

Effects of NMDA-Receptor Antagonist on the Expressions of Bcl-2 and Bax in the Subventricular Zone of Neonatal Rats with Hypoxia–Ischemia Brain Damage

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Abstract Neonatal hypoxia–ischemia brain damage is an important cause of death by affecting prognosis of neural diseases. It is difficult to find effective methods of prevention and treatment due to the complexity of its pathogenesis. *N*-methyl-D-aspartate (NMDA), as an excitotoxicity amino acids, has proven to play an important role in hypoxic–ischemic. However, the exact effects of the NMDA subunits, NR2A and NR2B, during hypoxic–ischemic have not been investigated in detail. Therefore, we sought to study whether the NMDA receptor antagonist could confer neuroprotective effects in a neonatal rat hypoxia–ischemia model. The effects of intraperitoneal injections of different drugs, namely MK-801 (0.5 mg/kg), NVP-AAM077 (5 mg/kg), and Ro25-6981 (5 mg/kg), on the expressions of anti-apoptotic protein Bcl-2 and apoptosis protein Bax in the subventricular zone were analyzed by immunohistochemical staining to explore the roles of NMDA subunits (NR2A and NR2B) in hypoxic–ischemic. We found that the NR2B antagonist (Ro25-6981) could inhibit hypoxic–ischemic with the increasing Bcl-2

expression. NR2A antagonists (NVP-AAM077) can increase cerebral hypoxia–ischemia in neonatal rats, promoting the expression of apoptotic protein Bax.

Keywords NMDA receptor antagonist · Hypoxia–ischemia · Bax · Bcl-2 · Apoptosis

Introduction

Acute hypoxic–ischemic encephalopathy caused by cerebral hypoxia–ischemia brain damage triggers a series of neurological and psychiatric symptoms of clinical syndrome. The interactions between multiple steps and multiple damage factors lead to inflammation, necrosis, and apoptosis in neurons, and secondary functional disorders of the nervous system. Neonatal hypoxia–ischemia brain damage has become one of today's hottest topics in neonatal medicine. Perinatal asphyxial hypoxia–ischemia brain damage, clinical performance of a series of anomalies of the central nervous system, is the important reason causing neonatal deaths, with symptoms including irreversible nervous system damage, cerebral palsy, and epilepsy with pain, often accompanied by severe neurological sequelae such as cognitive impairments [1, 4]. Research has shown that the expressions of Bcl-2 and Bax apoptosis-related proteins and cell apoptosis are closely related. Bcl-2 and Bax protein expressions have been used as apoptosis level indicator [32]. The *N*-methyl-D-aspartate (NMDA) receptor is a type of glutamate receptor. Under physiological conditions, NMDA receptors play a key role in nervous system development, learning and memory, and in glutamate neurotoxicity during hypoxic–ischemic [24]. It has been shown that excessive activation or loss of activation of NMDA receptors has been involved in many

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acute and chronic diseases of central nervous system, such as cerebral ischemia, traumatic brain injury, and mediated excitotoxic neuronal death [7–9, 15, 21, 23, 27]. However, NMDA receptor subunits may play different role in hypoxia–ischemia brain damage. To exam this, expressions of apoptosis-related proteins Bcl-2 and Bax in subventricular zone (SVZ) in hypoxia–ischemia rats were analyzed after intraperitoneal injection of NMDA receptor antagonists. The effects of NMDA receptor antagonist on the SVZ hypoxic-ischemic in rats were also illuminated.

Materials and Methods

Animals and Grouping

One hundred 7-day-old new born Sprague–Dawley rats were provided by the laboratory animal Center of Xuzhou Medical College according to the animal research principles and procedures of the university's Animal Care Committee and National Institute of Health Guide for the Care and Use of Laboratory Animals. P7 Sprague–Dawley rat pups of either sex that weighed between 12 and 18 g were randomly divided into five groups ($n = 20$): Sham group, Model group, MK-801 (MK) group, NVP-AAM077 (NVP) group, and Ro25-6981 (Ro) group.

Equipment and Reagents

MK-801 (M107), Ro25-6981 (R7150), and NVP-AAM077 (P1999) were purchased from Sigma (St-Louis, Missouri). Immunohistochemical SP Kit (SP-9001 Kit) was purchased from Beijing Jin Qiao biotechnology company. Anti-Bax antibody (ab7977) and anti-Bcl-2 antibody (ab7973) were purchased from Abcam(England,United Kingdom), antibody titer of 1:100. Paraffin wax slicing machine (RM2235; Leica, Germany), Photomicrography system (DP25; Olympus, Osaka, Japan), and Image analysis software (Image Pro Plus 6.0; Media Cybernetics, Chicago,USA) were used for sample preparation and analysis.

