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Original Research

Glycine Receptor Beta Subunit (GlyR-β) Promotes Potential Angiogenesis and Neurological Regeneration during Early-Stage Recovery after Cerebral Ischemia Stroke/Reperfusion in Mice

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Abstract

Background: Ischemic stroke is mainly caused by cerebral artery thrombosis. This study investigated the role of glycine receptor beta subunit (GlyR-β) in the recovery from cerebral ischemia stroke/reperfusion. **Methods**: The oxygen glucose deprivation and recovery (OGD/R) bEnd3 cell model and the middle cerebral artery occlusion/reperfusion (MCAO/R) mouse model were used in this study. **Results**: Expression of both the *GlyR-β* gene and vascular endothelial growth factor (*Vegf*), cell proliferation, and tube formation ability was decreased in bEnd3 cells after OGD/R, and was reversed by overexpression of GlyR-β. Neurological function, asindicated by Zea Longa scores, area of cerebral ischemia, and pathological changes were increased in mice after MCAO/R, and were ameliorated by overexpression of the glycine receptor beta (*Glrb*) gene at 24 h and 7 d after MCAO/R. Expression of GlyR-β and Gap-43 was decreased, and the expression of CD34, Vegf, and Bdnf, and cell growth as determined by a bromodeoxyuridine (BrdU) assay, increased in the affected brain tissue of MCAO/R mice in a time-dependent manner. GlyR-β overexpression resulted in enhanced expression of CD34, Vegf, Growth association protein 43 (Gap-43), and brain-derived neurotrophic factor (Bdnf) and cell growth in affected brain tissue of MCAO/R mice in a time-dependent manner. **Conclusions**: GlyR-β promoted potential angiogenesis and neurological regeneration in affected brain tissue, thus promoting recovery from cerebral ischemia stroke/reperfusion.

Keywords: stroke; ischemia and reperfusion injury; recovery; GlyR-β

1. Introduction

Ischemic stroke is mainly caused by cerebral artery thrombosis [1]. Cerebral artery occlusion leads to secondary thrombosis of downstream microvessels, leading to dysfunction of brain endothelial cells, nerve cells, and astrocytes; destruction of the blood brain barrier; and ischemic cell damage. The primary goal of the treatment of ischemic stroke is to rapidly reopen the occluded vessels, reinstate cerebral vascular flow in the ischemic cerebral microvascular bed, maintain the integrity of the vessels, and reduce the necrosis of neurons to promote recovery. Recovery from ischemic stroke is a dynamic and complex process, involving angiogenesis [2,3], nerve regeneration [4–6], mobilization of the immune system [7–9], and signaling pathways such as phosphatidylinositol 3-kinase/threonine protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) [10]. The underlying molecular mechanism is not completely clear.

The glycine receptor (GlyR) is a pentameric ligand-gated ion-channel that mediates fast inhibitory neurotransmission in the brain stem, spinal cord, and retina [11]. GlyR could also control sensory functions and motor functions, including audition and vision [12]. GlyR is an inhibitory pentamer complex formed by alpha- (α-) and beta- (β-) subunits. While α-subunits bind ligands, β-subunits play a structural role. The glycine receptor beta subunits (GlyR-β) not only regulate and maintain structural integrity but may also play a functional role in binding of the GlyR ligand [13]. A study by Fujita *et al*. [14] revealed that GlyR-β is widely and abundantly expressed in the rat brain. A mouse glycine receptor beta (*Glrb*) mutation leads to a spastic phenotype [15]. A polymorphism in exon 8 has been found in both healthy people and families affected by hyperventilation syndrome [16]. In patients with diseases carrying *Glrb* mutations, loss or acquisition of related functions or changes in synaptic GlyR aggregation are observed [17,18]. *Glrb* mRNA levels are continuously decreased in neurovascular units at 24 h and on the 7th day after stroke in a cerebral ischemia/reperfusion mouse model [19]. Accordingly, *GlyR* might play a critical role in the development of cerebral ischemia. However, the role of the *Glrb* gene in neurovascular injury and regeneration after stroke is still unclear.

In this study, we investigated the *GlyR-β* expression profile and the role of *GlyR-β* expression during the recovery period from stroke ischemia/reperfusion using the oxygen glucose deprivation and recovery (OGD/R) mouse brain microvascular endothelial cell line (bEnd3) and the

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middle cerebral artery occlusion/reperfusion (MCAO/R) mouse model. We found that the expression of the *Glrb* gene continuously decreases in damaged brain areas after reperfusion from cerebral ischemic stroke. Overexpression of the *Glrb* gene promoted potential angiogenesis and neurological regeneration, thus promoting brain tissue repair after cerebral ischemic stroke/reperfusion. Our findings provide a clue to understanding underlying mechanisms and identifying a target for intervention to promote recovery after cerebral ischemic stroke reperfusion.

2. Materials and Methods

2.1 Constructs

The plasmid pcDNA3.1-EGFP-Glrb was constructed for the overexpression of the *Glrb* gene based on the full-length coding sequence (CDS) of the mouse *Glrb* gene (Gene ID: 14658). The CDS DNA fragment with an NheI restriction endonuclease site (gctagc) at the 5′ end and an EcoRI restriction endonuclease site (gaattc) at the 3′ end was synthesized (Sangon, Shanghai, China) and cloned into the plasmid vector pcDNA3.1-EGFP (enhanced green fluorescent protein) (catalogue number P1038, Miaoling Biology, Wuhan, China) after modification using NheI (catalogue number 1622, TaKaRa, Tokyo, Japan) and EcoRI (catalogue number 1611, TaKaRa, Japan). The construct was verified by Sanger sequencing. The expression efficiency of the construct was tested in bEnd3 cells.

The adenoviral construct pDC316-mCMV-EGFP-Glrb for the overexpression of the *Glrb* gene was constructed using the full-length CDS of the mouse *Glrb* gene (Gene ID: 14658). The CDS DNA fragment with an ApaI site (gggccc) at the 5′ end and a PstI site (ctgcag) at the 3′ end was synthesized by Sangon (Shanghai) and cloned into the vector pDC316-mCMV-EGFP (catalogue number P1691, Miaoling Biology, China) after modification using ApaI (catalogue number 1604, TaKaRa, Japan) and PstI (catalogue number 1624, TaKaRa, Japan). The construct was verified by Sanger sequencing. A two-plasmid system consisting of the shuttle plasmid pDC316-mCMV-EGFP and the skeleton plasmid pBHGlox (delta) E1, 3cre, and 293A cells was used for virus packaging. The titers of pDC316-mCMV-EGFP and pDC316-mCMV-EGFP-Glrb were 1.2×10^9 TU/mL and 1.0×10^9 TU/mL, respectively.

2.2 Culture and Treatment of Cells

The mouse brain microvascular endothelial cell line bEnd3 was purchased from Beina Bio (Beijing, China). Cells were cultured in dulbecco's modified eagle medium (DMEM) medium (catalogue number 11995-065, Gibco, Grand Island, NY, USA) supplemented with 1% penicillin-streptomycin (catalogue number C0222, Beyotime, Shanghai, China) and containing 10% fetal bovine serum (catalogue number 10099-141, Gibco, USA) at 37 °C and 5% CO_2. The bEnd3 cell line was validated and tested negative for mycoplasma.

The bEnd3 cells were divided into three groups: the Control group (CTL; bEnd3 cells were cultured normally and then transfected with a blank vector pcDNA3.1-EGFP), the OGD/R group (bEnd3 cells were subjected to oxygen glucose deprivation and recovery (OGD/R) and then transfected with a blank vector), and the OGD/R+Glrb group (bEnd3 cells were subjected to OGD/R and then transfected with the pcDNA3.1-EGFP-Glrbvector). To establish the OGD/R cell model, bEnd3 cells in the logarithmic growth phase were cultured in glucose-free medium under hypoxic conditions for 2 h and then cultured in normal medium under normal conditions. After 24 h, the bEnd3 cells were transfected with GlyR-β overexpression and/or blank vectors using Lipofectamine 2000 (catalogue number 31985-070, Invitrogen, Carlsbad, CA, USA) for 4–6 h. The cells were then cultured using completely fresh medium for 48 h prior to use.

2.3 Quantitative Polymerase Chain Reaction (qPCR)

The levels of mRNA of *Glrb*, *Vegf*, *Bdnf*, and Growth association protein 43 (*Gap-43*) were determined using quantitative polymerase chain reaction (qPCR). Total RNA was extracted from cell and tissue samples using RNAiso (catalogue number 9109, Takara, Tokyo, Japan). Complementary DNA (cDNA) was synthesized using random primers and the Hifair ® II kit (catalogue number 11121ES60, Yeasen, Shanghai, China). The qPCR reaction was carried out using specific primers, SYBR green I fluorescent dye (catalogue number 10222ES60, Yeasen, China), and Hieff UNICON Universal Blue qPCR SYBR Green Master Mix (catalogue number 11184ES03, Yeasen, China) on a qPCR system (Model Stepone plus, ABI, Norwalk, CT, USA). The qPCR reaction conditions were as follows: Initial, 95 °C, 2 min; Denaturation, 95 °C, 10 s, 40 cycles; Annealing/extension, 60 °C, 30 s; In the melting curve stage, the instrument was set to default mode. The primers included *Gapdh*-F, CAGAAGGGGCGGAGATGAT; *Gapdh*-R, AGGCCGGTGCTGAGTATGTC; *Glrb*-F, GTTGTTTGAAGACGCTTGTGCT; *Glrb*-R, AGGAATGCCTTTGAAGTTTGGT; *Vegf*-F, ATCTTCAAGCCGTCCTGTGTGC; *Vegf*-R, ACTCCAGGGCTTCATCGTTA; *Bdnf*-F, TCTGACGACGACATCACTGGCT; *Bdnf*-R, CCTTATGGTTTTCTTCGTTGGG; *Gap-43*-F, CCAAACAGGTTGAAAAGAATGAT; and *Gap-43*-R, AGCTTTTTCCTTGTTATGTGTCCA. *Gapdh* was used as an internal reference gene. The fold changes were calculated using $2^{(-\Delta\Delta Ct)}$.

2.4 Western Blotting

The bEnd3 cells were lysed in cell lysis buffer (catalogue number P0013, Beyotime, Shanghai, China) after treatment. Concentrations of protein were verified using a bicinchoninic acid assay (BCA) kit (catalogue number P0010, Beyotime, China). The samples were

loaded and resolved on an sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel by electrophoresis. Protein in the SDS-PAGE gel was transferred onto a polyvinylidene fluoride (PVDF) membrane (catalogue number FFP24, Beyotime, China). After blocking using 5% skimmed milk, the PVDF membranes were incubated using the rabbit anti-Glrb antibody (catalogue number 15371-1-AP, Protentech, Wuhan, China) and the rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (catalogue number ET1601-4, Huabio, Zhejiang, China) (1:1000 dilution) at 4 °C overnight. After being probed with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (catalogue number A0208, Beyotime, China) (1:1000 dilution) at room temperature for 1 h, the membrane was incubated with enhanced chemiluminescence (ECL) solution (catalogue number P1050, Applygen, Beijing, China) for signal development. The signal was detected using a Tanon-4200 Gel Imaging System (Model Tanon-4200, Tanon, Shanghai, China).

2.5 CCK-8 Assay

Proliferation activity of the cells in each group was determined using the cell counting kit-8 (CCK-8) assay. After OGD/R and transfection, the bEnd3 cells were inoculated in 96-well plates at a density of 5000–10,000 cells per well. After culturing for 72 h, CCK-8 reagent (catalogue number C0039, Beyotime, China) was added, followed by incubation for 1 h. Absorbance was measured at a wavelength of 450 nm. Cell survival rates were calculated.

