Brain cell apoptosis and enhancement of nervous excitability in pregnant rats with high plasma levels of homocysteine☆

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Abstract

Hyperhomocysteinemia is an important risk factor for preeclampsia-eclampsia. This study established a pregnant rat model of hyperhomocysteinemia, in which blood plasma homocysteine concentrations were twice or three times greater than that of normal pregnant rats. TUNEL revealed an increase in the number of apoptotic cells in the frontal cortex of pregnant rats with hyperhomocysteinemia. In addition, immunohistochemical staining detected activated nuclear factor-κB-positve cells in the frontal cortex. Reverse transcription-PCR detected that mRNA expression of the anti-apoptotic gene bcl-2 diminished in the frontal cortex. *In situ* hybridization and western blotting revealed that N-methyl-D-aspartate receptor 1 mRNA and protein expression was upregulated in the frontal cortex and hippocampus. These results indicate that hyperhomocysteinemia can induce brain cell apoptosis, increase nerve excitability, and promote the occurrence of preeclampsia in pregnant rats.

Key Words

hyperhomocysteinemia; homocysteine; preeclampsia; frontal cortex; N-methyl-D-aspartate receptor; nerve excitability; cell apoptosis; pregnancy; brain; neural regeneration

Research Highlights

(1) Hyperhomocysteinemia increased the number of apoptotic cells in the frontal cortex of pregnant rats.
(2) Hyperhomocysteinemia induced the translocation of nuclear factor-κB to the nuclei of cells in pregnant rats.
(3) Hyperhomocysteinemia downregulated bcl-2 mRNA and upregulated N-methyl-D-aspartate receptor 1 expression in the frontal cortex of pregnant rats.
(4) Hyperhomocysteinemia increased nervous excitability in pregnant rats, and promoted the occurrence of preeclampsia.

Abbreviations

HHcy, hyperhomocysteinemia; NF-κB, nuclear factor-κB; NMDAR, N-methyl-D-aspartate receptor

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INTRODUCTION

Preeclampsia-eclampsia occurs in pregnant women with gestational hypertension and is the leading cause of maternal and neonatal death[1-2]. Hyperhomocysteinemia (HHcy) is an important risk factor for preeclampsia-eclampsia[3-4]. HHcy is an independent risk factor for cerebrovascular disease[5-6]. The correlation of HHcy and preeclampsia-eclampsia has become of great focus in

vwomen who had twice or three times higher plasma homocysteine levels than normal pregnant women suffered from preeclampsia or eclampsia[9]. In the clinic, some patients did not suffer from severe hypertension or proteinuria, but experienced eclampsia, which suggested that its pathogenesis is probably associated with its effects on the nervous system[10]. Neural cell apoptosis is an important form of cell injury, which is correlated with many nervous system diseases[11]. Bcl-2 is an anti-apoptotic gene[12], and nuclear factor-κB (NF-κB), a eukaryotic cell transcription factor, is distributed throughout the body. Neural cell apoptosis frequently occurs when NF-κB is activated[13]. Glutamate is an excitatory neurotransmitter in the central nervous system. The N-methyl-D-aspartate receptor (NMDAR), a glutamate receptor, exists throughout the nervous system and participates in excitatory synaptic transmission[14].

This study sought to investigate the effects of high concentrations of homocysteine on apoptotic cell death, bcl-2 and NF-κB expression, and NMDAR1 mRNA and protein expression, and to explore the possible mechanism of pregnancy-induced preeclampsia-eclampsia.