Hypoxia–Ischemia Model

Hypoxia–ischemia model was induced as previously described with slight modifications [29]. Sham controls were randomly chosen from the same generation of hypoxia–ischemia rats and were given anesthesia but not subjected to hypoxia–ischemia, and the sham control group did not receive any treatment. Members of the Model group were pretreated with an equal volume of saline. After 30 min, the left common carotid artery was clamped. Drug intervention groups (the MK group, NVP group, and Ro group) were injected intraperitoneally with selective

noncompetitive NMDAR antagonist MK-801 (0.5 mg/kg), NR2B antagonist Ro25-6981 (5 mg/kg), and NR2A antagonist NVP-AAM077 (5 mg/kg) 30 min prior to the induction of hypoxia–ischemia. The pups of rats (model group and drug intervention group) were then placed in a container at 37 °C, perfused with the mixed gases of 8 % oxygen and 92 % nitrogen 1.5–2.5 L/min for 2 h. Neonatal rats were taken out after hypoxic treatment and continued for breastfeeding.

Immunohistochemical Staining

Rats were deeply anesthetized by chloral hydrate and intracardially perfused with physiological saline solution followed by 4 % paraformaldehyde. At the end of perfusion, the brains were removed and post-fixed overnight at 4 °C. The tissues were rinsed with water, dehydrated through an ascending series of alcohol, and embedded in paraffin through xylene. Sections were cut at 4- μ m thickness using a Leica paraffin wax slicing machine. The antigen retrieval was performed in citrate antigen retrieval solution in a microwave oven. The sections were cooled to room temperature and transferred to 3 % hydrogen peroxide for 10 min to block endogenous peroxidase. The sections were rinsed with 0.01 M phosphate buffered saline (PBS; pH 7.4) for three times and for 5 min each time, and then placed in blocking buffer containing 10 % goat serum for 1 h at 37 °C. The tissues were incubated with antibody overnight at 4 °C with the dilutions: anti-Bcl-2, 1:100 (Rabbit polyclonal anti-Bcl-2; Abcam); and anti-Bax, 1:100 (Rabbit polyclonal anti-Bcl-2 (Abcam). The sections were rewarmed to room temperature (25 °C), washed in 0.01 M PBS (pH 7.4) for 5 min, and the secondary antibodies (pv-9001) were applied and incubated at 37 °C for 30 min, then washed in PBS three times and each time for 5 min. The sections were treated with DAB solution as recommended by the manufacturer. The stained histochemical reaction product forms within about 5 min. The tissues were washed with distilled water to remove the surplus staining reagent. Immunolabeled sections were microscopically examined, and the images were captured using an Olympus DP25 photomicrography system. Controls received identical treatment except for incubation of primary antibodies.

Statistical Analysis

Two-tailed Student's *t* test and one-way analysis of variance (ANOVA) were used for statistical comparison. Student Newman–Keuls and Dunnet tests were used for post hoc analysis. Data are presented as mean \pm SD; all *P* values <0.05 were considered significant.

Results

Expression of Bax in the SVZ in Each Group

Bax-positive cells showed the typical morphological features of apoptosis, including chromatin condensation and apoptotic bodies after hypoxia–ischemia insults (Fig. 1, a1–a4). Sham group showed no significant neuronal damage, no obvious Bax-positive cells, and no significant cell apoptosis (Fig. 1, a1–a4). The pro-apoptotic protein Bax is normally expressed at a low level in SVZ (Fig. 1, a1–a4). Compared with the model group, the Ro group and the MK group showed the reduced number of Bax protein-positive cells in the SVZ of (Fig. 2a); however, there were more positive cells in the MK group than those in the Ro group (Figs. 1, e1–e4, 2a). Quantification of SVZ neurons revealed that the number of Bax cells in the SVZ of Ro-treated group had an obviously significant difference (Fig. 1, d1–d4, $P < 0.05$, Fig. 2a). The NVP group showed more Bax protein-positive cells than that of any other group, and the number of positive cell had increased constantly during 48 h, with plasma membrane or cytoplasm showing brownish yellow, irregular cell morphology (Fig. 1, c1–c4, $P < 0.05$, Fig. 2a).