2.6 Tubing Formation Assay

The bEnd3 cells that had been cultured for 72 h after OGD/R and transfection treatment in 6-well plates were collected and diluted to a density 8×10^5 cell/mL in medium. An aliquot of 50 µL cell suspension was added to Matrigel (catalogue number 354230, BD Biosciences, San Jose, CA, USA) and cultured overnight. Tubing formation was observed and recorded after 24 h. Quantitative analysis was carried out using Image-J software (Version 1.8.0.345, National Institutes of Health, Bethesda, MD, USA), following published protocols [20,21]. Three representative images from different field views in each treatment group were analyzed.

2.7 Immunocytochemistry and Immunohistochemistry

Vegf expression in bEnd3 cells was detected using immunocytochemistry. After treatment, bEnd3 cells were fixed on cover slips using 4% paraformaldehyde (catalogue number C104188, Aladdin, Shanghai, China) and incubated with 0.5% Triton X-100 (catalogue number A600198, Sangon, Shanghai, China) for 20 min, then incubated with 3% H_2O_2 for 15 min at room temperature. After washing with phosphate buffered saline (PBS), cells were treated with Vegf antibody (dilution ratio 1:200, catalogue number bs-1665R, Bioss, Beijing, China) at 4 °C overnight. After rewarming for 30 min at room temperature and washing with PBS, the cells were incubated with the secondary antibody (HRP-labeled, dilution ratio 1:50, goat anti-rabbit IgG, catalogue number A0208, Beyotime, China) at room temperature for 1 h. After washing with PBS, 3,3′-diaminobenzidine (DAB) was applied to the cells for 2 min and then they were washed with PBS. The cells were stained using Hematoxylin (catalogue number C0107, Beyotime, Shanghai, China) for 5 min and then washed with tap water. The stain was sealed with neutral gum and observed and recorded under a microscope (Model DM750, Leica, Wetzlar, Germany).

Expression of the GlyR-β, CD34, Vegf, Bdnf, and Gap-43 proteins in the mouse brain tissue was determined using immunohistochemistry. The brain tissue was washed with PBS, fixed with 4% paraformaldehyde, sequentially dehydrated with gradient ethanol, treated with xylene, embedded in paraffin, and cut into 5-µm slices. After being placed on coverslips, the slices were dewaxed using xylene, rehydrated with ethanol, and then treated with citrate buffer at between 92 °C and 98 °C for 10–15 min. After cooling to room temperature and washing with PBS, 0.5% TritonX-100 was added to the sliced tissue and they were incubated at room temperature for 20 min. Following, the slices were incubated with 3% H_2O_2 at room temperature for 15 min and then incubated with the primary antibody (dilution ratio 1:200) at 4 °C overnight and then incubated with the secondary antibody (HRP-labeled, dilution ratio 1:50) for 60 min. The slices were washed with PBS after each treatment. DAB was applied to the slices for 1–10 min and then they were washed with distilled water twice for 5 min. The slices were then stained using hematoxylin for 5 min, washed with tap water, sequentially dehydrated with ethanol (75, 85, 95, 100%), treated with xylene, and finally sealed with neutral gum. The slices were observed and recorded under a microscope (Model DM750, Leica, Germany). The following antibodies were used: Bdnf (rabbit, catalogue number 25699-1-AP, Protentech, Wuhan, China), CD34 (rat, catalogue number ab8158, Abcam, Cambridge, UK), Gap-43 (rabbit, catalogue number ab75810, Abcam, Cambridge, UK), Vegf (rabbit, catalogue number bs-1665R, Bioss, China), and HRP-labeled goat anti-rat IgG (catalogue number A0192, Beyotime, China).

2.8 Animal Model and Treatment

Specific pathogen-free (SPF) healthy adult male C57BL/6 mice (6–8 weeks old, 20–25 g in weight, n = 56) were purchased from Chengdu Yaokang Biotechnology Co., Ltd. (Sichuan, China) and kept in the internal animal facility with a 12 h/12 h light/dark cycle, free access to food and water, at 23–25 °C for 1 week. All animals were injected with 50 mg/kg BrdU (Cat. No. 40204ES60, YEASEN, Shanghai, China) intraperitoneally 7 days prior to operation. The animals were divided into three groups: the Control group (CTL, n = 16) (the

mice were sham-operated and injected with the blank vector pDC316-mCMV-EGFP), the MCAO/R group (n = 16) (the mice were subjected to MCAO/R and then injected with the blank vector pDC316-mCMV-EGFP), and the MCAO/R+GlyR-β group (n = 16) (mice were subjected to MCAO/R and then injected with the vector pDC316-mCMV-EGFP-Glrb). According to occlusion of the right middle cerebral artery (MCA) in mice using the Longa thread embolism method, an improved model of acute local cerebral ischemia/reperfusion was established. Reperfusion was performed 2 h after ischemia. A total of 40 mice were subjected to MCAO/R. After 3–4 h, the mice were subjected to Zea Longa Grade 5 neurological deficit evaluation. Mice with scores 2–3 were selected and randomly divided into the MCAO/R group and the MCAO/R+GlyR-β group. The expression vector pDC316-mCMV-EGFP-Glrb (titer $\geq 1 \times 10^{11}$ PFU/mL) was then injected at 1 µL/min into the right ventricle (ischemic) at a dose of 5 µL/mouse. The CTL group and the MCAO/R group were injected with equal amounts of the blank vector pDC316-mCMV-EGFP. Zea Longa Grade 5 neurological deficit was evaluated at 24 h and on the 7th day after ischemia reperfusion. After anesthesia with 1.25% tribromoethanol (0.2 mL/10 g; Cat. No. T161626, Aladdin, Shanghai, China), the abdominal and thoracic cavities of the mice were opened. After perfusing the heart with physiological saline, it was then perfused with polyformaldehyde. After perfusion, mice were killed by cervical dislocation, and the skull was opened to remove brain tissues for pathological and molecular examination.

2.9 TTC Staining of Mouse Brain Tissues

The mice were anesthetized by intraperitoneal injection of 1.25% tribromoethanol at a dose of 0.2 mL/10 g and the cerebral tissue was rapidly collected. After rapid freezing at –80 °C for 5 min, the brain tissue was cut into 2-mm thick slices. The slices were placed in 1% triphenyltetrazolium chloride (TTC) (catalogue number T8877, Sigma, St. Louis, MO, USA) and incubated in a 37 °C water bath for 15 min. The stained sections were removed and fixed in 4% paraformaldehyde (catalogue number A500684, Sangon, Shanghai, China) for 24 h. Infarcted areas were evaluated and recorded photographically. Normal tissue was stained red, while ischemic tissue was paler. Quantitative analysis was performed using Image-J software (National Institutes of Health, USA) following the published protocol [21,22].

2.10 Hematoxylin and Eosin Staining

The mouse brain tissue sections were stained with hematoxylin dye (catalogue number D005-1-3, Nanjing Jiancheng, Jiangsu, China) for 10 min and then rinsed with tap water for approximately 5 min and with distilled water for several seconds. After incubation with 1% hydrochloric acid alcohol for decolorization, the sections were stained with eosin dye (catalogue number C0109, Beyotime, China) for 30 s, and then rinsed with tap water for 3 min and with distilled water for seconds. The stained tissue sections were sealed with neutral resin (catalogue number E675007, Sangon, China) and then examined and recorded under a microscope (Model DM750, Leica, Germany). Histopathological damage scores were determined following the published literature [23]: 0, without morphological damage; 1, slight damage with edema or dark neurons; 2, moderate damage with edema or hemorrhages; and 3, severe damage with local damage.

2.11 BrdU Assay

The regeneration of mouse brain cells was determined using the BrdU assay. BrdU (50 mg/kg) was injected intraperitoneally 7 days before the operation. The mouse brain tissue was rapidly frozen at –80 °C, embedded with optimal cutting temperature compound (OCT), and frozen. The embedded tissue blocks were cut to obtain 5-µm slices using a freezing microtome (CM3050 S, Leica). Slices were incubated with adequate HCl (2 M) drops at 37 °C for 30 min. Tissue sections were subjected to immunohistochemistry using the primary antibody for BrdU (dilution ratio 1:200, mouse anti-BrdU, catalogue number 66241-1-Ig, Proteintech, Wuhan, China) and the secondary fluorescent antibody Cy3 (dilution ratio 1:800, goat anti-mouse IgG H&L, Cy3®, catalogue number ab97035, Abcam, UK). 4,6-diamino-2-phenyl indole (DAPI) (catalogue number C1006, Beyotime, China) was applied to stain the nuclei of cells. The samples were sealed with anti-fluorescence quenching agent and then examined and recorded under a laser scanning confocal microscope (TCS SP2 AOBS, Leica, Germany). BrdU positive cells were counted. Three representative images from different field views from each treatment group and time point were analyzed.

2.12 Statistical Analysis

Quantitative data were statistically analyzed using Prism Graphpad 8.0 (GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used to analyze statistical differences among the cell model experiment groups. Two-way ANOVA was used to analyze statistical differences among the animal model experiment groups. Tukey's multiple comparison test was used for the single time point treatment groups. Sidak's multiple comparison test was used for the different time points of the same group. $p < 0.05$ was defined as indicating a significant difference.

3. Results

3.1 Expression of the Glrb Gene and Proliferation of bEnd3 Cells Decreased After OGD/R and Was Reversed by Overexpression of GlyR-β

To investigate the expression and role of *Glrb* during ischemic stroke recovery, we cultured bEnd3 cells under normal conditions (the CTL group) or subjected the cells

to OGD/R treatment, which mimicked the ischemic stroke condition, and then transfected them with the pcDNA3.1-EGFP (the OGD/R group) or pcDNA3.1-EGFP Glrb plasmid (the OGD/R+GlyR-β group), and determined the *Glrb* mRNA and protein levels, and cell proliferation. The results showed that pcDNA3.1-EGFP Glrb was overexpressed in bEnd3 cells (Fig. 1a). The levels of *Glrb* mRNA (Fig. 1b) and protein (Fig. 1c,d) in the OGD/R group were decreased compared with the CTL group. They were significantly higher in the OGD/R+GlyR-β group than in the OGD/R group. The proliferation of bEnd3 cells decreased after OGD/R and was reversed by *GlyR-β* overexpression (Fig. 1e). The results suggested that expression of the *Glrb* gene and proliferation of bEnd3 cells decreased after OGD/R. Transfection of the pcDNA3.1-EGFP Glrb plasmid reversed these decreases.

3.2 Overexpression of the Glrb Gene Promoted Tube Formation Ability and Vegf Expression in bEnd3 Cells After OGD/R

To investigate the role of *GlyR-β* during recovery from ischemic stroke, we determined the tube formation ability and expression of Vegf of bEnd3 cells in the CTL group, the OGD/R group, and the OGD/R+GlyR-β group. The results showed that tube formation abilities in the OGD/R group decreased compared with those of the CTL group, and were significantly higher in the OGD/R+GlyR-β group than in the OGD/R group (Fig. 2a–c). The immunocytochemical results showed that the expression of Vegf protein in the OGD/R group also decreased compared with the CTL group. However, expression was significantly higher in the OGD/R+GlyR-β group than in the OGD/R group (Fig. 2d). The results suggested that the tube formation ability and Vegf expression of bEnd3 cells decreased significantly after OGD/R, and transfection of the pcDNA3.1-EGFP Glrb plasmid reversed those decreases.

