience limited excitotoxicity compared to D group rats. Early c-Jun expression (protein and mRNA) was significantly enhanced in DH rats compared to group D rats, while late c-Jun expression was significantly lower in group DH rats compared to group D. Early expression of c-Jun, therefore, may be associated with enhanced endogenous neuroprotective capacity in young rats, while the second wave of c-Jun, together with enhanced NMDAR1 expression, may promote brain injury following SE. In summary, mild hypothermia can partially reverse neuronal necrosis and apoptosis by regulating NMDAR1 and c-Jun expression.

Hypothermia is perhaps the most reliable inducer of neuroprotection. Here, we show that these effects can be mediated by downregulation of NMDAR1 expression and by time-dependent up- and downregulation of c-Jun. However, the signaling pathways involved remain unknown. Similarly, it is not clear how hypothermia induces such biphasic changes in c-Jun expression. We propose that neuroprotection results from (at least) 2 hypothermia-related mechanisms: (1) downregulation of NMDAR1 expression by mild hypothermia reduces activation of downstream excitotoxic signals (calcium dysregulation, ROS generation) and may also enhance c-Jun activation; and (2) by reducing energy consumption, mild hypothermia will maintain the neuronal energy charge to rebalance ionic gradients, suppress free radicals, and respond to external stimuli that activate endogenous neuroprotective mechanisms (including c-Jun and c-Jun-regulated genes). Further studies are required to investigate these c-Jun-dependent neuroprotective mechanisms.

6. CONCLUSION

Mild post-ictal hypothermia partially rescued/delayed neuronal cell death in the hippocampus following SE. Our findings suggest that elevated NMDAR1 expression exacerbates the delayed SE-induced neuronal death in pilocarpine-treated rats. Comparatively, we propose that early c-Jun overexpression, concomitant with hypothermia, suppresses subsequent neuronal death.

7. ACKNOWLEDGEMENTS

The author would like to thank our research colleagues, Dr. Xiao-mei Shao, Dr. Lian Chen and Dr.Ying Wang for their encouragement and suggestions over the years in developing this work. The encouragement and support of Dr. Yi Wang were greatly appreciated.

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Abbreviations: NMDAR1: NMDA receptor subunit 1, SE: status epilepticus, NS: normal saline, DH: diazepam plus hypothermia, D: diazepam, HE: hematoxylin-eosin, qRT-PCR: quantitative real-time PCR

Key Words: Poliocarpine, Status epilepticus, Brain injury, Mild hypothermia, Neuroprotection, NMDAR1, C-JUN

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