Expression of Bcl-2 in the SVZ Cells in Each Group at Different Times

In the Sham group, photographs showed that there was overexpression of positive cells of Bcl-2 in the SVZ zone, more Bcl-2-positive cells than the other groups (Fig. 3, a1–a4, $P < 0.05$, Fig. 4a). A few Bcl-2-positive cells in the SVZ were found in the Model group after hypoxic–ischemic (Fig. 3, b1–b4). But in comparison with Model group, the expression of Bcl-2-positive cells was further decreased; it suggests that hypoxic–ischemic increased in the NVP group (Fig. 3, c1–c4, $P < 0.05$, Fig. 4a). Compared with the MK group, the expressions of Bcl-2-positive cells were increased in the NVP group and in the Model group ($P < 0.05$, Fig. 4a). Further, the Bcl-2 staining results revealed that pretreatment with Ro attenuated neuronal cell apoptosis in the SVZ at 48 h after hypoxia–ischemia insult (Figs. 3, d1–d4, 4a). Also, the number of Bcl-2-positive cells increases in the MK group (Figs. 3, e1–e4, 4a). This indicates that the MK and Ro have some protective effects.

Discussion

The fatality rate of neonatal hypoxia–ischemia brain damage is very high. Neuronal apoptosis leading to loss of neurons is the main pathological features of hypoxia–

ischemia [10], which leads to many nervous system sequelae for patients after treatment. The mechanism of hypoxia–ischemia brain damage is very complex, which is related to excitatory amino acid neurotoxicity, free radical damage, intracellular Ca^{2+} overload, energy metabolism disorder, apoptosis, and so on [33]. In mammals, there are at least two neurogenesis regions in the adult brain, the hippocampal dentate gyrus (HDG) and the SVZ [2]. These two regions are able to produce neural stem cells (NSCs). Neural stem cells are self-renewing, multipotent cells that can differentiate into all the cell types found in the nervous system (including neurons, astrocytes, and oligodendrocytes), which are ideal for repair of nervous system injury cells. Unilateral carotid artery ligation followed by supply of mixed gases of 8 % oxygen and 92 % nitrogen has been used to generate hypoxic–ischemic brain damage model. After 48 h of hypoxia–ischemia, the number of apoptosis-positive cells was increased significantly, and the damage was found mainly in oligodendrocyte precursor cells and NSCs [16]. While leading to ischemic necrosis in the SVZ, the cerebral hypoxia–ischemia will have a greater impact on survival and prognosis for patients. Current means of treatment for hypoxia–ischemia brain damage are still dominated by symptomatic treatment and rehabilitation training on clinical treatment. An effective treatment with high specificity for promoting nerve regeneration is still missing. Survivors of hypoxic–ischemic brain damage may commonly suffer neurological injury [12, 28], which can be devastating to the patient.

Bcl-2 family gained the most attention among a variety of related proteins functioning in apoptosis regulation [30]. In the Bcl-2 family, Bax and Bcl-2 were the most studied. Healthy cells express all classes of Bcl-2 proteins; it was proposed that anti-apoptotic proteins must continually inhibit the function of Bax to ensure the mitochondrial integrity [6].

In normal cells, Bax is distributed in the cytoplasm or bound on the cytoskeleton. Under the hypoxia–ischemia brain damage circumstances, activation of c-Jun proteins regulates the signal transduction pathway and therefore upregulates the Bax promoter [11]. Overexpression of Bax accelerates the mitochondria and endoplasmic reticulum collapse [25]. Bax-mediated promotion of IP3-mediated efflux increases Ca^{2+} concentration of vicinal mitochondria and endoplasmic reticulum, and favors cardiolipin oxidation and promotes cytochrome *c* release [31]. Ca^{2+} is released from the mitochondrial inter-membrane space, and then promotes the activity of endonuclease. Apoptosis is induced by calcium and interaction with anti-apoptotic proteins within the membranes. The inactivation of anti-apoptotic proteins by calcium leads to loss of cell function and