3.3 Expression of Glrb Gene Decreased in the Brain Tissues of MCAO/R Mice in a Time-Dependent Manner

To investigate the expression and role of *GlyR-β* during ischemic stroke recovery, we examined *Glrb* mRNA and protein expression in mouse brain tissue in the CTL group, the MCAO/R group, and the MCAO/R+GlyR-β group using qPCR and immunohistochemical assay. The results showed that the level of *Glrb* mRNA (Fig. 3a) and the expression of proteins (Fig. 3b) in the brain tissue of the MCAO/R group were significantly lower than those of the CTL group at 24 h and on the 7th day. They were significantly higher in the MCAO/R+GlyR-β group than in the MCAO/R group at 24 h and on the 7th day, but still significantly lower than in the CTL group. They were significantly or tended to be lower on the 7th day than those at 24 h in the MCAO/R group and the MCAO/R+GlyR-β group. The results suggested that the expression of the *Glrb* gene in the damaged brain tissue of MCAO/R mice decreased continuously in a time-dependent manner. This decrease was reversed by infection with the pDC316-mCMV-EGFP Glrb adenovirus.

3.4 Overexpression of the Glrb Gene Improved Neurological Function, the Area of Cerebral Ischemia, and Pathological Changes in MCAO/R Mice

We examined the neurological function of mice in the CTL group, the MCAO/R group, and the MCAO/R+GlyR-β group using Zea Longa Grade 5 neurological deficit scores. The results showed that the Zea Longa score in the MCAO/R group was significantly higher than that in the CTL group at 24 h and on the 7th day (Fig. 4a). The score was significantly lower in the MCAO/R+GlyR-β group than in the MCAO/R group at 24 h, and lower than that in the MCAO/R group on the 7th day, but still higher than that in the CTL group (Fig. 4a). The score was significantly lower on the 7th day than that at 24 h in the MCAO/R group or the MCAO/R+GlyR-β group (Fig. 4a). The results suggested that MCAO/R induced a neurological deficit in the mouse brain tissue, and overexpression of *GlyR-β* significantly ameliorated the neurological impairment induced by MCAO/R.

We examined the area of cerebral ischemia in mice in the CTL group, the MCAO/R group, and the MCAO/R+GlyR-β group using TTC staining. The results showed that there was no pale area in the brain tissue of the CTL group. Pale areas were present in the brain tissue of the MCAO/R group at 24 h and on the 7th day, and a small number of red spots appeared in the pale areas at 7 d (Fig. 4b,c). Pale areas were also present in the MCAO/R+GlyR-β group at 24 h and on the 7th day after MCAO/R. However, unlike the MCAO/R group, there were obvious red patches in the pale areas of the MCAO/R+GlyR-β group, and there were more red patches in the pale areas on the 7th day than in those at 24 h after MCAO/R. Compared with the CTL group, the ischemic area of brain tissue in the MCAO/R group and the MCAO/R+GlyR-β group appeared significantly larger at 24 h and on the 7th day (Fig. 4b,c), and the ischemic areas on the 7th day were smaller than those at 24 h (Fig. 4b,c). Compared with the MCAO/R group, the ischemic areas of the MCAO/R+GlyR-β group at 24 h and on the 7th day were smaller (Fig. 4b,c). The results suggested that MCAO/R induced cerebral ischemia in mice, and *GlyR-β* overexpression continuously reduced the cerebral ischemic areas in mice after MCAO/R.

We examined the pathological changes of the mice in the CTL group, the MCAO/R group, and the MCAO/R+GlyR-β group using hematoxylin-eosin (H&E) staining. The results showed that at 24 h and on the 7th day in the CTL group, the cells were ordered, with large nuclei, uniform chromatin distribution, clear nucleoli, and no degeneration. Cell damage and other lesions were found (Fig. 5a,b). In the MCAO/R group, cells were arranged disorderly. The nuclei were fragmented, dissolved, and

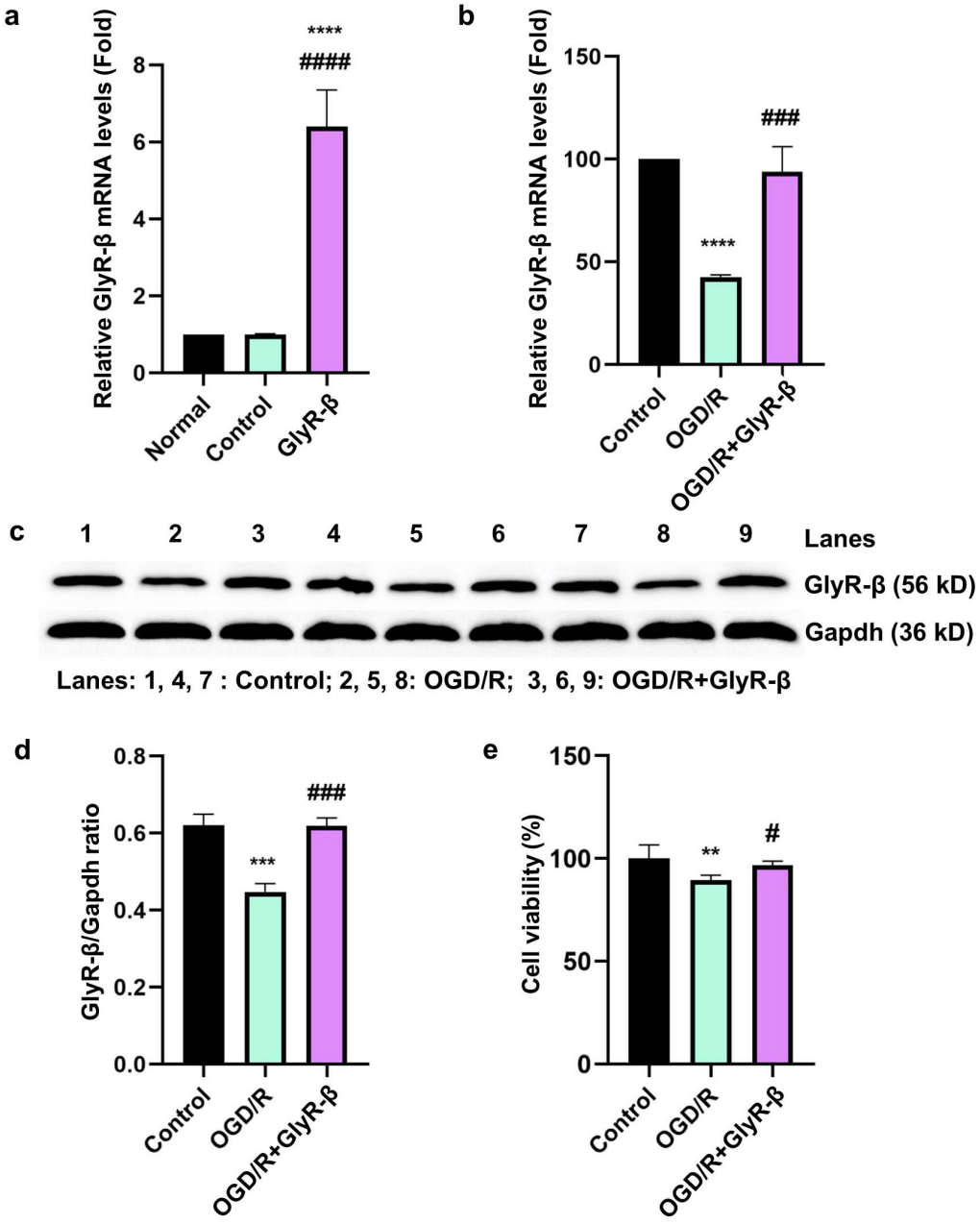

Fig. 1. *GlyR-β* expression and cell viability of bEnd3 cells decreased after oxygen glucose deprivation and recovery (OGD/R) and was reversed by transfection with the pcDNA3.1-EGFP-Glrb vector. (a) The level of *Glrb* mRNA was significantly increased in bEnd3 cells transfected with the pcDNA3.1-EGFP-Glrb vector. In the Normal group, the bEnd3 cells were normally cultured; in the CTL group, the bEnd3 cells, were transfected with vector pcDNA3.1-EGFP; in the GlyR-β group, the bEnd3 cells were transfected with pcDNA3.1-EGFP-Glrb. ****, $p < 0.0001$ vs Normal; $^{\#\#\#\#}$, $p < 0.0001$ vs Control. (b,c) *Glrb* mRNA (b) and protein (c) expression decreased in the bEnd3 cells after OGD/R and was reversed by transfection with the pcDNA3.1-EGFP-Glrb vector. In the CTL group, the bEnd3 cells were cultured normally, transfected with blank vector pcDNA3.1-EGFP, and then cultured normally; in the OGD/R group, the bEnd3 cells were subjected to OGD/R, then transfected with blank vector pcDNA3.1-EGFP, and then cultured normally; in the OGD/R+GlyR-β group, the bEnd3 cells were subjected to OGD/R, then transfected with the *GlyR-β* overexpression vector pcDNA3.1-EGFP-Glrb, and then cultured normally. qPCR was used to detect the levels of *Glrb* mRNA (a,b). Western blotting was used to detect levels of the GlyR-β protein (c). The *Gapdh* gene was used as the internal reference gene. (d) Quantitative analysis of the western blot results in (c). (e) Cell proliferation activity was examined using CCK-8. **, $p = 0.002$, ***, $p = 0.0003$, ****, $p = 0.0001$, vs Control; $^{\#}$, $p = 0.026$, $^{\#\#\#}$, $p = 0.0003$, vs OGD/R. GlyR-β, glycine receptor beta subunit; Glrb, glycine receptor beta; OGD/R, oxygen glucose deprivation and recovery; CTL, control group; qPCR, quantitative polymerase chain reaction; CCK-8, cell counting kit-8.

