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GluR1 protects hypoxic ischemic brain damage via activating Akt signaling pathway in neonatal rats

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Abstract. – OBJECTIVE: To investigate the role of glutamic acid receptor 1 (*GluR1*) in hypoxic-ischemic brain damage (HIBD) in neonatal rats and its underlying mechanism.

MATERIALS AND METHODS: 7-day-old neonatal rats received right common carotid artery (CCA) ligation for the establishment of HIBD. After the operation, rats were sacrificed at different time points (0, 4, 6, 12, 24, 48, and 72 h), respectively. Meanwhile, rats in Sham group underwent similar procedures without ligation. Lentivirus-*GLUR1*-shRNA (LV-*GLUR1* shRNA group) was constructed and then transfected into the right lateral ventricles of rats to inhibit *GluR1 in vivo*. Rats received LV-control injection were selected in the control group (LV-control group). After injection of Lentivirus-*GLUR1*-shRNA, CCA ligation was performed in rats for HIBD construction. Western blot was performed to detect the protein levels of *GLUR1*, *Akt*, *p-Akt*, and vascular endothelial growth factor (*VEGF*) in brain tissues. Cell apoptosis was measured by TUNEL staining assay.

RESULTS: After hypoxic ischemia (HI), *GLUR1* expression increased gradually and reached a peak at 24 h. Meanwhile, *p-Akt* expression increased immediately and then gradually decreased. 24 h later, *p-Akt* expression increased again and peaked at 48 h. *VEGF* expression increased at 4 h after HI and reached a peak at 12 h. The expression levels of *GLUR1*, *p-Akt*, and *VEGF* in the brain tissues derived from rats transfected with LV-*GLUR1* shRNA significantly decreased at both 4 h and 24 h after HI. In addition, results indicated that cell apoptosis was enhanced after LV-*GLUR1* shRNA administration, suggesting the role of *GLUR1* in protecting against HIBD.

CONCLUSIONS: *GLUR1* exhibits a remarkable protective role in HIBD, which may be related to the activation of the Akt signaling pathway and the upregulation of *VEGF* after HI.

Key Words:
Hypoxic-ischemic brain damage (HIBD), Lentivirus, *GLUR1*, VEGF, Akt.

Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is the neonatal brain injury contributed by partial or complete anoxia and reduced or ceased cerebral blood flow caused by asphyxia in perinatal period[1,2]. HIE is known as the most important cause of neonatal death and disability[3]. Brain damage resulted from hypoxic ischemia involves multiple biological processes, such as oxygen radicals[4], intracellular overload of calcium[5,6], glutamate (Glu) excitotoxicity[7], inflammatory reaction[8], cell apoptosis[9], and autophagy[10]. Among them, Glu receptor-mediated excitotoxity is extremely important among the above processes.

Glu receptors are divided into two categories, including metabotropic receptor and ionotropic receptor. The latter can be further divided into three subtypes, namely AMPA (Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor, NMDA (N-methyl-D-aspartate) receptor, and KA (kainite) receptor[11]. Some studies[12,13] have shown that the expression of *GLUR1* significantly increased in neonatal rats with HIBD. However, the exact role of *GLUR1* in HIBD still remains unclear.

In this work, we first detected the protein expression of *GLUR1* in the neonatal rat after the onset of HIBD. We aim to explore the correlation between *GLUR1* and the expression levels of *Akt* and vascular endothelial growth factor (*VEGF*) for further elucidating the effect of the *GLUR1*/*Akt* signaling pathway in the recovery of HIBD.

Materials and Methods

Animals

A total of 120 Sprague Dawley (SD) rats aged 7 days (10-12 g in weight) were obtained from Bei-

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jing Vital River Experimental Animal Technology Co., Ltd. (Beijing, China). All SD rats were randomly divided into four groups: the Sham group, the ischemic hypoxic group (HIBD group), the lentivirus-empty group (LV-empty group), and the lentivirus-*GLUR1*-shRNA group (LV-*GLUR1* shRNA group). The Animal Ethics Committee of the Third Affiliated Hospital of Soochow University Animal Center approved this investigation.