RESULTS

Quantitative analysis of experimental animals

A total of 20 pregnant and 20 non-pregnant Wistar rats were equally and randomly assigned to control and HHcy groups. Rats from the HHcy groups were given drinking water supplemented with DL-homocysteine. Rats from the control groups were given drinking water without DL-homocysteine. All 40 animals were included in the final analysis.
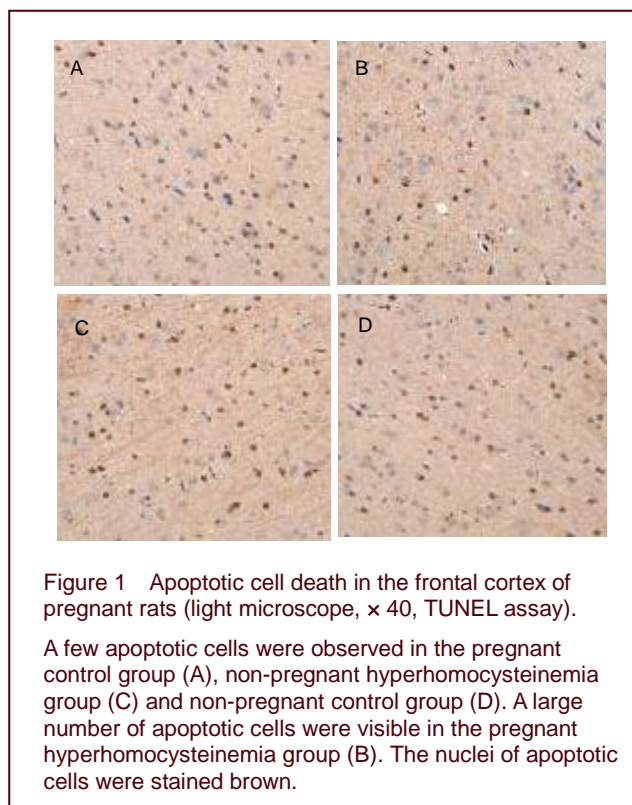
Homocysteine concentrations in the blood plasma

The concentration of blood plasma homocysteine was significantly lower in the non-pregnant control group than in the non-pregnant HHcy group (6.1 ± 1.8 μM *vs.* 18.7± 2.3 μM, $P < 0.05$). Homocysteine concentrations were significantly lower in the pregnant control group than in the pregnant HHcy group (5.2 ± 1.7 μM *vs.* 18.9 ± 3.1 μM, $P < 0.05$). These suggested that homocysteine concentrations produced in this study were two to three times that of normal concentrations, which was required for our experimental study.

Apoptosis in the frontal cortex

TUNEL-positive cells were detectable in the rat frontal cortex in each group (Figure 1). The number of TUNEL-positive cells in the pregnant HHcy group (79.5 ± 9.3 cells/400-fold visual field) was significantly greater

compared with the pregnant control group, non-pregnant HHcy group, and non-pregnant control group (48.7 ± 3.1, 59.6 ± 2.1, 56.3 ± 3.2 cells/400-fold visual field, respectively; $P < 0.05$).

Figure 1 Apoptotic cell death in the frontal cortex of pregnant rats (light microscope, × 40, TUNEL assay).

A few apoptotic cells were observed in the pregnant control group (A), non-pregnant hyperhomocysteinemia group (C) and non-pregnant control group (D). A large number of apoptotic cells were visible in the pregnant hyperhomocysteinemia group (B). The nuclei of apoptotic cells were stained brown.

NF-κB activation in the frontal cortex

Immunohistochemical staining revealed that NF-κB translocated to the nuclei of cells in the pregnant HHcy group. NF-κB was visible in the cytoplasm in the pregnant control group. Significant differences were detected between the pregnant HHcy and pregnant control groups (67± 6.5 cells/400-fold visual field *vs.* 123 ± 16.5 cells/400-fold visual field, $P < 0.05$). Translocation of NF-κB to the nuclei was found in the non-pregnant HHcy group (51 ± 8.7 cells/400-fold visual field) and non-pregnant control group (48 ± 7.5 cells/400-fold visual field, $P > 0.05$; Figure 2).