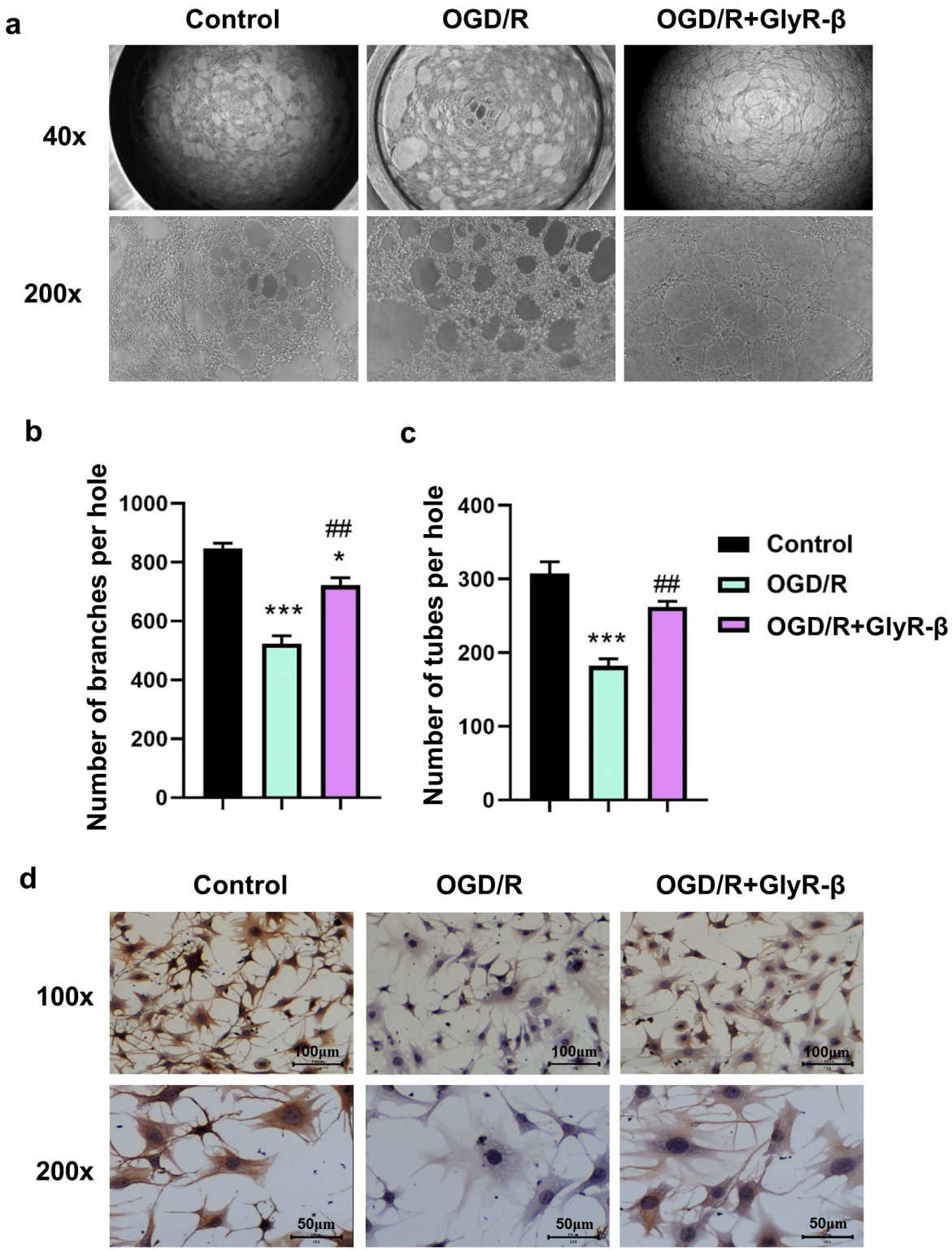

Fig. 2. *Glrb* gene overexpression promoted the ability of bEnd3 cells to form tubes and Vegf expression after OGD/R. The bEnd3 cells were divided into three groups: the CTL group, the OGD/R group, and the OGD/R+GlyR-β group. The bEnd3 cells were cultured for 72 h after transfection with the corresponding plasmids. (a) The tube formation ability of the cells was tested using a tube formation assay. Microscopic observations and photographic record. (b) and (c) Quantitative analysis and comparison of the tube formation capabilities in all groups. (b) *, $p = 0.024$, ***, $p = 0.0002$ vs Control; $^{\#\#}$, $p = 0.0028$ vs OGD/R. (c) ***, $p = 0.0007$ vs Control; $^{\#\#}$, $p = 0.0075$ vs OGD/R. (d) Vegf expression was detected using immunocytochemistry. Microscopic observation and photographic record.

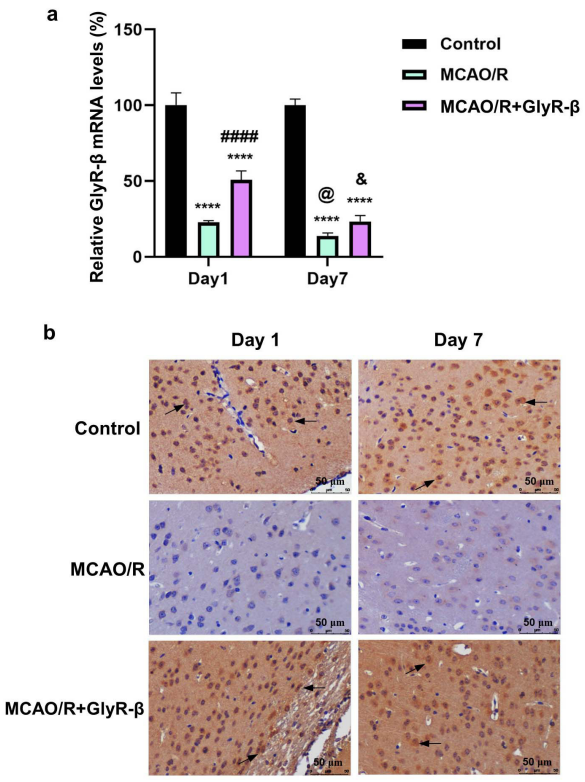

Fig. 3. Expression of the *Glrb* gene was decreased in the brain tissue of MCAO/R mice in a time-dependent manner. C57BL/6 mice were divided into three groups: The CTL group, MCAO/R group, and the MCAO/R+GlyR-β group. At 24 h and on the 7th day, brain tissue was collected. (a) *Glrb* mRNA levels. Total RNA was extracted from each sample, and the level of *Glrb* mRNA was detected using qPCR. The *Gapdh* gene was used as an internal reference gene. ****, $p < 0.0001$ vs Control; $^{\#\#\#\#}$, $p < 0.0001$ vs MCAO/R; $^{@}$, $p = 0.012$ vs MCAO/R at Day 1; $^{\&}$, $p = 0.013$, vs MCAO/R+GlyR-β at Day 1. (b) Expression of the GlyR-β protein. Expression of the GlyR-β protein in tissue was detected using immunohistochemistry. The black arrows indicate GlyR-β expression (brown stained areas). Microscopic observation and photographic record. MCAO/R, middle cerebral artery occlusion/reperfusion.

had disappeared. Cell damage was obvious at 24 h. Cell damage was still visible on the 7th day, and the range was even slightly larger than that at 24 h (Fig. 5a,b). In the MCAO/R+GlyR-β group, cells were arranged disorderly at 24 h, and the nuclei were fragmented, dissolved, and had disappeared. Cell damage was obvious and the magnitude was slightly smaller than that in the MCAO/R group (Fig. 5a,b). Cells were disorderly arranged on the 7th day, but the range of cell damage was significantly lower than that at 24 h or that on the 7th day in the MCAO/R group. Neuronal pyknosis remained, but cell damage on the infarcted side was significantly reduced (Fig. 5a,b). The results suggested that MCAO/R caused pathological damage to the mouse brain tissue, which lasts at least 7 days after injury. *GlyR-β* overexpression promoted the pathological recovery of damaged brain tissue after injury.

3.5 Overexpression of the Glrb Gene Promoted the Expression of CD34 and Vegf in the Ischemic Brain Tissues of MCAO/R Mice

We examined the expression of the *CD34* and *Vegf* genes in the ischemic infarcted areas of mouse brain tissue in the CTL group, the MCAO/R group, and the MCAO/R+GlyR-β group using immunohistochemistry and qPCR. The results showed that the expression of the CD34 protein in the ischemic brain tissue of the MCAO/R group was higher than that of the CTL group at 24 h and on the 7th day (Fig. 6a). They were higher in the MCAO/R+GlyR-β group than in the MCAO/R group at 24 h and on the 7th day (Fig. 6a). They were higher on the 7th day than at 24 h in the MCAO/R group and in the MCAO/R+GlyR-β group (Fig. 6a). The results suggested that MCAO/R caused a continuous increase in expression of the CD34 protein in a time-dependent manner, and overexpression of *GlyR-β* promoted expression of the CD34 protein in damaged brain tissue.

Vegf mRNA levels (Fig. 6b) and protein expression (Fig. 6c) in damaged brain tissue of the MCAO/R group and the MCAO/R+GlyR-β group were significantly higher than in the CTL group at 24 h and on the 7th day. They were significantly higher in the MCAO/R+GlyR-β group than the MCAO/R group at 24 h and on the 7th day. *Vegf* mRNA levels (Fig. 6b) and/or protein expression (Fig. 6c) increased significantly or slightly on the 7th day compared with at 24 h in the MCAO/R group and the MCAO/R+GlyR-β group. The results suggested that MCAO/R caused a continuous increase in the expression of the *Vegf* gene. *GlyR-β* overexpression promoted expression of the *Vegf* gene in damaged brain tissue, and the degree of promotion did not change dramatically over time.

3.6 Overexpression of the Glrb Gene Promoted Cell Regeneration, and Bdnf and Gap-43 Expression in the Ischemic Brain Tissues of MCAO/R Mice

We examined cellular regeneration in mouse brain tissue in the CTL group, MCAO/R group, and MCAO/R+GlyR-β group using the BrdU assay. The results showed that the number of BrdU positive cells in the brain tissue of the MCAO/R group and the MCAO/R+GlyR-β group was higher than in the CTL group at 24 h and on the 7th day (Fig. 7a,b). The number was higher in the MCAO/R+GlyR-β group than in the MCAO/R group both at 24 h and on the 7th day (Fig. 7a,b). The number was higher on the 7th day than at 24 h in the MCAO/R group and in the MCAO/R+GlyR-β group (Fig. 7a,b). The results suggested that there was cellular regeneration in the mouse brain tissue after MCAO/R. Overexpression of *GlyR-β* promoted cell regeneration in damaged brain tissue.

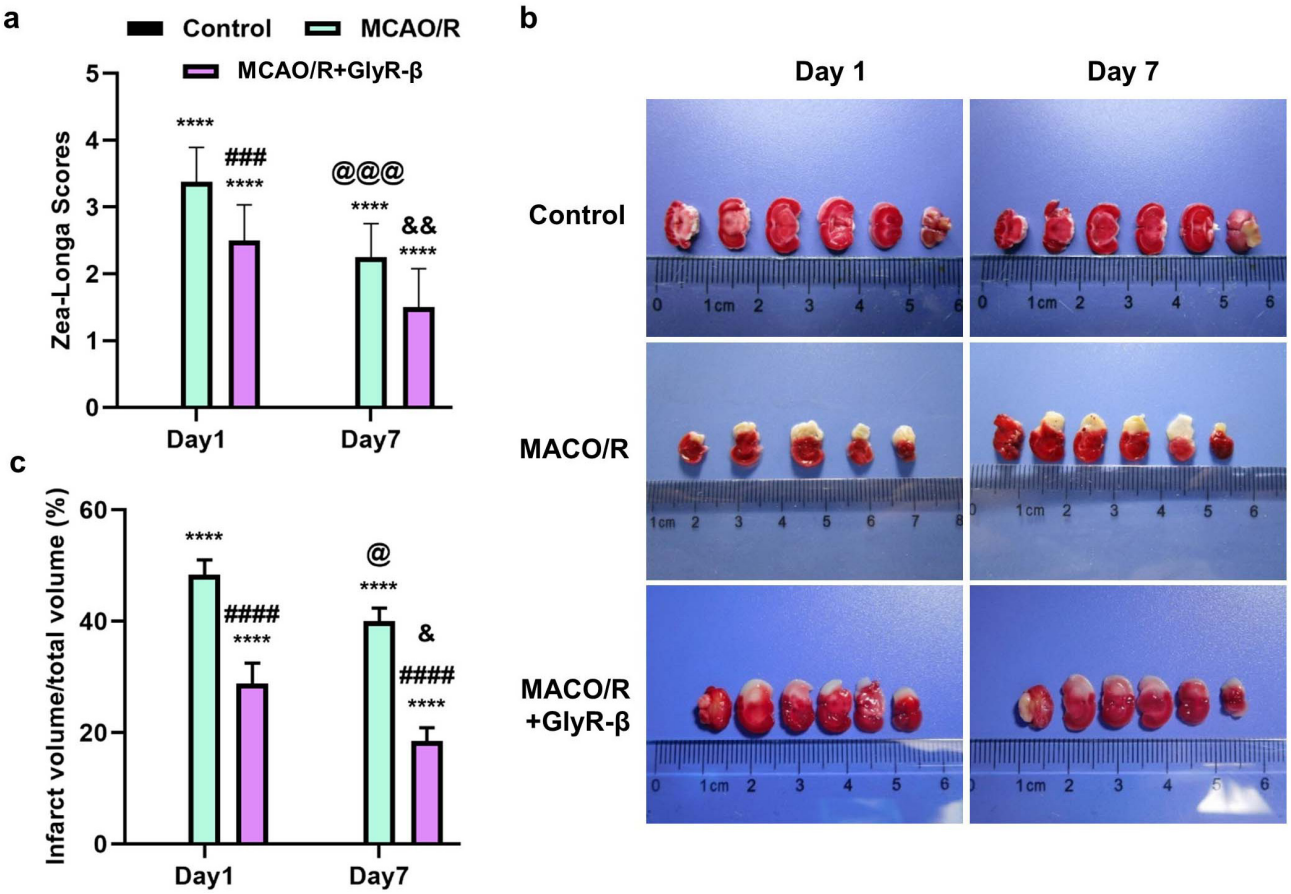

Fig. 4. Overexpression of the *Glrb* gene promoted neurological function recovery and reduced the area of cerebral ischemia of MCAO/R mice. C57BL/6 mice were divided into three groups: the CTL group, the MCAO/R group, and the MCAO/R+GlyR-β group. (a) Zea Longa Grade 5 neurological deficit scores were measured at 24 h and on the 7th day after MCAO/R. ****, $p < 0.0001$ vs Control; $^{\#\#\#}$, $p = 0.001$ vs MCAO/R; $^{@@@}$, $p = 0.0006$ vs MCAO/R at Day 1; $^{\&\&}$, $p = 0.0021$ vs MCAO/R+GlyR-β at Day 1. (b) Brain tissue was stained with tetrazolium chloride (TTC). (c) Quantitative analysis of changes in the ratio of cerebral ischemia in all groups. ****, $p < 0.0001$ vs Control; $^{\#\#\#\#}$, $p < 0.0001$ vs MCAO/R; $^{@}$, $p = 0.0399$ vs MCAO/R at Day 1; $^{\&}$, $p < 0.0158$ vs MCAO/R+GlyR-β at Day 1.