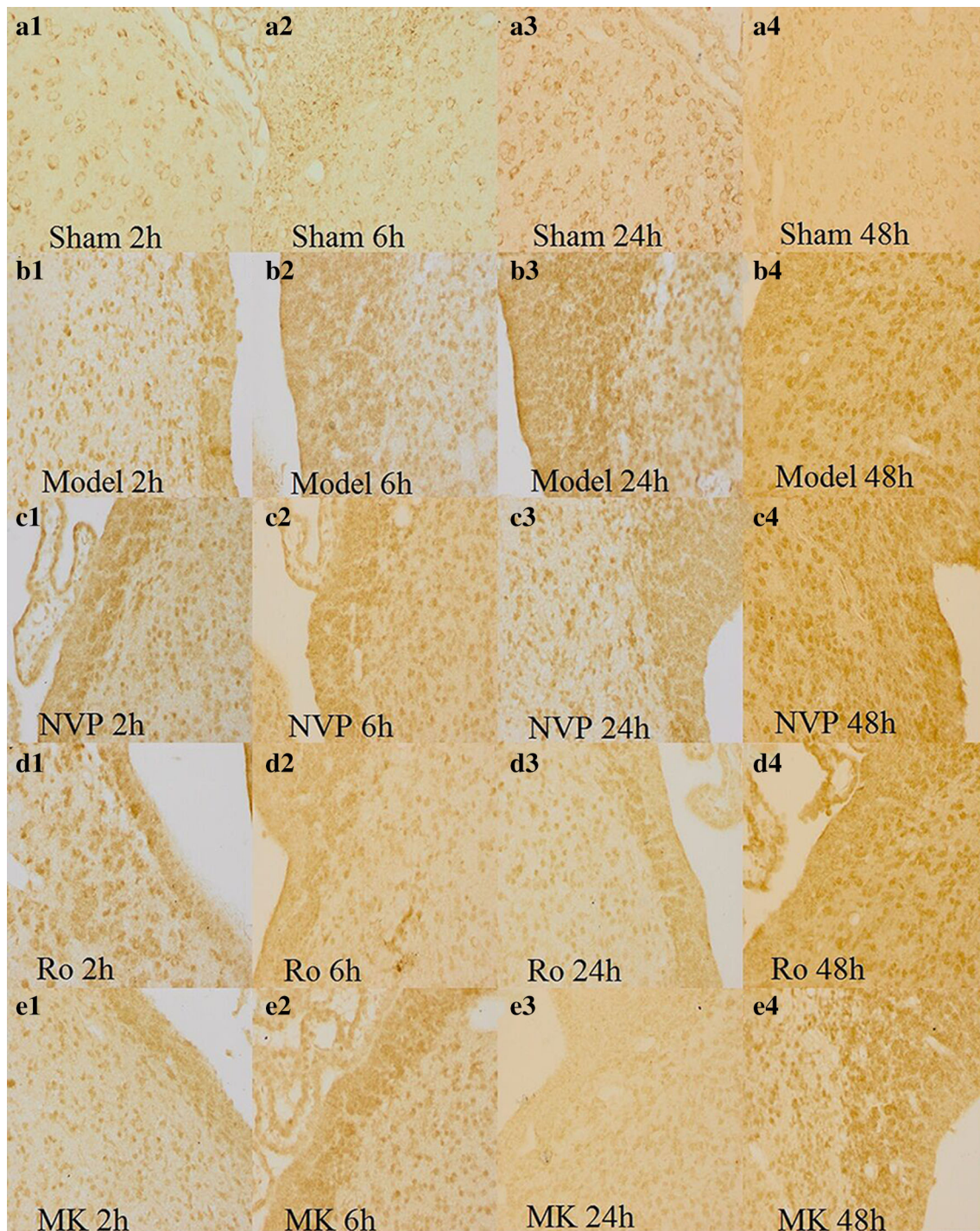


Fig. 1 Bax immune expression in the SVZ of rat brain at postnatal day 7 after hypoxic–ischemic. Brain sections from rat subjected to 2, 6, 24, and 48 h of hypoxic–ischemic following 7 days of apoptosis were used for single immunostaining. There was no significant neuronal damage and no positive cell of Bax in the SVZ (**a1–a4**). However, positive cells appear after hypoxia–ischemia, with gradual increase in the number of positive cells (**b1–b4**). Photographs show more Bax positive cells in the SVZ compared to those of other group