Establishment of the HIBD Rat Model

All experiments were performed according to the Institutional Animal Care guidelines. HIBD was induced by the combination of common carotid artery occlusion and hypoxia in postnatal day 7 (P7) rat pups[14]. All rat pups were placed in a 37°C incubator. Under deep ether anesthesia, the right common carotid artery was isolated, double ligated, and cut between the two ligations. After surgery, the rats were first sent to the temperature-controlled incubator to recover for 2 h. Subsequently, they were induced for hypoxia in an enclosed and vented chamber that was partially submerged in water at 37°C for 2 h.

A continuous flow of warmed, humidified gas with 8% oxygen, which was balanced with 92% nitrogen at the flow rate of 0.5 L/mL, was used for hypoxia induction. All HI animals exhibited hemiplegia and anoxia.

HE Staining in Brain Tissues

Slides of brain tissues were stained with hematoxylin-eosin and examined under a light microscope (Olympus BH-2, Tokyo, Japan) by a pathologist in a blinded way. According to the Foster's pathological standard[15], pathological conditions were graded.

Lentivirus-GLUR1-shRNA Transfection

Lentivirus-*GLUR1*-shRNA was purchased from Gemma Gene Company (Shanghai, China). Totally 15 rats were enrolled in the LV-*GLUR1* shRNA group. Lentivirus containing *GLUR1* shRNA was injected into the right lateral ventricles of rats, and then, the HIBD rat model was constructed. Meanwhile, rats that received LV-empty vector injection were used as the LV-control group (n=3).

Western Blot

Proteins were first extracted from brain tissues as previously reported. Protein concentration was measured using the BCA kit purchased from Beyotime (Shanghai, China). Extracted proteins were degenerated and cooled on ice. Subsequently, 20 μg extracted proteins were separated on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with 5% fat-free milk to block non-specific protein interactions at 4°C for 1 h. After that, the membranes were incubated with primary antibodies at 4°C overnight. Then, the membranes were washed three times with Tris-Buffered Saline and Tween 20 (TBST) to remove the unbound antibody (10 min each time). Membranes were incubated with the corresponding secondary antibody conjugated with horseradish peroxide at 25°C for 1 h. After washing three times with TBST, the membranes were visualized by using enhanced chemiluminescence (ECL; Millipore, Billerica, MA, USA) according to the instructions.

TUNEL Assay

A commercial TUNEL kit (Apop Tag Peroxidase In Situ; Chemicon International, Billerica, MA, USA) was used for the fast detection of fragmented DNA in the nucleus by red fluorescence probe labels during cell apoptosis. The apoptotic cells in brain tissue sections were analyzed according to the manufacturer's instructions. The results were expressed as the number of apoptotic cells per mm^2 from selected brain fields or the percentage of apoptotic cells. The number of TUNEL-positive cells in the area surrounding the lesion was counted in six randomly selected sections per mouse using the Image J Software.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 Software (IBM, Armonk, NY, USA) was used for all statistical analysis. Quantitative data were expressed as mean ± standard deviations. Independent samples *t*-test was performed to compare the difference between groups. $p < 0.05$ was considered statistically significant.

Results

Histological Changes After HIBD Establishment

After the construction of an animal model, we first evaluated the behavioral changes of rats in the Sham and the HIBD group, respectively. Rats in the HIBD group showed reduced kinematic flexibility, as well as slow and uncoordinated move-

ment. Meanwhile, these rats also showed tonic convulsion on the opposite side (left). However, no significant behavioral changes were observed in the Sham group after animal procedures.

Subsequently, HE staining was performed in the sections of sub-cortex, hippocampus, and lateral ventricle to verify the successful modeling of HIBD. As shown in Figure 1, normal structure, regular cell arrangement and complete cell outline with intact, center-positioned nucleus and clear entoblast were observed in the Sham group. However, in HIBD group, brain tissues exhibited significant leukoaraiosis, abnormal cell arrangement, and partial or complete neuron degeneration at 1 day and 3 days after HIBD modeling.

Expression of GLUR1 after HIBD

Brain tissues were collected from the right side of rats in the Sham Group. Meanwhile, tissues were collected from the same part of rats in the HIBD group at 0 h, 4 h, 6 h, 12 h, 24 h, 48 h, and 72 h after HI, respectively. Western blot showed that the protein expression of *ILK* increased gradually and reached a peak at 24 h after HI, which was then downregulated. The expression of *GLUR1* was also detected in the brain tissues of rats in the Sham group (Figure 2). The Wes was used as an internal reference.