Expression of bcl-2 mRNA and NMDAR1 protein in the frontal cortex

Reverse transcription-PCR revealed that bcl-2 mRNA expression was detectable in the frontal cortex of pregnant rats in each group, and significantly visible in the pregnant control group. Bcl-2 mRNA expression was lowest in the pregnant HHcy group when compared with the other three groups ($P < 0.01$). NMDAR1 protein expression was significantly greater in the pregnant HHcy group than in the other three groups ($P < 0.01$; Figures 3, 4; Table 1).
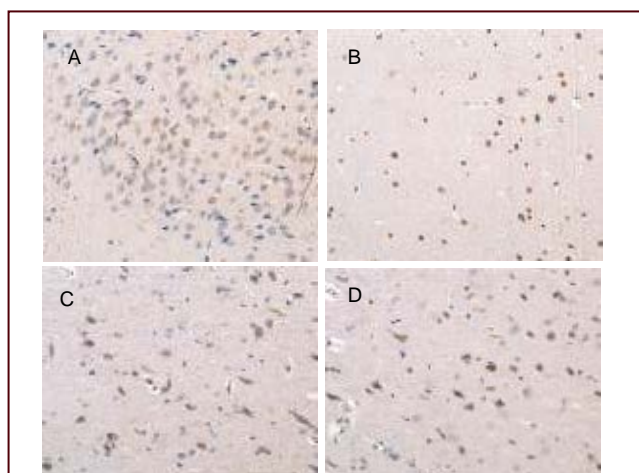
Figure 2　Nuclear factor-κB (NF-κB) expression in the frontal cortex of pregnant rats (light microscope, × 40, immunohistochemistry).

Cells with stained nuclei and an unstained cytoplasm were NF-κB-positive cells. NF-κB was observed in the cytoplasm in the pregnant control group (A). NF-κB translocated to the nucleus in the pregnant hyperhomocysteinemia group (B). NF-κB slightly translocated to the nucleus in the non-pregnant hyperhomocysteinemia group (C) and non-pregnant control group (D).
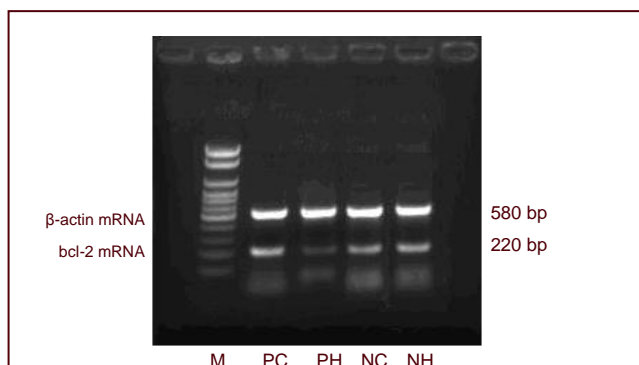

Figure 3　mRNA expression of bcl-2 in the frontal cortex of pregnant rats (reverse transcription-PCR).

M: Marker; PC, PH, NC, and NH: pregnant control, pregnant hyperhomocysteinemia (HHcy), non-pregnant group, non-pregnant HHcy groups, respectively.
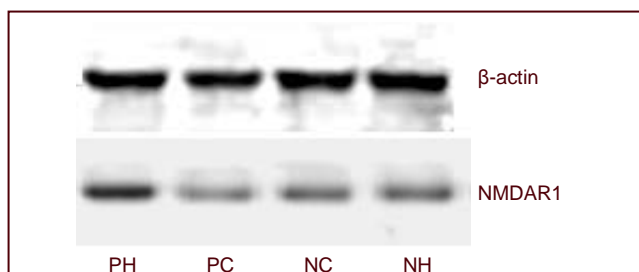

Figure 4　N-methyl-D-aspartate receptor 1 (NMDAR1) protein expression in the frontal cortex of pregnant rats. (western blot assay).

PH, PC, NC, and NH: Pregnant hyperhomocysteinemia, pregnant control, non-pregnant control, and non-pregnant hyperhomocysteinemia groups, respectively.