(**c1–c4**). NVP (NR2A antagonist NVP-AAM077) increases the expression of activated Bax after hypoxic–ischemic. The expression of Bax was inhibited by MK (selective noncompetitive NMDAR antagonist MK-801) and Ro (NR2B antagonist Ro25-6981), and the positive cells in the Ro group are less than MK group in the SVZ (**d1–d4**, **e1–e4**). Bar 20 μm . A, mean values of cell counting (from 100 rats). Cell number was counted in one vision under microscope ($\times 400$). * $P < 0.05$ versus sham group; ** $P < 0.05$ versus MK group

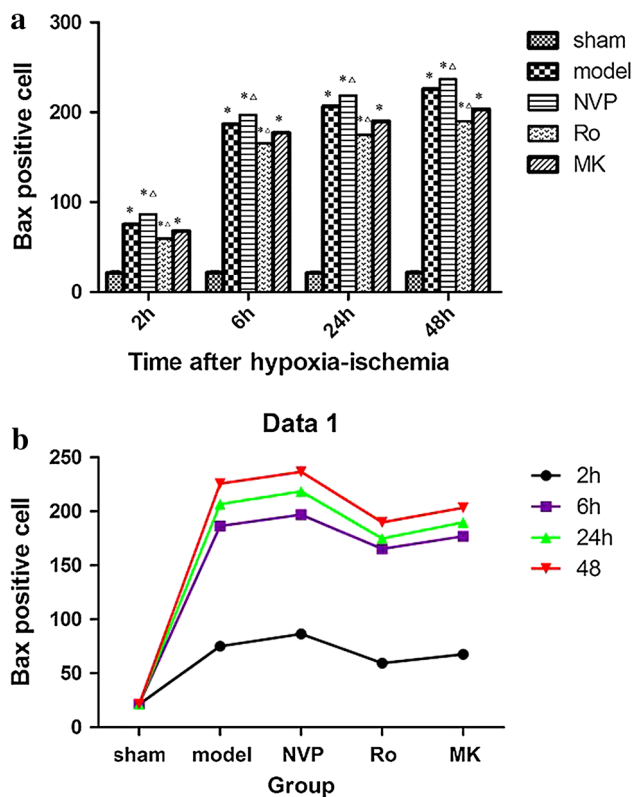


Fig. 2 The comparison of immunohistochemical staining for Bax in the SVZ on coronal section at rats after hypoxic–ischemic with different periods in each group

release of apoptosis factors, eventually leading to apoptosis [19].

Bcl-2 is expressed at low levels in the maturation of brain tissue, but is highly expressed during the development of newborn immature neurons [34]. Bcl-2 is mainly localized at the endoplasmic reticulum membrane and mitochondrial outer membrane in healthy cells and adjusts the permeability of the membranes [17]. When hypoxia–ischemia injury occurs, Bcl-2 acts as an anti-apoptotic protein and interferes with stimuli leading to endoplasmic reticulum Ca^{2+} depletion, thereby contributing to keep the luminal Ca^{2+} concentration at physiological levels [14]. Bcl-2 can adjust cellular redox state after damage stimulus occurs to block oxidation destruction of cell components. Bcl-2 decreases the cellular Ca^{2+} concentration by blocking Ca^{2+} release. Activities of endonuclease and glutamyl transferase are inhibited, and the release of cytochrome *c* from the mitochondria is blocked, which results in decrease in the cell apoptosis [11]. SVZ is a specialized region for neurogenesis in the brain of rats. Bcl-2 overproduction could reduce the apoptosis rate of newborn neurons after hypoxic–ischemic. Bcl-2 may play an important role in growth, development, and differentiation of nerve cells as well as the protection of neurons [13].

There are abundant of excitatory amino acids including NMDA in central nervous system. NMDAR-mediated signals are critical for the survival of developing neurons and for several forms of synaptic plasticity [26]. However, excessive glutamate causes excitotoxicity, which has been implicated in a diversity of neurodegenerative disorders. NMDA receptors belong to ligand-gated ion channels, which are tetramers or pentamers formed by different subunits: the NR1, NR2 (NR2A–NR2D), and NR3 subunits. NR1 is the functional unit, while NR2 is the regulatory unit to modulate the expression of NR1 [5, 18, 20]. After hypoxia–ischemia brain injury, a large number of glutamates were released. Accumulation of glutamate in the synaptic cleft leads to activation of NMDA receptors, and then Ca^{2+} was released from mitochondria and endoplasmic reticulum. When cytosol Ca^{2+} is increased, mitochondria accumulate Ca^{2+} , which leads to cytochrome *c* release from mitochondria to cytosol and activates apoptotic functions, which is an early event in excitotoxic neuronal death [22].