Expression of p-Akt and VEGF after HIBD

After HI, the protein expression level of *p-Akt* in rats of the HIBD group significantly increased at 4 h. However, it gradually decreased, and the minimal level was attained at 24 h. Subsequently, an elevated expression was found again, and the peak level was reached at 48 h. After HI, the expression of *VEGF* in the HIBD group increased at 4 h and the peak level was attained at 12 h, which was then maintained at a high level. The *VEGF* expression in the HIBD group was significantly higher than that of the Sham group (Figure 3).

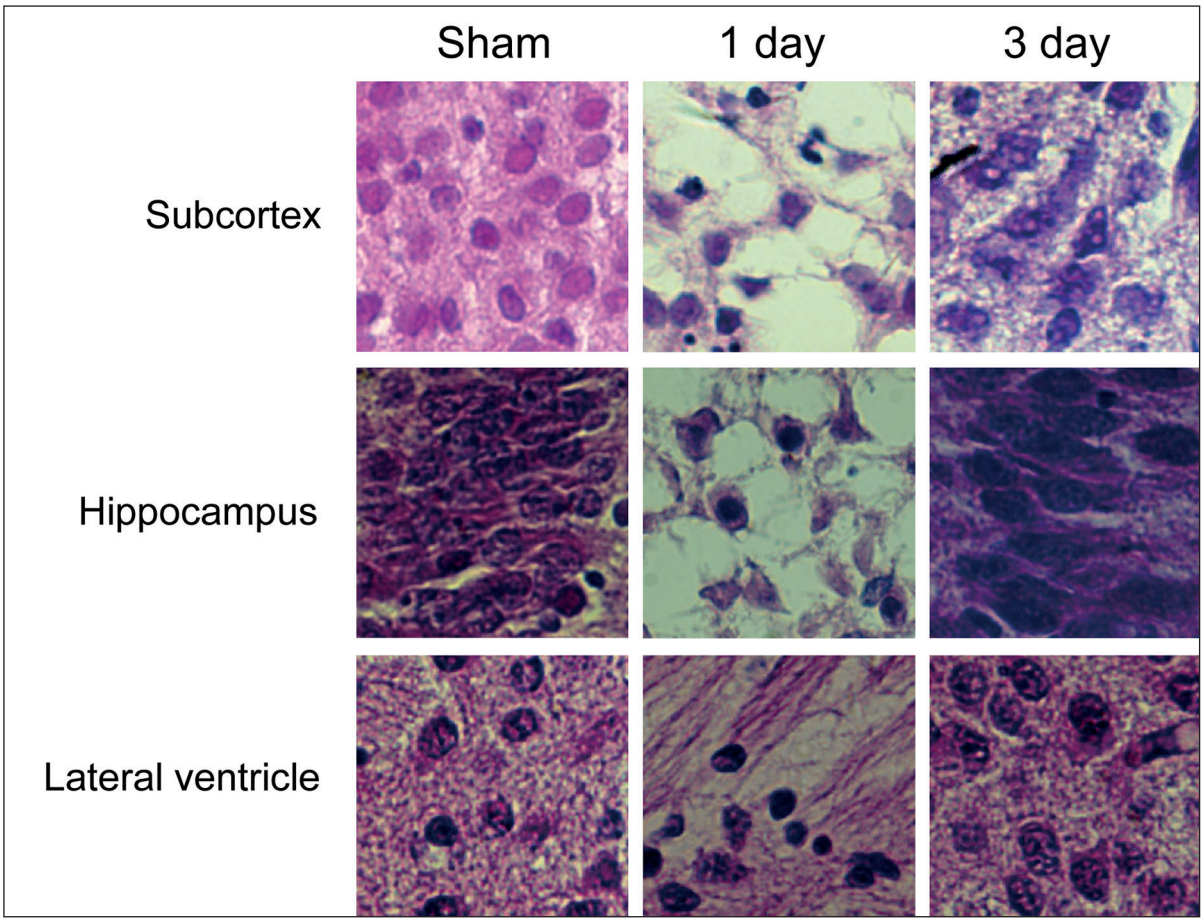

Figure 1. Histological changes after HIBD establishment. HE-staining on the sections of sub-cortex, hippocampus, and lateral ventricle in the Sham group and the HIBD group after 1 d and 3 d, respectively (magnification 200×). HIBD: hypoxic ischemic brain damage.