Table 1　Effects of hyperhomocysteinemia (HHcy) on bcl-2 mRNA and N-methyl-D-aspartate receptor 1 (NMDAR1) protein expression in the frontal cortex of pregnant rats

Group	bcl-2 mRNA (absorbance ratio to β-actin)	NMDAR1 (absorbance ratio to β-actin)
Pregnant control	1.10±0.51[a]	0.98±0.44[a]
Pregnant HHcy	0.62±0.20	0.45±0.37
Non-pregnant control	0.98±0.49[a]	0.89±0.52[a]
Non-pregnant HHcy	0.95±0.38[a]	0.92±0.47[a]

Data are expressed as mean ± SD. [a]$P < 0.01$, *vs.* pregnant HHcy group. Data in each group were analyzed using one-way analysis of variance. Intergroup variables were analyzed using least-significant-difference method for paired comparisons.

NMDAR1 mRNA expression in the frontal cortex and hippocampus

Under the light microscope, NMDAR1 mRNA-positive cells presented brown in color, and round or irregular in shape. Staining was mainly observed in the cell membrane and cytoplasm. NMDAR1 mRNA expression in the frontal cortex and hippocampus of pregnant rats was significantly greater in the pregnant HHcy group compared with the other three groups ($P < 0.05$; Figures 5, 6, and Table 2).
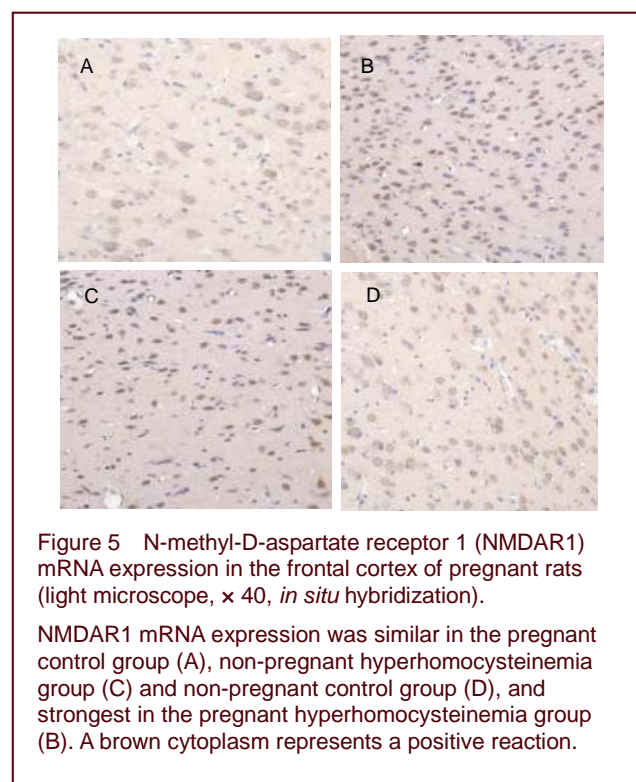

Figure 5　N-methyl-D-aspartate receptor 1 (NMDAR1) mRNA expression in the frontal cortex of pregnant rats (light microscope, × 40, *in situ* hybridization).

NMDAR1 mRNA expression was similar in the pregnant control group (A), non-pregnant hyperhomocysteinemia group (C) and non-pregnant control group (D), and strongest in the pregnant hyperhomocysteinemia group (B). A brown cytoplasm represents a positive reaction.

DISCUSSION

In this study, homocysteine concentrations in pregnant rats with HHcy were similar to that in a previous study[9]. A