In this study, NMDA receptor antagonists were administered by intraperitoneal injection, and the expressions of Bax and Bcl-2 in SVZ after hypoxia–ischemia were analyzed. Our findings indicated that Bcl-2 was overexpressed in the cells of SVZ of the rat brain at each tested postnatal at different times in the sham group. This result was in agreement with that of a previous report [34]. There are a large number of apoptotic cells in the NVP group and the Model group. Compared with the model group, Bax-positive cells were increased in the NVP group. NVP pretreatment significantly increased the infarction volume in hypoxia–ischemia rats and aggravated brain cell damage. In the NVP group, the expressions of positive cells of Bax pro-protein were increased, and the number of Bcl-2 positive cells was reduced in SVZ. We demonstrated here that blocking NR2A-containing NMDA receptors enhanced neuronal death after hypoxia–ischemia. After Ro pretreatment, the Bcl-2-positive cells were increased significantly, and the number of Bax protein positive cells was reduced in the SVZ. The Ro pretreatment blocked NR2B-containing NMDA receptors, which attenuated hypoxia–ischemia cell death. Our study showed differential roles of NMDA receptor subtypes in hypoxia–ischemia neuronal cell death and hypoxia–ischemia tolerance. Previous studies have shown that complete block of NR2A abolished the neuroprotective effect, but block of NR2B enhanced the neuroprotective effect [5]. NR2A subunit plays a more prominent role in mediating the increase of neuronal vulnerability than NR2B [3]. In this experiment, we have given the rats, NVP 5 mg/kg by intraperitoneal injection. All rats were found dead after hypoxia–ischemia brain injury, and some neonatal rats were dead after intraperitoneal injection of MK-801, which also confirmed previous