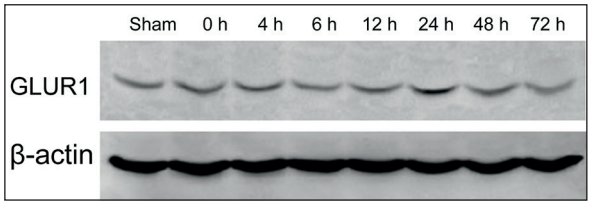

Figure 2. Expression of *GLUR1* after HIBD. Protein expression of *GLUR1* in the Sham group and the HIBD group at 0 h, 4 h, 6 h, 12 h, 24 h, 48 h, and 72 h after HI, respectively. GLUR1: glutamic acid receptor 1. HIBD: hypoxic ischemic brain damage.

Effect of LV-GLUR1 shRNA on the Expression of GLUR1

We then detected the protein expression of *GLUR1* in the right brain tissues after injection of LV-*GLUR1*-shRNA. Results indicated that in the LV-*GLUR1*-shRNA group, *GLUR1* expression first significantly decreased at 2 days and 3 days after the operation, and then increased gradually. Subsequently, it was gradually returned to a similar level as the LV-Empty group. On the third day after the intracerebroventricular injection of LV- GLUR1 shRNA, a minimal level of *GLUR1* expression was observed (Figure 4).

Effect of LV-GLUR1 shRNA on the Expression of p-Akt/Akt and VEGF

Western blot results at 4 h and 24 h after HIBD showed that compared with the Sham group, the protein expression levels of *GLUR1*, *pAkt/Akt,* and *VEGF* were significantly elevated after the intracerebroventricular injection of LV-*GLUR1* shRNA. Meanwhile, the trend in variation was