We examined the expression of the *Bdnf* and *Gap-43* genes in mouse brain tissue in the CTL group, the MCAO/R group, and the MCAO/R+GlyR-β group using qPCR and immunohistochemistry. The results showed that the levels of *Bdnf* gene mRNA (Fig. 8a) and protein expression (Fig. 8b) in the MCAO/R group and the MCAO/R+GlyR-β group were significantly higher than those of the CTL group at 24 h and on the 7th day. They were significantly higher in the MCAO/R+GlyR-β group than in the MCAO/R group at 24 h and on the 7th day. The increases in *Bdnf* gene mRNA levels (Fig. 8a) and protein expression (Fig. 8b) on the 7th day were significantly lower than those of 24 h in the MCAO/R group and the MCAO/R+GlyR-β group. The results suggested that MCAO/R resulted in increases in the expression of the *Bdnf* gene in damaged mouse brain tissue, and the degree of increase decreased with time. *GlyR-β* overexpression continuously promoted the expression of the *Bdnf* gene in brain tissue damaged by MCAO/R, and the degree of increase decreased over time.

Conversely, the mRNA levels (Fig. 8c) and protein expression (Fig. 8d) of the *Gap-43* gene in the brains of the MCAO/R group and the MCAO/R+GlyR-β group were significantly lower than those of the CTL group both at 24 h and on the 7th day. However, they were significantly higher in the MCAO/R+GlyR-β group than in the MCAO/R group at 24 h and on the 7th day (Fig. 8c,d). In the MCAO/R group, the *Gap-43* mRNA levels (Fig. 8c) and protein (Fig. 8d) expression were significantly or slightly higher on the 7th day than at 24 h. In the MCAO/R+GlyR-β group, the *Gap-43* mRNA levels (Fig. 8c) and protein expression (Fig. 8d) were almost the same at 24 h and on the 7th day. The results suggested that MCAO/R caused a reduction in the expression of the *Gap-43* gene in the damaged mouse brain tissue, and the expression of the *Gap-43* gene increased with time after reduction. *GlyR-β* overexpression promoted the expression of the *Gap-43* gene for at least 7 days in damaged mouse brain tissue after MCAO/R.

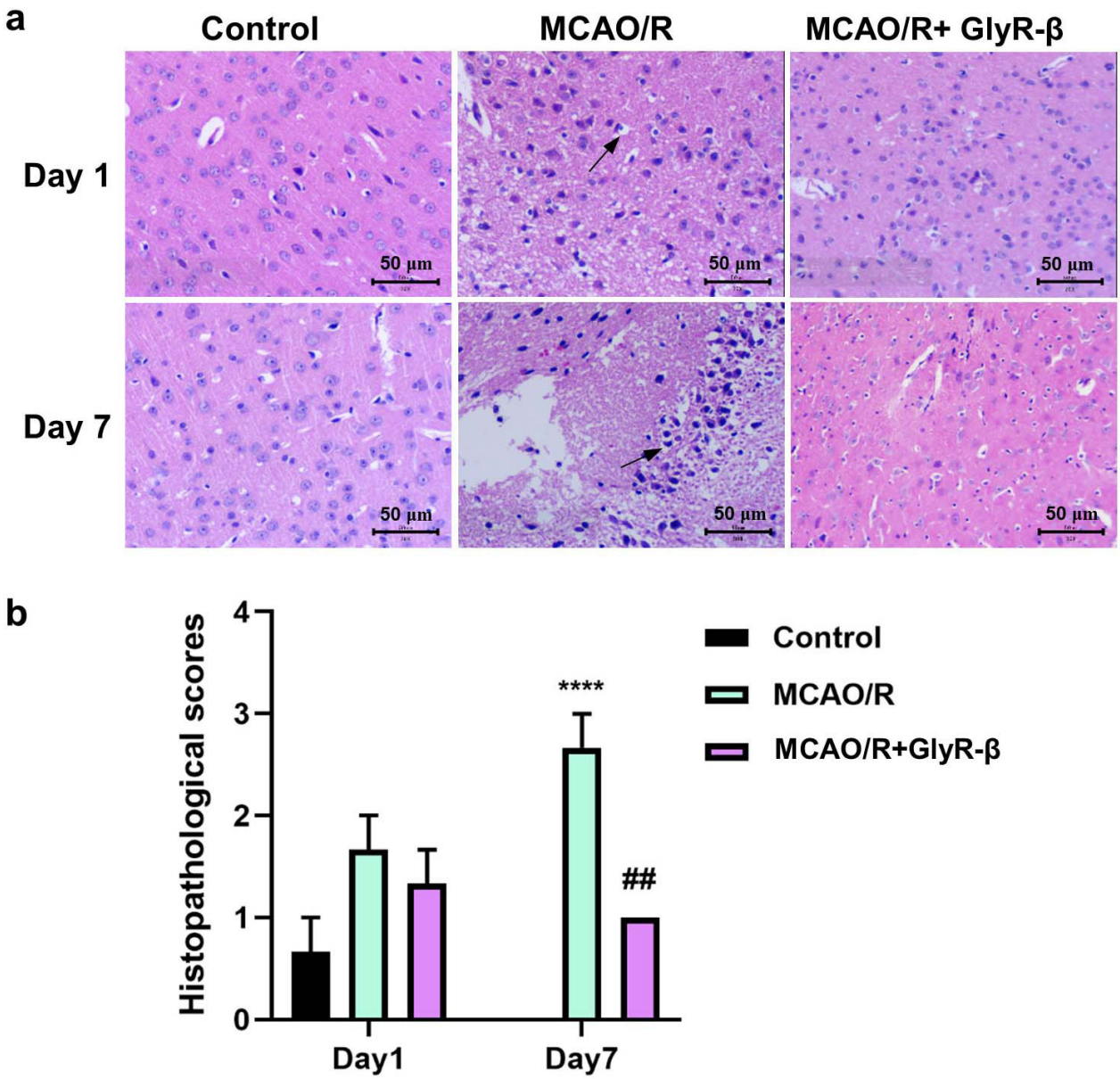

Fig. 5. *Glrb* gene overexpression improved pathological changes in MCAO/R mice. C57BL/6 mice were divided into three groups: the CTL group, the MCAO/R group, and the MCAO/R+GlyR-β group. Brain tissue was subjected to hematoxylin-eosin (H&E) staining at 24 h and on the 7th day. (a) Microscopic observation and photo recording. The black arrows indicate areas of lesion (the ischemic central area is lightly stained and shows a mesh-like pattern). (b) Quantitative analysis of pathological changes in all groups. ****, $p <$ 0.0001 vs Control; $^{\#\#}$, $p = 0.0026$ vs MCAO/R.

4. Discussion

The results of our investigation prove that GlyR-β expression was decreased in mouse bEnd3 cells after OGD/R. These findings are consistent with the results of the *in vivo* study by Kestner *et al.* [19]. We observed that GlyR-β was expressed in the normal cerebra of mice, which is consistent with Fujita *et al.*'s [14] study in a rat model. Furthermore, we found that GlyR-β expression in the damaged cerebral area was continuously decreased using the C57BL/6J model after MCAO/R. It is likely that GlyR-β expression is decreased in the damaged cerebral area, especially in the endothelial cells after ischemic stroke/reperfusion. As we only studied 24 h and 7 d after MCAO/R, whether GlyR-β continues to decline after 1 week of ischemic stroke/reperfusion remains to be further studied.

OGD/R and MCAO/R are often used to mimic the situation after reperfusion of ischemic stroke/reperfusion *in vitro* and *in vivo*, respectively. In the current study, we found that the proliferation, tube formation ability, and Vegf expression of bEnd3 cells are significantly decreased after OGD/R, and that neurological deficit, the ischemic area,