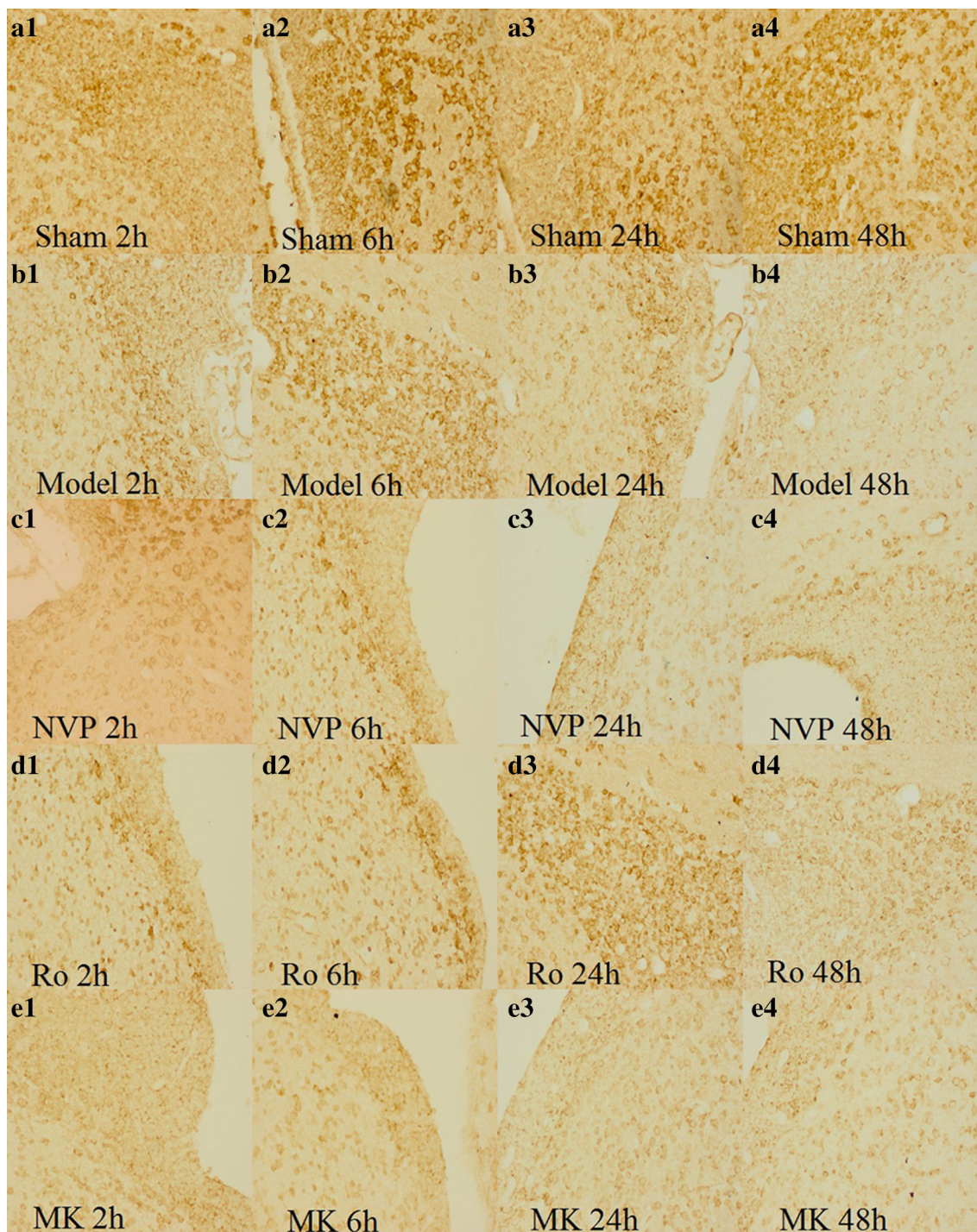


Fig. 3 Bcl-2 immune expression in the SVZ of rat brain at postnatal day 7 after hypoxic-ischemic. The Bcl-2 immune reactivity was very strong at postnatal day 7 compared with the other group, and the positive cell density increased significantly (**a1–a4**). After hypoxic-ischemic, the positive cell density decreased significantly (**b1–b4**), and the Bcl-2 immune reactivity was very low in the NVP group compared to that of the other group (**c1–c4**). However, in the Ro

group and MK group, the cell densities in the SVZ were higher than those in the Model group and NVP group (**d1–d4**, **e1–e4**). Photographs showed more Bcl-2 positive cells in the SVZ compared with the Model group, the NVP group, and the MK group (**d1–d4**). Bar 20 μm . B, mean values of cell counting (from 100 rats). Cell number was counted in one vision under microscope ($\times 400$). * $P < 0.05$ versus sham group; ** $P < 0.05$ versus MK group

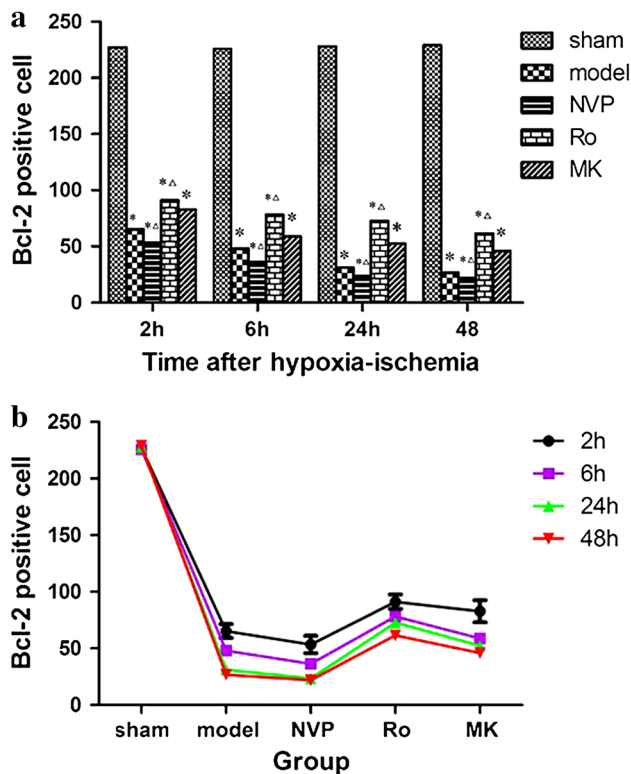


Fig. 4 The comparison of immunohistochemical stainings for Bcl-2 in the SVZ on coronal section at rats after hypoxic-ischemic for different periods in each group

findings. In this study, the effects of NMDA receptor antagonist on the SVZ hypoxic-ischemic in rats were analyzed, we will further investigate its underlying mechanisms.

In conclusion, NVP-AAM077 increased hypoxia-ischemia brain damage, while Ro25-6981 and MK-801 mitigated the SVZ neuron death.

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Conflict of interest The authors declare that they have no conflicts of interest.

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