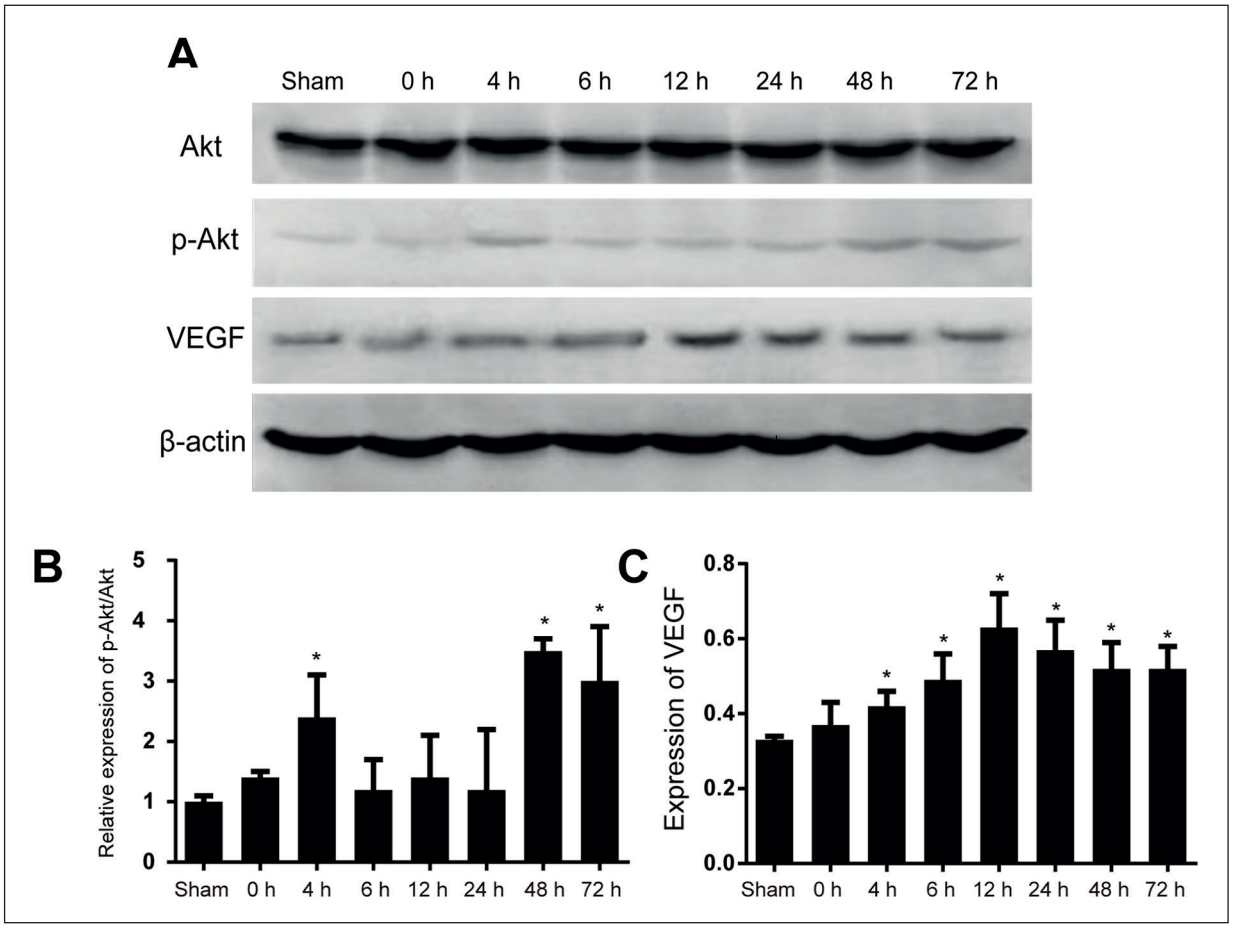

Figure 3. Expressions of *Akt*, *p-Akt* and *VEGF* after HIBD. ***A,*** Protein expressions of *Akt*, *p-Akt* and *VEGF* in the Sham group and the HIBD group at 0 h, 4 h, 6 h, 12 h, 24 h, 48 h, and 72 h after HI, respectively. ***B,*** Relative expressions of *p-Akt*/*Akt* in the Sham group and the HIBD group at 0 h, 4 h, 6 h, 12 h, 24 h, 48 h, and 72 h after HI, respectively. *$p < 0.05$, compared with the Sham group. ***C,*** Expression of *VEGF* in the Sham group and the HIBD group at 0 h, 4 h, 6 h, 12 h, 24 h, 48 h, and 72 h after HI, respectively. *$p < 0.05$, compared with the Sham group. VEGF: vascular endothelial growth factor. HIBD: hypoxic ischemic brain damage.

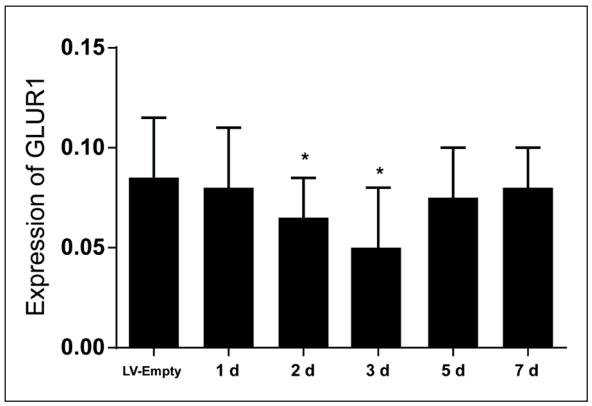

Figure 4. Effect of LV-*GLUR1*-shRNA on the expression of *GLUR1*. Protein expression of *GLUR1* in the LV-Empty group and the LV-*GLUR1* shRNA group at 1 d, 2 d, 3 d, 5 d, and 7 d after LV-*GLUR1*-shRNA injection, respectively.

consistent with that before LV-*GLUR1* shRNA injection. At the same time points after HI, the protein levels of *GLUR1*, *p-Akt,* and *VEGF* in the LV-*GLUR1*-shRNA group were markedly lower than those of the LV-Empty group (Figure 5).

Effect of LV-GLUR1 shRNA on Cell Apoptosis

Cell apoptosis was only identified in a small fraction of cells in the right brain tissues of the Sham group. The apoptotic index in the right brain tissues that received an intracerebroventricular injection of LV-empty shRNA was remarkably higher than that of the Sham group ($p < 0.05$). Moreover, the apoptotic index of cells in the right brain tissues of the LV-*GLUR1*-shRNA group was significantly higher than that of the LV-empty group ($p < 0.05$) (Figure 6).