previous study has shown that pregnant women who had twice or three times greater blood plasma homocysteine levels than normal pregnant women did not suffer from hypertension, proteinuria or endothelin changes[9].
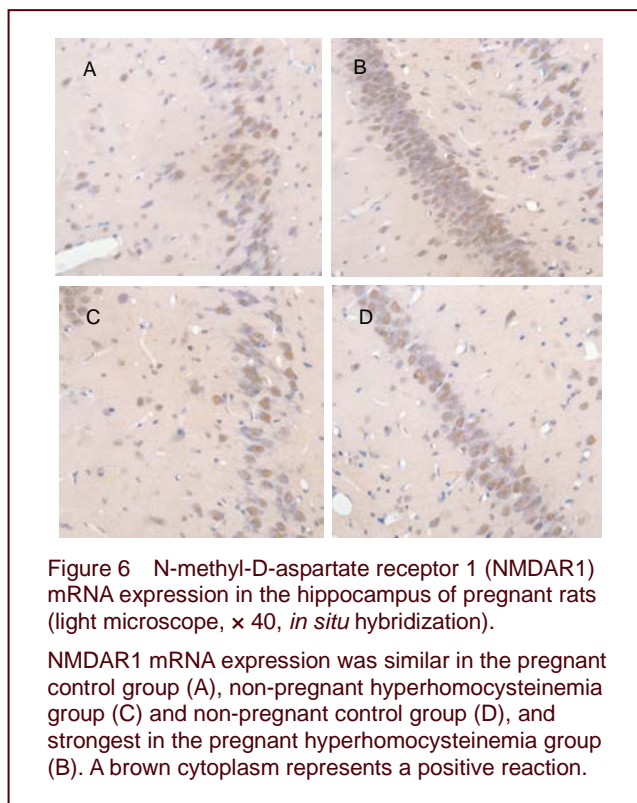

Figure 6 N-methyl-D-aspartate receptor 1 (NMDAR1) mRNA expression in the hippocampus of pregnant rats (light microscope, × 40, *in situ* hybridization).

NMDAR1 mRNA expression was similar in the pregnant control group (A), non-pregnant hyperhomocysteinemia group (C) and non-pregnant control group (D), and strongest in the pregnant hyperhomocysteinemia group (B). A brown cytoplasm represents a positive reaction.

Table 2 N-methyl-D-aspartate receptor 1 mRNA expression (absorbance) in the frontal cortex and hippocampus of pregnant rats

Region	NC	NH	PC	PH
Frontal cortex	0.42±0.14[a]	0.48±0.02[a]	0.49±0.04[a]	0.72±0.13
Hippocampus	0.35±0.07[a]	0.46±0.06[a]	0.51±0.09[a]	0.76±0.06

Data are expressed as mean ± SD (n = 10 per group). [a]$P < 0.05$, *vs.* PH group. Data in each group were analyzed using one-way analysis of variance. Intergroup variables were analyzed using least-significant- difference method for paired comparisons. NC, NH, PC, and PH: Non-pregnant control, non-pregnant HHcy, pregnant control, and pregnant HHcy groups, respectively.

However, HHcy probably damaged neurons. Therefore, patients with preeclampsia-eclampsia not only experienced hypertension and proteinuria, but also suffered from headache, dizziness and spasms. Results from the present study confirmed that homocysteine concentrations in the pregnant HHcy group were approximately three times that of the pregnant control group, which was slightly higher than in a previous study[10]. Thus, HHcy was an independent risk factor for cerebrovascular disease. The effects of HHcy on vascular injury and its direct neurotoxic effects contribute to neuronal damage.

Neural cell apoptosis is an important form of cell injury, and is associated with many nervous system diseases[11]. In this study, the number of apoptotic cells in the cerebral cortex of rats from the pregnant HHcy group was greater when compared with the pregnant control group, which suggested that HHcy damaged neural cells of pregnant rats. Moreover, the number of apoptotic cells in the non-pregnant HHcy group was not higher than that in the non-pregnant control group, suggesting that HHcy did not obviously damage neural cells of non-pregnant rats because HHcy concentrations were similar. This may be due to the possibility that the sensitivity of neural cells of pregnant mothers to changes in homocysteine concentrations are stronger, which may correlate with placental hormones[15], or with immunologic mechanisms[16]. Nevertheless, the changes were not remarkable during the non-pregnant period, which is probably associated with the following reasons: concentration changes did not induce endothelin changes; mild injury or non-injury of endothelial cells did not lead to neural cell lesions; the protective effect of antiapoptotic genes decreased. Bcl-2 has an important anti-apoptotic effect, and protein expression can only be detected in cells that have not died, resulting in suppression of neural cell apoptosis[12]. Results from this study demonstrated that the number of apoptotic cells significantly increased, but that bcl-2 gene expression decreased following HHcy stimulation.