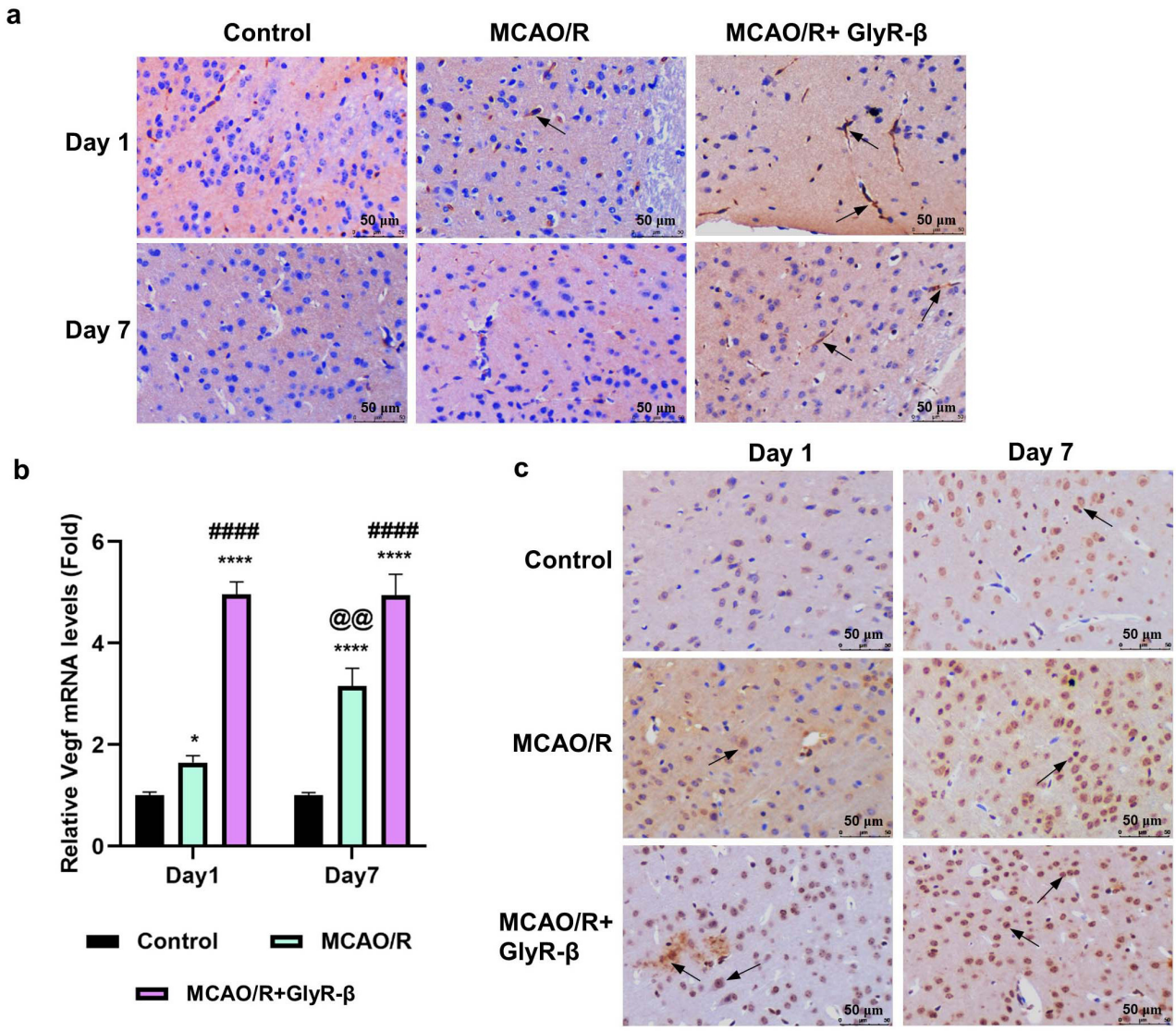

Fig. 6. Overexpression of the *Glrb* gene promoted the expression of CD34 and *Vegf* in damaged brain tissue of MCAO/R mice. C57BL/6 mice were divided into three groups: the CTL group, the MCAO/R group, and the MCAO/R+GlyR-β group. At 24 h and on the 7th day, brain tissue was collected. (a) CD34 expression in the collected tissue was detected using an immunohistochemical assay. Microscopic observation and photo recording. The black arrows indicate expressed CD34. (b) The levels of *Vegf* mRNA in the collected tissue was determined using qPCR. Here, the value of the CTL group was set to "1", while the values of the other groups are multiples (folds) of "1". Therefore, this graph represents the significant differences in *Vegf* expression among the groups. The *Gapdh* gene was used as an internal reference gene. *, $p = 0.021$, ****, $p < 0.0001$ vs Control; $^{\#\#\#\#}$, $p < 0.0001$ vs MCAO/R; $^{@@}$, $p = 0.0027$ vs MCAO/R on Day 1. (c) The expression of the Vegf protein in tissue was detected using immunohistochemistry. Microscopic observation and photographic record. The black arrows indicate expressed Vegf.

and pathological changes occur in mice after MCAO/R. These findings are consistent with the published literature [24–30]. We found that neurological function is repaired and pathological changes occur in the cerebral ischemic areas from 24 h to 7 days after MCAO/R. This is consistent with published findings [19,31]. *GlyR-β* overexpression results in significant reduction of the area of cerebral ischemia, and improvements in pathological changes and neurological function in mice after MCAO/R. Overexpression of *GlyR-β* also results in increases in proliferation, tube formation ability, and Vegf expression of bEnd3 cells after OGD/R. Therefore, decreased GlyR-β expression can contribute to delayed recovery after cerebral ischemia stroke/reperfusion. Enhanced overexpression of *GlyR-β* promotes recovery.

Angiogenesis can improve the blood supply of the cerebral ischemic region, promote neurogenesis, and improve neurological function [32–34]. Regulating vascu-

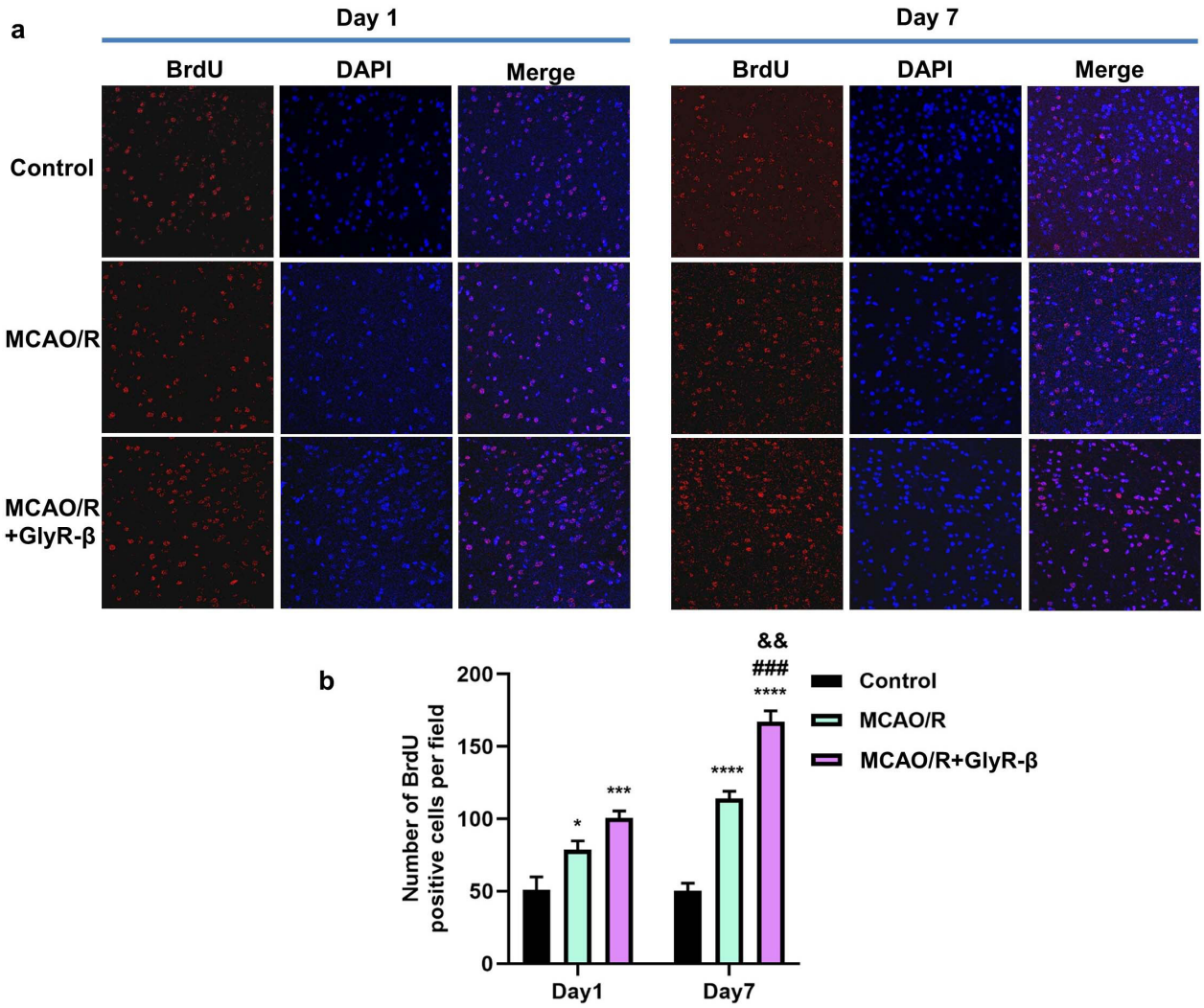

Fig. 7. Overexpression of the *Glrb* gene promoted cellular regeneration in damaged brain tissue of MCAO/R mice. C57BL/6 mice were divided into three groups: the CTL group, the MCAO/R group, and the MCAO/R+GlyR-β group. Brain tissue was collected at 24 h and on the 7th day after MCAO/R. BrdU immunofluorescence was used to detect cell regeneration. (a) Microscopic observation and photographic record (magnification: $\times$400). (b) Quantitative analysis of the number of BrdU positive cells in all groups. *, $p = 0.0252$, ***, $p = 0.0004$, ****, $p < 0.0001$ vs Control; $^{\#\#\#}$, $p = 0.0002$ vs MCAO/R; $^{\&\&}$, $p = 0.0036$ vs MCAO/R+GlyR-β on Day 1. DAPI, 4,6-diamino-2-phenyl indole; BrdU, bromodeoxyuridine.

lar growth in ischemic regions is an important measure for the treatment of ischemic stroke [35]. CD34 is an important marker of angiogenesis. Endothelial cells expressing CD34 are essential for angiogenesis and participate in initial angiogenesis [36–38]. Vegf is the most important factor in regulating angiogenesis. In the ischemic area, Vegf is up-regulated by hypoxia and other factors, thus starting the process of angiogenesis. Vegf can promote vasorelaxation and increase vascular permeability, promote endothelial cell proliferation and migration, accelerate the formation of new blood vessels, improve cerebral blood flow, and reduce brain damage [39–43]. In the present study, CD34 expression, *Vegf* mRNA levels, and Vegf expression in damaged brain tissue after MCAO/R were higher than those in the normal CTL group, indicating that there is potentially angiogenesis in the damaged brain after MCAO/R. Overexpression of *GlyR-*β could enhance proliferation activity and the cell tube formation ability of bEnd3 cells after OGD/R. *GlyR-*β overexpression enhances expression of Vegf and CD34 in OGD/R bEnd3 cells and MCAO/R mice. It is likely that *GlyR-*β overexpression potentially promotes angiogenesis during recovery after ischemic stroke/reperfusion.

Our data show that the expression of the *Vegf* gene and CD34 in mouse brain tissue is continuously increased in a time-dependent manner in mice after MCAO/R. *GlyR-*β overexpression stably promotes the expression of the *Vegf* gene after MCAO/R, while it has a continuous promot-