Discussion

Multiple mechanisms are involved in the occurrence of HIBD, such as oxygen radicals, intracellular overload of calcium, glutamate excitotoxicity, inflammatory reaction, cell apoptosis, and autophagy. Moreover, energy failure, cell membrane depolarization, Glu receptor-mediated excitotoxity, and the voltage-gated Glu excitatory calcium channel play key roles in the above processes[16].

Glu receptors are divided into two categories, including metabotropic receptor and ionotropic receptor. Meanwhile, the ionotropic receptor can be further divided into three subtypes, namely NMDA receptor, AMPA receptor, and KA receptor. As a kind of ligand-gated cation channel, AMPA receptor is characterized by a tetrameric

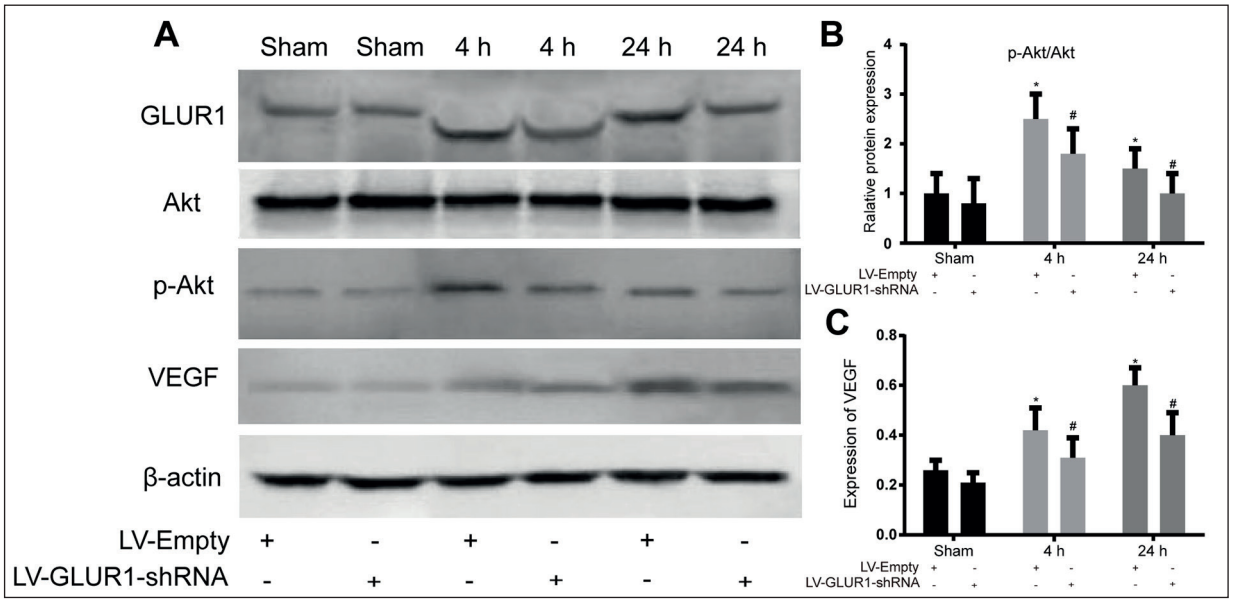

Figure 5. Effect of LV-*GLUR1*-shRNA on the expression of *p-Akt*/*Akt* and *VEGF*. ***A,*** Expressions of *GLUR1*, *p-Akt*/*Akt,* and *VEGF* in the Sham group and the HIBD group at 4h and 24 h after HIBD, treated with LV-Empty and LV-*GLUR1*-shRNA, respectively. ***B,*** Relative protein expressions of *p-Akt*/*Akt*. $^{*}p < 0.05$, compared with the Sham group treated with LV-Empty. $^{\#}p < 0.05$, compared with the Sham group treated with LV-*GLUR1*-shRNA. ***C,*** Expression of *VEGF*. $^{*}p < 0.05$, compared with the Sham group treated with LV-Empty. $^{\#}p < 0.05$, compared with the Sham group treated with LV-*GLUR1*-shRNA. VEGF: vascular endothelial growth factor. HIBD: hypoxic ischemic brain damage.