The transcription factor NF-κB can directly affect gene transcription in various neural cells. Neural cells after stimulation induce NF-κB activation, which is accompanied by neural cell apoptosis[13]. Results from the present study indicated that HHcy promoted NF-κB activation, resulting in neural cell apoptosis in pregnant rats.

Changes in neurotransmitter levels play an important role in the onset of encephalopathy. The imbalance of excitatory and inhibitory transmitters is one reason for epileptiform discharge[17]. Glutamate is the principal excitatory transmitter in the central nervous system. NMDAR is believed to play a major role in learning and excitotoxic neuronal damage. NMDAR mRNA expression is observed in all neurons, and is abundant in the hippocampus and cerebral cortex[14]. Results from this study suggested that HHcy induced the upregulation of excitatory neurotransmitter receptor expression in pregnant rats and an increase in nervous excitability in pregnant rats with HHcy, which resulted in eclampsia. Results suggested that high expression of NMDAR is one of the possible mechanisms involved in cell apoptosis. HHcy induced an increase in NMDAR mRNA and protein expression, excessive opening of Ca^{2+} channels, and overloading of cytoplasmic Ca^{2+}, which may combine with calmodulin, resulting in excessive phosphorylation of calmodulin kinase. Phosphorylated calmodulin kinase can

promote the opening of NMDAR channels, which leads to a feedback loop and the formation of a vicious cycle[18]. Overloaded Ca^{2+}-mediated excitotoxic injury produces a large number of free radicals, which leads to oxidative stress and oxidative damage, and causes cell apoptosis *via* the mitochondrial pathway[18]. An *in vitro* study demonstrated that HHcy elevates the permeability of the blood-brain barrier and is the potentiator of NMDAR[19]. Therefore, we presumed that HHcy-induced neural cell apoptosis is mediated by NMDARs and elevates the excitability of the nervous system, resulting in the occurrence of preeclampsia-eclampsia. Taken together, a decrease in homocysteine levels may prevent or delay the occurrence of preeclampsia-eclampsia.

MATERIALS AND METHODS

Design
A randomized controlled animal study.

Time and setting
Experiments were conducted at the Medical Experimental Animal Center, General Hospital of Shenyang Military Area Command of Chinese PLA from May 2007 to May 2008.

Materials
The protocols were performed in accordance with the rules of Experimental Animal Protection. Grade II adult healthy Wistar rats at sexual maturity of both genders without mating were supplied by the Experimental Animal Center, General Hospital of Shenyang Military Area Command of Chinese PLA (license No. SYXK (Jun) 2007-001), weighing 200–240 g. The male and female rats were housed at a ratio of 5: 1 (female to male) in each cage. Vaginal secretions were daily obtained at 8:00 in the morning for microscopic examination. The sperm was found on day 0 of pregnancy. Following pregnancy, the rats were housed in an individual cage.

Methods
Production of HHcy
In accordance with a previous published method[10], 0.67 mg/mL DL-homocysteine (Sigma, St. Louis, MO, USA) was added to the drinking water on day 7 before mating in the pregnant HHcy group until day 19 of pregnancy to establish animal models of HHcy. DL-homocysteine (0.67 mg/mL) was added to the drinking water in the non-pregnant HHcy group for 26 days. The rats from the pregnant control group were given water without DL-homocysteine until day 19 of pregnancy.

Management of animals was identical between the non-pregnant control and pregnant control groups.