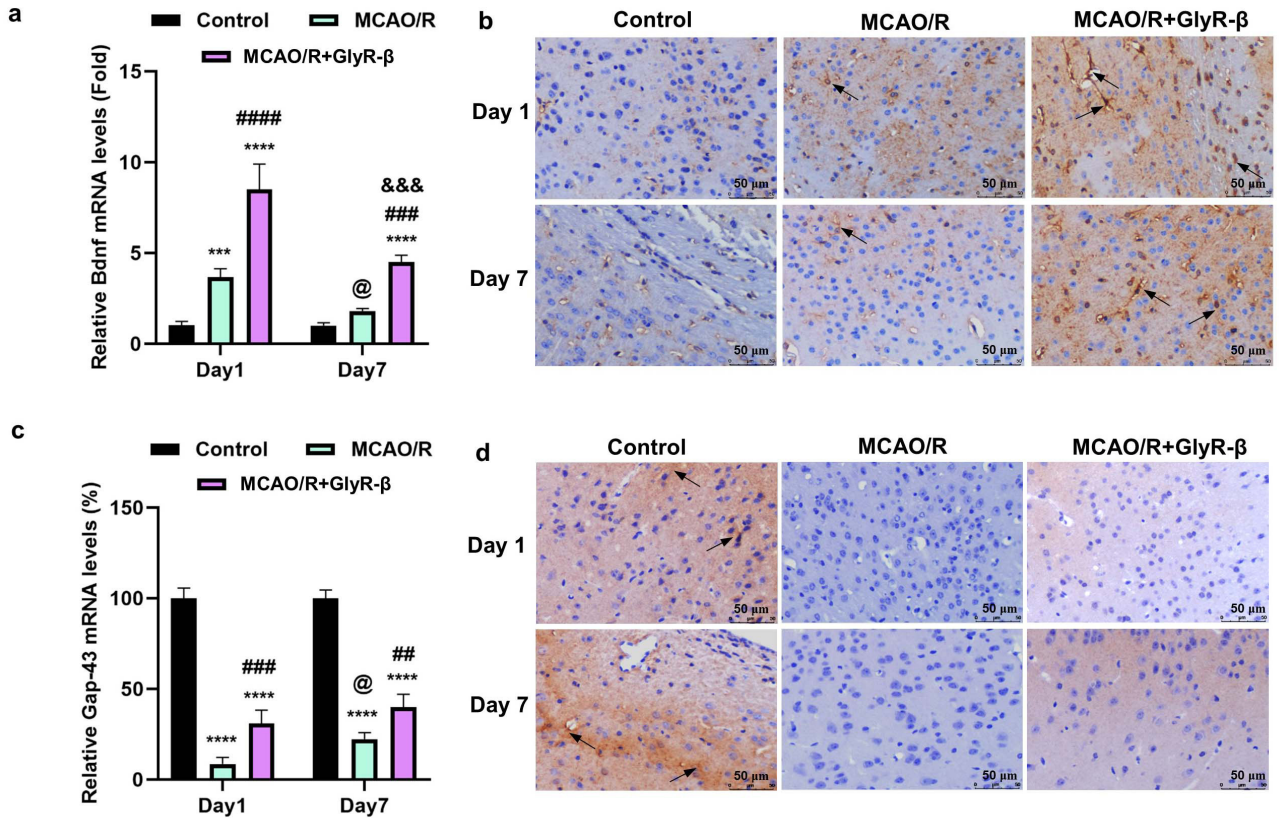

Fig. 8. Overexpression of the *Glrb* gene promoted the expression of the *Bdnf* and *Gap-43* in damaged brain tissue of MCAO/R mice. C57BL/6 mice were divided into three groups: the CTL group, the MCAO/R group, and the MCAO/R+GlyR-β group. Brain tissue was collected at 24 h and on the 7th day after MCAO/R. (a) Levels of the *Bdnf* mRNA were determined using qPCR. The *Gapdh* gene was used as an internal reference gene. ***, $p = 0.0007$, ****, $p < 0.0001$ vs Control; $^{###}$, $p = 0.0006$, $^{####}$, $p < 0.0001$ vs MCAO/R; $^{@}$, $p = 0.011$ vs MCAO/R at Day 1; $^{\&\&\&}$, $p = 0.0002$ vs MCAO/R+GlyR-β at Day 1. (b) The expression of Bdnf protein in tissue was determined using immunohistochemistry. Microscopic observation and photographic record. The black arrows indicate expressed Bdnf. (c) *Gap-43* mRNA levels were determined using qPCR. The images show all infarcted areas of brain tissue, and the images of the three experimental groups were from the same location. The *Gapdh* gene was used as an internal reference gene. ****, $p < 0.0001$ vs Control; $^{##}$, $p = 0.0052$, $^{###}$, $p = 0.0008$ vs MCAO/R; $^{@}$, $p = 0.038$ vs MCAO/R at Day 1. (d) The expression of the Gap-43 protein in tissue was detected using immunohistochemistry. Images show all infarcted areas of brain tissue from the same location. Microscopic observation and photographic record. The black arrows indicate expressed Gap-43. Gap-43, growth associated protein 43; Bdnf, brain-derived neurotrophic factor.

ing effect on CD34 increase. Therefore, GlyR-β may promote the expression of Vegf and CD34 in different and time-dependent ways. Early intervention using *GlyR-β* overexpression may enhance potential angiogenesis earlier and be more conducive to recovery from cerebral ischemic stroke/reperfusion.

Damaged brain tissue is repaired by generating new cells that include living cells such as neurons, macrophages/monocytes, lymphocytes, neovascularized smooth muscle cells, endothelium, and reactive astrocytes in the damaged brain areas of the cerebral cortex and the hippocampus [31,44–49]. However, due to pathological conditions, such as local ischemia and hypoxia and the release of inflammatory factors, the survival rate of regenerated cells, including neurons, is low and the repair ability is very limited [44,45]. Our BrdU assay data showed that the regeneration process is stimulated, but the degree of regeneration was very limited in the MCAO/R group. Overexpression of *GlyR-β* promotes continuous cellular regeneration in damaged brain tissue both at 24 h and on the 7th day. We also observed red regions in the pale area of the infarcted brain tissue in mice treated after MCAO/R by TTC staining on the 7th day in the MACO/R+GlyR-β group. We speculate that it could be regenerated tissue that contains living cells in the damaged area. This needs further verification by other means. Brain tissue regeneration is likely to occur in damaged brain tissue 24 h after cerebral ischemic stroke/reperfusion, and it may last for at least 1 week. *GlyR-β* overexpression promotes cellular regeneration in damaged brain tissue.

It is useful to use drugs to promote and enhance endogenous brain tissue regeneration, including neurogenesis, to relieve neurological dysfunction caused by cerebral ischemia [50,51]. In the present study, our findings showed that *GlyR-β* overexpression promotes brain tissue regeneration in damaged brain tissue. Expression of the *Bdnf* gene increases and expression of the *Gap-43* gene decreases in damaged brain tissue in mice after MCAO/R. Overexpression of *GlyR-β* continuously promotes *Bdnf* and *Gap-43* expression in MCAO/R damaged brain tissue. Brain-derived neurotrophic factor (Bdnf) is a small and basic secreted protein, which is the most abundant neurotrophic factor in the mammalian brain. The protein is involved in the regulation of apoptosis and survival of neurons after stroke, and changes in its expression can reflect changes in the number and function of neurons. Bdnf can not only protect neurons in ischemic-damaged areas, but also inhibit delayed neuronal necrosis, and is a key regulator of post stroke rehabilitation [52–57]. Gap-43 is a membrane phosphate protein, a specific protein of the nervous system, and widely distributed in the brain, cerebellum, hippocampus, spinal cord, dorsal root ganglion, and autonomic nervous system. Gap-43 is a key protein involved in axon growth and synaptic reconstruction, a marker protein of neuronal growth and development, and plays a key role in the process of repair and regeneration after nerve injury. This protein can promote the growth, development, regeneration, and synaptic reconstruction of neurons [58–65]. Therefore, overexpression of GlyR-*β* may participate in the process of brain tissue regeneration, including potential neuron regeneration regulated by Bdnf and Gap-43. Early intervention by GlyR-*β* overexpression would be conducive to potential neuron regeneration in damaged brain tissue.

This study has a limitation that H&E staining was used to demonstrate the pathological changes in the brain tissue from MCAO/R. However, it could be that the H&E staining indicated the basic structures of the brain tissue only. Therefore, in the future, we would combine H&E staining with other staining methods to support the specific benefits of GlyR-*β* overexpression.

5. Conclusions

Expression of the *Glrb* gene is continuously decreased after cerebral ischemic stroke/reperfusion. Overexpression of the *Glrb* gene promotes potential neurological and vascular regeneration, thus promoting recovery from cerebral ischemic stroke/reperfusion injury. Our findings reveal one of the potential mechanisms of recovery after cerebral ischemic stroke/reperfusion and provide an intervention target for promoting recovery after cerebral ischemic stroke/reperfusion.

Abbreviations

GlyR-*β*, glycine receptor beta subunit; OGD/R, oxygen glucose deprivation and recovery; MCAO/R, middle cerebral artery occlusion/reperfusion; GlyR, glycine receptor; CDS, coding sequence; Bdnf, brain derived neurotrophic factor.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Conceptualization: YX, HY; Data acquisition and analysis: YX, JYang, YSY, JSC, JYan, JNJ, ZWY. Writing and editing: YX, HY; Supervision: HY. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experiments were approved by the Institutional Animal Care and Use Committee of Guizhou Medical University (Approval Number. 2201038) and the Guide for the Care and Use of Laboratory Animals.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

References

[1] Yan X, Chen H, Shang X. Human urinary kallidinogenase decreases the incidence of post-stroke cognitive impairment in acute ischemic stroke patients. Journal of Integrative Neuroscience. 2022; 21: 80.

[2] Arenillas JF, Sobrino T, Castillo J, Dávalos A. The role of angiogenesis in damage and recovery from ischemic stroke. Current Treatment Options in Cardiovascular Medicine. 2007; 9: 205–212.

[3] Pedragosa J, Miró-Mur F, Otxoa-de-Amezaga A, Justicia C, Ruíz-Jaén F, Ponsaerts P, *et al*. CCR2 deficiency in monocytes impairs angiogenesis and functional recovery after ischemic stroke in mice. Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism. 2020; 40: S98–S116.

[4] Hu KH, Li YA, Jia W, Wu GY, Sun L, Wang SR, *et al*. Chemogenetic activation of glutamatergic neurons in the motor cortex