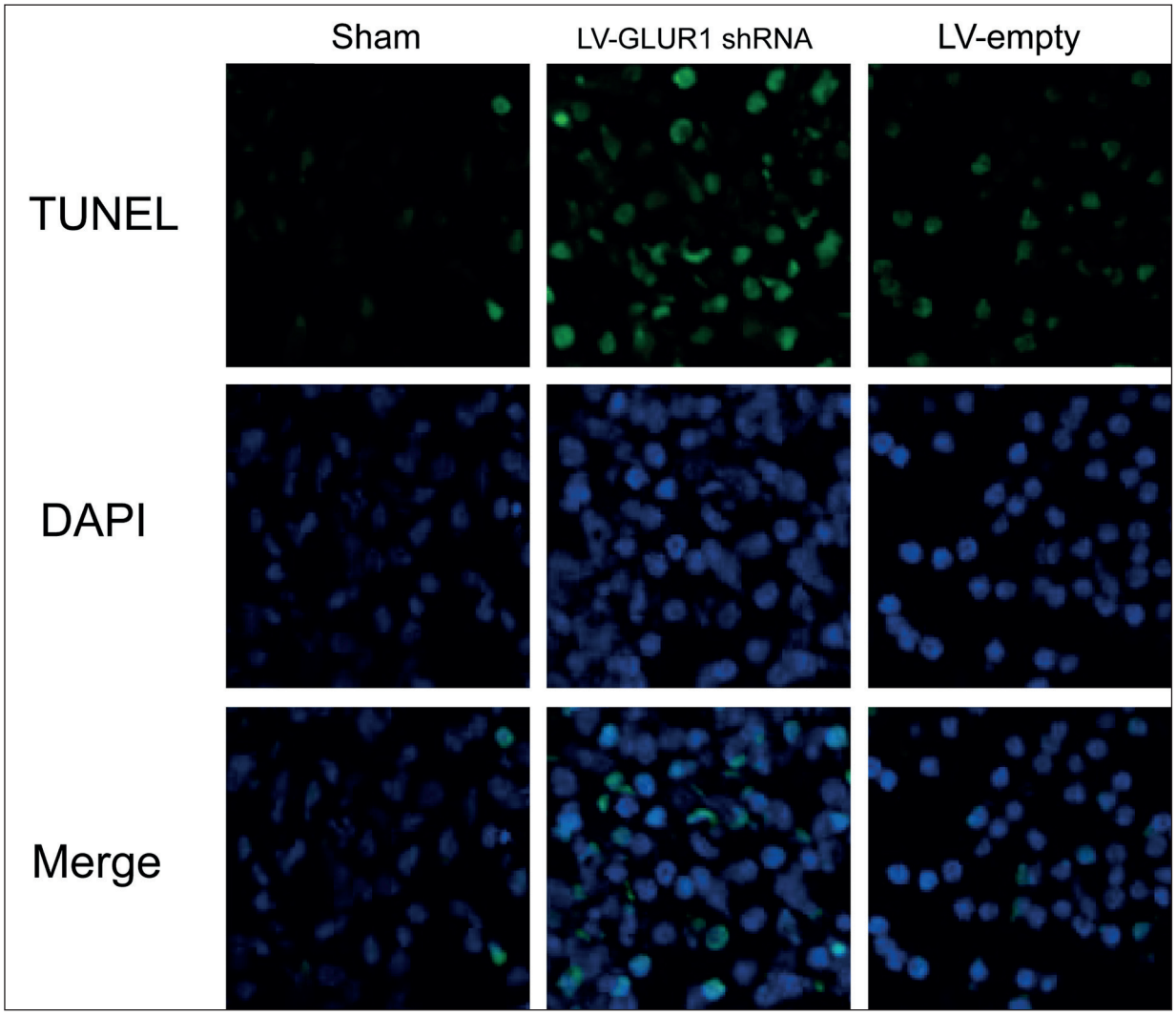

Figure 6. Effect of LV-*GLUR1*-shRNA on cell apoptosis. Apoptosis of brain cells in the Sham group, the LV-Empty group, and the LV-GLUR1-shRNA group, respectively.

structure, where the central hydrophilic channel is surrounded by 4 subunits of Glu receptor (1 to 4)[11]. Generally, high expression of *GluR1* subunit can be found in the AMPA receptor of most neurons[17].