Analysis of plasma homocysteine
Approximately 1 mL of blood was collected from the orbital venous plexus of rats. Total plasma homocysteine was measured by fluorescence high-performance liquid chromatography as described previously[20]. Briefly, blood samples of 1 mL were placed into vacutainer tubes containing sodium heparin (Becton Dickinson, Shanghai, China) and immediately centrifuged at 1 000 × *g* for 10 minutes at 4°C. Plasma samples (100 µL) or solutions mixed with 10 µL internal standard (2.0 mM 2-mercaptoethylamine) were treated with 10 µL of 10% (w/v) tri-n-butylphosphine in dimethylformamide at 4°C for 30 minutes. Subsequently, 100 µL of the supernatant was transferred into a solution containing 20 µL of 1.55 M sodium hydroxide, 250 µL of 0.125 M borate buffer (pH 9.5), and 100 µL of 1.0 mg/mL 4-fluoro-7-sulfamoylbenzofurazan solution. The resulting mixture was incubated at 60°C for 30 minutes to accomplish derivatization of plasma thiols. High-performance liquid chromatography was performed with a Hewlett-Packard Model 1090 Series II system (AIR Analytical Instrument Recycle, Vermont, USA) with an autosampler. Separation was carried out at ambient temperature on an analytical column, Supelco LC-18-DB (150 × 4.6 mm ID, 5 µm) with a Supelcosil LC-18 guard column (20 × 4.6 mm ID, 5 µm). Fluorescence intensities were measured spectrophotometrically (Hewlett-Packard Model 1046A, Vermont, USA) at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. The peak area of the chromatographs was quantified with a Hewlett- Packard 3392 integrator (AIR Analytical Instrument Recycle, Inc.). The analytical column was eluted with 0.1 M potassium dihydrogen phosphate buffer (pH 2.1) containing 6% (v/v) acetonitrile as mobile phase with a flow rate of 2.0 mL/minute.

Preparation of brain tissue sections
On day 20 of pregnancy, the rats were intraperitoneally anesthetized with 10% (v/v) chloral hydrate (3 mL/kg), and then decapitated. The brain was dissected in accordance with stereotaxic coordinates[21]. After cerebral cortex and hippocampal tissues were dissociated, some tissues were fixed in 4% (w/v) neutral paraformaldehyde at 4°C for 5 hours for TUNEL and immunohistochemistry. The remaining tissues were stored in liquid nitrogen for reverse transcription-PCR and western blotting.

TUNEL for cell apoptosis in the frontal cortex
The cerebral cortex was sliced into sections, deparaffinized,

hydrated, placed in proteinase K for 5 minutes, and washed with tris-buffered saline (TBS) at room temperature. The sections were placed in TUNEL reaction mixture (Beijing Zhongshan Golden Bridge Biological Technology, Beijing, China) in a wet box at room temperature for 30 minutes, and then for a further 1 hour in the dark. After a wash in TBS, the sections were developed in diaminobenzidine, counterstained with hematoxylin, dehydrated, permeabilized, mounted, and observed under a light microscope (Olympus, Japan). Enzyme was not added to the blank controls. TUNEL-positive cells represented apoptotic cells. Five nonoverlapping visual fields of each section were randomly observed under a high power lens (× 400). Images were collected using an image analysis system. The nuclei of apoptotic cells were stained brown. The number of positive cells was calculated and the average value was obtained.

Immunohistochemistry for NF-κB activation

In accordance with an instruction for a two-step kit (Beijing Zhongshan Golden Bridge Biological Technology), sections were deparaffinized, hydrated, and washed in 0.01 M potassium phosphate buffer. Antigen retrieval was performed using a microwave. The sections were cooled at room temperature, washed, and incubated in 3% (v/v) H_2O_2 at room temperature for 10 minutes. Following inactivation of endogenous peroxidase, the sections were incubated in NF-κB mouse anti-rat monoclonal antibody (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at room temperature overnight, followed by incubation in horseradish peroxidase-labeled rabbit anti-mouse IgG (Beijing Zhongshan Golden Bridge Biological Technology) at room temperature for 30 minutes. After washing, the sections were developed with diaminobenzidine for 2–3 minutes. The reaction was terminated with distilled water. After counterstaining, the sections were mounted. Phosphate buffered saline was used as a primary antibody for negative controls. The number of NF-κB-positive cells was calculated as performed for TUNEL.