promotes functional recovery after ischemic stroke in rats. Behavioural Brain Research. 2019; 359: 81–88.
[5] Liu Y, Fu N, Su J, Wang X, Li X. Rapid Enkephalin Delivery Using Exosomes to Promote Neurons Recovery in Ischemic Stroke by Inhibiting Neuronal p53/Caspase-3. BioMed Research International. 2019; 2019: 4273290.
[6] Jiang MQ, Yu SP, Wei ZZ, Zhong W, Cao W, Gu X, *et al*. Conversion of Reactive Astrocytes to Induced Neurons Enhances Neuronal Repair and Functional Recovery After Ischemic Stroke. Frontier of Aging Neuroscience. 2021; 13: 612856.
[7] Wang S, Zhang H, Xu Y. Crosstalk between microglia and T cells contributes to brain damage and recovery after ischemic stroke. Neurological Research. 2016; 38: 495–503.
[8] Santamaría-Cadavid M, Rodríguez-Castro E, Rodríguez-Yáñez M, Arias-Rivas S, López-Dequidt I, Pérez-Mato M, *et al*. Regulatory T cells participate in the recovery of ischemic stroke patients. BMC Neurology. 2020; 20: 68.
[9] Hermann DM, Liesz A, Dzyubenko E. Implications of immune responses for ischemic brain injury and stroke recovery. Brain, Behavior, and Immunity. 2021; 96: 292–294.
[10] Yan BC, Wang J, Rui Y, Cao J, Xu P, Jiang D, *et al*. Neuroprotective Effects of Gabapentin Against Cerebral Ischemia Reperfusion-Induced Neuronal Autophagic Injury via Regulation of the PI3K/Akt/mTOR Signaling Pathways. Journal of Neuropathology & Experimental Neurology. 2019; 78: 157–171.
[11] Cerdan AH, Sisquellas M, Pereira G, Barreto Gomes DE, Changeux JP, Cecchini M. The Glycine Receptor Allosteric Ligands Library (GRALL). Bioinformatics (Oxford, England). 2020; 36: 3379–3384.
[12] Du J, Lü W, Wu S, Cheng Y, Gouaux E. Glycine receptor mechanism elucidated by electron cryo-microscopy. Nature. 2015; 526: 224–229.
[13] Rees MI, Lewis TM, Kwok JBJ, Mortier GR, Govaert P, Snell RG, *et al*. Hyperekplexia associated with compound heterozygote mutations in the beta-subunit of the human inhibitory glycine receptor (GLRB). Human Molecular Genetics. 2002; 11: 853–860.
[14] Fujita M, Sato K, Sato M, Inoue T, Kozuka T, Tohyama M. Regional distribution of the cells expressing glycine receptor beta subunit mRNA in the rat brain. Brain Research. 1991; 560: 23–37.
[15] Handford CA, Lynch JW, Baker E, Webb GC, Ford JH, Sutherland GR, *et al*. The human glycine receptor beta subunit: primary structure, functional characterisation and chromosomal localisation of the human and murine genes. Brain Research. Molecular Brain Research. 1996; 35: 211–219.
[16] Milani N, Mülhardt C, Weber RG, Lichter P, Kioschis P, Poustka A, *et al*. The human glycine receptor beta subunit gene (GLRB): structure, refined chromosomal localization, and population polymorphism. Genomics. 1998; 50: 341–345.
[17] Schaefer N, Signoret-Genest J, von Collenberg CR, Wachter B, Deckert J, Tovote P, *et al*. Anxiety and Startle Phenotypes in *Glrb Spastic* and *Glra1 Spasmodic* Mouse Mutants. Frontiers in Molecular Neuroscience. 2020; 13: 152.
[18] Piro I, Eckes AL, Kasaragod VB, Sommer C, Harvey RJ, Schaefer N, *et al*. Novel Functional Properties of Missense Mutations in the Glycine Receptor β Subunit in Startle Disease. Frontiers in Molecular Neuroscience. 2021; 14: 745275.
[19] Kestner RI, Mayser F, Vutukuri R, Hansen L, Günther S, Brunkhorst R, *et al*. Gene Expression Dynamics at the Neurovascular Unit During Early Regeneration After Cerebral Ischemia/Reperfusion Injury in Mice. Frontiers in Neuroscience. 2020; 14: 280.
[20] Brown RM, Meah CJ, Heath VL, Styles IB, Bicknell R. Tube-Forming Assays. Methods in Molecular Biology (Clifton, N.J.). 2016; 1430: 149–157.
[21] Schroeder AB, Dobson ETA, Rueden CT, Tomancak P, Jug F, Eliceiri KW. The ImageJ ecosystem: Open-source software for image visualization, processing, and analysis. Protein Science: a Publication of the Protein Society. 2021; 30: 234–249.
[22] Türeyen K, Vemuganti R, Sailor KA, Dempsey RJ. Infarct volume quantification in mouse focal cerebral ischemia: a comparison of triphenyltetrazolium chloride and cresyl violet staining techniques. Journal of Neuroscience Methods. 2004; 139: 203–207.
[23] Huang S, Tan Z, Cai J, Wang Z, Tian Y. Myrtenol improves brain damage and promotes angiogenesis in rats with cerebral infarction by activating the ERK1/2 signalling pathway. Pharmaceutical Biology. 2021; 59: 584–593.
[24] Cai Q, Xu G, Liu J, Wang L, Deng G, Liu J, *et al*. A modification of intraluminal middle cerebral artery occlusion/reperfusion model for ischemic stroke with laser Doppler flowmetry guidance in mice. Neuropsychiatric Disease and Treatment. 2016; 12: 2851–2858.
[25] Feng SQ, Zong SY, Liu JX, Chen Y, Xu R, Yin X, *et al*. VEGF Antagonism Attenuates Cerebral Ischemia/Reperfusion-Induced Injury via Inhibiting Endoplasmic Reticulum Stress-Mediated Apoptosis. Biological & Pharmaceutical Bulletin. 2019; 42: 692–702.
[26] Yao X, Yao R, Huang F, Yi J. LncRNA SNHG12 as a potent autophagy inducer exerts neuroprotective effects against cerebral ischemia/reperfusion injury. Biochemical and Biophysical Research Communications. 2019; 514: 490–496.
[27] Zhao H, Tang J, Chen H, Gu W, Geng H, Wang L, *et al*. 14,15-EET Reduced Brain Injury from Cerebral Ischemia and Reperfusion via Suppressing Neuronal Parthanatos. International Journal of Molecular Sciences. 2021; 22: 9660.
[28] Zhou X, Xu B, Gu Y, Ji N, Meng P, Dong L. Long noncoding RNA SNHG1 protects brain microvascular endothelial cells against oxygen-glucose deprivation/reoxygenation-induced injury by sponging miR-298 and upregulating SIK1 expression. Biotechnology Letters. 2021; 43: 1163–1174.
[29] Wang G, Chen Z, Song Y, Wu H, Chen M, Lai S, *et al*. Xueshuantong injection alleviates cerebral microcirculation disorder in middle cerebral artery occlusion/reperfusion rats by suppressing inflammation via JNK mediated JAK2/STAT3 and NF-κB signaling pathways. Journal of Ethnopharmacology. 2022; 298: 115592.
[30] Zhao J, Dong L, Huo T, Cheng J, Li X, Huangfu X, *et al*. O-GlcNAc Transferase (OGT) Protects Cerebral Neurons from Death During Ischemia/Reperfusion (I/R) Injury by Modulating Drp1 in Mice. Neuromolecular Medicine. 2022; 24: 299–310.
[31] Lee JM, Fernandez-Cadenas I, Lindgren AG. Using Human Genetics to Understand Mechanisms in Ischemic Stroke Outcome: From Early Brain Injury to Long-Term Recovery. Stroke. 2021; 52: 3013–3024.
[32] Font MA, Arboix A, Krupinski J. Angiogenesis, neurogenesis and neuroplasticity in ischemic stroke. Current Cardiology Reviews. 2010; 6: 238–244.
[33] Ruan L, Wang B, ZhuGe Q, Jin K. Coupling of neurogenesis and angiogenesis after ischemic stroke. Brain Research. 2015; 1623: 166–173.
[34] Hatakeyama M, Ninomiya I, Kanazawa M. Angiogenesis and neuronal remodeling after ischemic stroke. Neural Regeneration Research. 2020; 15: 16–19.
[35] Benedek A, Cernica D, Mester A, Opincariu D, Hodas R, Rodean I, *et al*. Modern Concepts in Regenerative Therapy for Ischemic Stroke: From Stem Cells for Promoting Angiogenesis to 3D-Bioprinted Scaffolds Customized via Carotid Shear Stress Analysis. International Journal of Molecular Sciences. 2019; 20:

2574.
[36] Kawamoto A, Iwasaki H, Kusano K, Murayama T, Oyamada A, Silver M, *et al*. CD34-positive cells exhibit increased potency and safety for therapeutic neovascularization after myocardial infarction compared with total mononuclear cells. Circulation. 2006; 114: 2163–2169.
[37] Popa ER, Harmsen MC, Tio RA, van der Strate BWA, Brouwer LA, Schipper M, *et al*. Circulating CD34+ progenitor cells modulate host angiogenesis and inflammation in vivo. Journal of Molecular and Cellular Cardiology. 2006; 41: 86–96.
[38] Guo X, Liu L, Zhang M, Bergeron A, Cui Z, Dong JF, *et al*. Correlation of CD34+ cells with tissue angiogenesis after traumatic brain injury in a rat model. Journal of Neurotrauma. 2009; 26: 1337–1344.
[39] Carmeliet P, Collen D. Molecular basis of angiogenesis. Role of VEGF and VE-cadherin. Annals of the New York Academy of Sciences. 2000; 902: 249–264.
[40] Ahluwalia A, Tarnawski AS. Critical role of hypoxia sensor–HIF-1α in VEGF gene activation. Implications for angiogenesis and tissue injury healing. Current Medicinal Chemistry. 2012; 19: 90–97.
[41] Breuss JM, Uhrin P. VEGF-initiated angiogenesis and the uPA/uPAR system. Cell Adhesion & Migration. 2012; 6: 535–615.
[42] Greenberg DA, Jin K. Vascular endothelial growth factors (VEGFs) and stroke. Cellular and Molecular Life Sciences: CMLS. 2013; 70: 1753–1761.
[43] Talwar T, Srivastava MVP. Role of vascular endothelial growth factor and other growth factors in post-stroke recovery. Annals of Indian Academy of Neurology. 2014; 17: 1–6.
[44] Wiltrout C, Lang B, Yan Y, Dempsey RJ, Vemuganti R. Repairing brain after stroke: a review on post-ischemic neurogenesis. Neurochemistry International. 2007; 50: 1028–1041.
[45] Cramer SC. Repairing the human brain after stroke: I. Mechanisms of spontaneous recovery. Annals of Neurology. 2008; 63: 272–287.
[46] Ohira K, Furuta T, Hioki H, Nakamura KC, Kuramoto E, Tanaka Y, *et al*. Ischemia-induced neurogenesis of neocortical layer 1 progenitor cells. Nature Neuroscience. 2010; 13: 173–179.
[47] Hayashi Y, Jinnou H, Sawamoto K, Hitoshi S. Adult neurogenesis and its role in brain injury and psychiatric diseases. Journal of Neurochemistry. 2018; 147: 584–594.
[48] Marques BL, Carvalho GA, Freitas EMM, Chiareli RA, Barbosa TG, Di Araújo AGP, *et al*. The role of neurogenesis in neurorepair after ischemic stroke. Seminars in Cell & Developmental Biology. 2019; 95: 98–110.
[49] Dillen Y, Kemps H, Gervois P, Wolfs E, Bronckaers A. Adult Neurogenesis in the Subventricular Zone and Its Regulation After Ischemic Stroke: Implications for Therapeutic Approaches. Translational Stroke Research. 2020; 11: 60–79.
[50] Caroni P. Neuro-regeneration: plasticity for repair and adaptation. Essays in Biochemistry. 1998; 33: 53–64.
[51] Cramer SC. Repairing the human brain after stroke. II. Restorative therapies. Annals of Neurology. 2008; 63: 549–560.
[52] Lindsay RM. Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 1988; 8: 2394–2405.
[53] Hiebert GW, Khodarahmi K, McGraw J, Steeves JD, Tetzlaff W. Brain-derived neurotrophic factor applied to the motor cortex promotes sprouting of corticospinal fibers but not regeneration into a peripheral nerve transplant. Journal of Neuroscience Research. 2002; 69: 160–168.
[54] Park J, Lim E, Back S, Na H, Park Y, Sun K. Nerve regeneration following spinal cord injury using matrix metalloproteinase-sensitive, hyaluronic acid-based biomimetic hydrogel scaffold containing brain-derived neurotrophic factor. Journal of Biomedical Materials Research. Part a. 2010; 93: 1091–1099.
[55] Lucini C, D'Angelo L, Cacialli P, Palladino A, de Girolamo P. BDNF, Brain, and Regeneration: Insights from Zebrafish. International Journal of Molecular Sciences. 2018; 19: 3155.
[56] Paro MR, Chakraborty AR, Angelo S, Nambiar S, Bulsara KR, Verma R. Molecular mediators of angiogenesis and neurogenesis after ischemic stroke. Reviews in the Neurosciences. 2022; 34: 425–442.
[57] Szarowicz CA, Steece-Collier K, Caulfield ME. New Frontiers in Neurodegeneration and Regeneration Associated with Brain-Derived Neurotrophic Factor and the rs6265 Single Nucleotide Polymorphism. International Journal of Molecular Sciences. 2022; 23: 8011.
[58] Gispen WH, Boonstra J, De Graan PN, Jennekens FG, Oestreicher AB, Schotman P, *et al*. B-50/GAP-43 in neuronal development and repair. Restorative Neurology and Neuroscience. 1990; 1: 237–244.
[59] Strittmatter SM, Vartanian T, Fishman MC. GAP-43 as a plasticity protein in neuronal form and repair. Journal of Neurobiology. 1992; 23: 507–520.
[60] Jain N, Florence SL, Kaas JH. GAP-43 expression in the medulla of macaque monkeys: changes during postnatal development and the effects of early median nerve repair. Brain Research. Developmental Brain Research. 1995; 90: 24–34.
[61] Benowitz LI, Routtenberg A. GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends in Neurosciences. 1997; 20: 84–91.
[62] Latchney SE, Masiulis I, Zaccaria KJ, Lagace DC, Powell CM, McCasland JS, *et al*. Developmental and adult GAP-43 deficiency in mice dynamically alters hippocampal neurogenesis and mossy fiber volume. Developmental Neuroscience. 2014; 36: 44–63.
[63] Holahan MR. A Shift from a Pivotal to Supporting Role for the Growth-Associated Protein (GAP-43) in the Coordination of Axonal Structural and Functional Plasticity. Frontiers in Cellular Neuroscience. 2017; 11: 266.
[64] Chung D, Shum A, Caraveo G. GAP-43 and BASP1 in Axon Regeneration: Implications for the Treatment of Neurodegenerative Diseases. Frontiers in Cell and Developmental Biology. 2020; 8: 567537.
[65] Huang R, Xiao H, Zhao J, Ju L, Wen Y, Xu Q, *et al*. GAP-43 is involved in the orientation of cell division by interacting with GAI during neurogenesis. The International Journal of Neuroscience. 2020; 130: 144–152.