Researches have confirmed that the protein expression of *GluR1* is upregulated at 30 min to 24 h after hypoxia induction in human umbilical vein endothelial cells. Meanwhile, the activity of kinase also increases[18]. After the onset of focal cerebral ischemia in adult rats, the protein expression of *GluR1* temporarily increased, and peak level is attained at 2 h. However, its expression is then downregulated to the level of the control group[19]. Our study revealed that the expression of *GluR1* significantly increased, which reached a peak at 24 h. However, a decrease in *GluR1* expression was detected. The increasing trend was consistent with previous studies, but there was a certain difference in the increasing duration and the time to reach the peak level of *GluR1* expression. This was considered to be related mainly to age, variety, and model of research animal.

Akt is a kind of serine/threonine kinase. Meanwhile, *PIP3* is the lipid metabolite of *PI3K*. The combination of *Akt* and *PIP3* alters the conformation of *Akt*, which subsequently phosphorylates the residues of Thr308 and Ser473, eventually activating *Akt*[20,21]. Activated *Akt* can increase the anti-apoptosis capability of cells and promote cell survival[22]. Currently, *PI3K*/*Akt* is

a widely accepted signaling pathway that regulates the survival and apoptosis of cells through multiple mechanisms, including the inactivation of tpro-apoptotic proteins and the phosphorylation of a series of transcription factors. Therefore, cell apoptosis is regulated at the transcriptional level[23,24]. Many studies have confirmed that hypoxic ischemia can induce the activation of the *PI3K*/*Akt* signaling pathway to upregulate the expressions of *p-Akt* and *HIF-1α*/*VEGF*, thus alleviating neuronal apoptosis and facilitating revascularization[25]. However, different patterns have been found in the variations of *p-Akt* in different focal ischemia rat models. Some models have found that *p-Akt* only increases temporarily at 0.5 h to 6 h after ischemic reperfusion[26,27], whereas other studies have indicated that *p-Akt* can be sustained to 24 h to 72 h after ischemic reperfusion[28]. We found that the expression of *p-Akt* in the HI group significantly increased at 4 h after HI, and then gradually decreased to a minimal level at 24 h, which was still higher than that of the Sham group. Meanwhile, the peak level of *p-Akt* expression was attained at 48 h. Besides, in the HIBD group, the expression of *VEGF* initially increased at 4 h after HI. Meanwhile, the peak level was attained at 12 h and then sustained for a long time, which was consistent with previous studies.

Activated *GLUR1* can activate the *PKB*/*Akt* signaling pathway through the phosphorylation of Ser473 residues, leading to variations in the expressions of downstream molecules such as *VEGF*, and facilitating revascularization[29]. Currently, it is believed that *VEGF* exerts a strong angiogenesis effect[30]. In this study, we found that the expression levels of *GLUR1*, *p-Akt,* and *VEGF* were upregulated in the HIBD rat model. Moreover, the variation trend was in consistency with previous studies, suggesting the existence of the *GLUR1*/*Akt*/*VEGF* signaling pathway after HI in neonatal rats. HI can induce the upregulation of *GLUR1* expression, activate the *PI3K*/*Akt* signaling pathway to increase the expression of *VEGF*, thereby facilitating the survival of neurons and revascularization. Cell apoptosis is also inhibited and the recovery of HIBD in neonatal rats is improved.

RNA inference is a kind of gene-blocking technologies, which is mediated by double-stranded RNA. The expressions of relevant genes may be blocked on the mRNA level after transcription. RNA inference can induce cells with the manifestation of specific genes deletion, which can be served as a very useful tool for researching gene functions and therapies[31]. In this study, we successfully constructed LV-*GLUR1*-shRNA, in which shRNA containing in a lentiviral vector was delivered into neurons to suppress the expression of *GLUR1* gene, thus inhibiting its protein expression. In this study, we found that at 4 h and 24 h after HIBD, the protein expression of *GLUR1* in the LV-GLUR1-shRNA group was significantly lower than that of the control group. Meanwhile, the protein expression levels of downstream molecules of *GLUR1* such as *p-Akt* and *VEGF* were also markedly lower than those of the control group. Besides, at 24 h after HI, the apoptotic index of neurons in the LV-GLUR1-shRNA group was remarkably higher than that of the control group. However, this is a pilot study. More animal and clinical studies are still needed in the future.

Conclusions

We found that *GLUR1* knockdown can decrease the protein expression levels of *p-Akt* and *VEGF*, as well as increase cell apoptosis. Our results confirmed that the *GLUR1*/*Akt* signal transduction pathway may regulate *VEGF* after HIBD, facilitate the survival of neurons and participate in the recovery of HIBD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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