Reverse transcription-PCR for bcl-2 mRNA expression

Total RNA from the frontal cortex was extracted according to the Trizol kit instructions (Shanghai Boya Biotechnology, Shanghai, China). RNA (20 µL) was reverse transcribed into cDNA. Products (2 µL) were used for target gene amplification. Bcl-2 primers were synthesized by Shanghai Boya Biotechnology. Amplified products were used for gel electrophoresis (Table 3). Images were photographed using the gel image analyzer (GenoSens 1510; Clinx Science Instruments, Shanghai, China) and analyzed using FlourChemV2.0 software

(BKM, USA). Expression levels were represented by the absorbance ratio of bcl-2 mRNA to β-actin. The experiment was repeated three times.

Table 3 Reverse transcription-PCR primers and reaction conditions		
Primer	Condition	Length (bp)
Bcl-2		220
Upstream 5'-GTC ATG TGC ATT TCC ACG TC -3'	94°C, 1 minute; 56°C, 1 minute	
Downstream 5'-ACA AAC CCC CCA CAC AGC AAA G -3'	72°C, 1 minute; 30 cycles	
β-actin		580
Upstream 5'- TCT GGA TCA CCT TCT GCT GG -3'	94°C, 1 minute; 56–58°C, 1 minute	
Downstream 5'- GAT TGC TCA GGA CAT TTC TG -3'	72°C, 1 minute; 30 cycles	

Western blot assay for NMDAR1 protein expression

Frontal cortex tissue (100 mg) was added to 1 mL of lysis buffer. The samples were immersed in an ice-water bath for ultrasonication. The specimens were centrifuged at 13 000 × *g* at 4°C for 1 hour, and total protein was quantified. Samples were heated and electrophoresed on a 12% (w/v) SDS-polyacrylamide gel for 1.5 hours, transferred onto nitrocellulose membrane and blocked with TBS containing 0.1% (v/v) Tween-20 and 5% (w/v) nonfat dry milk. The membrane was washed three times with TBS, each for 5 minutes, and incubated with rabbit anti-NMDAR1 polyclonal antibody and anti-b-actin antibody (1:250; Beijing Zhongshan Golden Bridge Biological Technology, Beijing, China) at 4°C overnight. Membranes were then incubated in alkaline phosphatase-labeled goat anti-rabbit IgG (1:20 000) at room temperature for 2 hours, and washed with TBS-Tween-20. Development was performed using the enzyme method. Developed bands were analyzed using FlourChemV2.0 software (BKM). Protein expression levels were determined as the absorbance ratio of NMDAR1 to β-actin. The experiment was repeated three times.

In situ hybridization for NMDAR1 mRNA expression

The frontal cortex and hippocampus were serially sliced into sections, deparaffinized, hydrated, immersed in drilling fluid at room temperature for 10 minutes, blocked in hydrogen peroxide at room temperature for 20 minutes, treated with compound digestion fluid, and incubated with prehybridization fluid at 37°C for 2 hours in a wet box. After washing, the specimens were incubated with hybridization fluid, with mouse anti-digoxin biotinylated antibody (Beijing Zhongshan Golden Bridge Biological Technology), and with high-sensitive peroxidase-streptavidin conjugate (Beijing

Zhongshan Golden Bridge Biological Technology). Following development with 3, 3'-diaminobenzidine, a brown cytoplasm represented a positive reaction. The sections were counterstained with hematoxylin, and nuclei were stained blue. Absorbance values were measured using an image analyzer (BKM). Corrected absorbance values were equal to the absorbance of reaction products – background absorbance, *i.e.*, actual absorbance value. Three sections from each specimen were selected, and three visual fields of each section were randomly selected to calculate the average value.

Statistical analysis

Data were analyzed using SPSS 13.0 and expressed as the mean ± SD. The measurement data were analyzed using one-way analysis of variance. Intergroup variables were compared with least-significant-difference paired comparison. Significance was accepted at $P < 0.05$.

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(Edited by Yu D, Lou Y/Qiu Y/Song LP)