Am J Transl Res 2020;12(10):6895-6907
www.ajtr.org /ISSN:1943-8141/AJTR0110530

Original Article

Glycine attenuates cerebrovascular remodeling via glycine receptor alpha 2 and vascular endothelial growth factor receptor 2 after stroke

Zheng Chen[1,2,3*], Xiang Wang[3*], Haikang Liao[6], Tao Sheng[1], Panhong Chen[1], Hongchang Zhou[1,2], Yongliang Pan[1,2], Weiqin Liu[5#], Hua Yao[4,6#]

[1]Schools of Medicine, Huzhou University, Huzhou Central Hospital, Huzhou, PR China; [2]Key Laboratory of Vector Biology and Pathogen Control of Zhejiang Province, Huzhou University, Huzhou Central Hospital, Huzhou, PR China; [3]Division of Vascular Surgery, East Hospital, Tongji University School of Medicine, Shanghai, 200120, PR China; [4]Guangxi Key Laboratory of Brain and Cognitive Neuroscience, Guilin Medical University, Guilin, PR China; [5]The Affiliated Ganzhou Hospital of Nanchang University, Ganzhou, PR China; [6]Institute of Life Sciences and Institute of Neuroscience, Wenzhou University, Wenzhou, PR China. [*]Equal contributors and co-first authors. [#]Equal contributors.

Received March 9, 2020; Accepted August 1, 2020; Epub October 15, 2020; Published October 30, 2020

Abstract: As a dual-acting neurotransmitter, glycine plays critical roles in cerebral ischemia by activating both glycine receptors (GlyRs) and N-methyl-D-aspartate acid receptors (NMDARs). However, the involvement of glycine receptor alpha 2 (GlyRa2) in cerebral ischemia has not been explored. The objective of this study was to determine the mechanism of action of GlyRa2 in cerebrovascular remodeling. After induction of rat tMCAO, levels of the *GLRA2* gene and GlyRa2 protein were examined using q-PCR, western blot, and immunohistochemical analyses. Blood-brain barrier permeability, and the presence of hemorrhage and arteriosclerosis were also analyzed. The underlying mechanism of vascular remodeling was examined using immunohistochemical and immunofluorescence analyses. Both the *GLRA2* gene and GlyRa2 protein were altered sharply after stroke. GlyRa2 of vascular origin appears to play a protective role after glycine treatment for ischemia. Blockade of GlyRa2 by the addition of cyclothiazide was found to abolish previous improvements in cerebrovascular survival after glycine treatment for tMCAO in rats. GlyRa2-dependent neurovascular remodeling was found to be correlated with the vascular endothelial growth factor receptor 2 (VEGFR2) pathways. These results suggest that vascular-derived GlyRa2 protects against post-ischemic injury. Vascular protection via GlyRa2 is due to VEGFR2/pSTAT3 signaling.

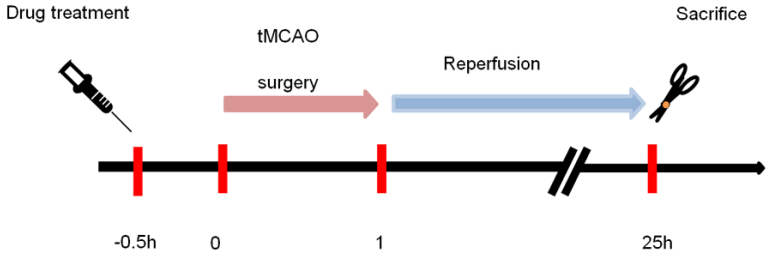
Keywords: GlyRa2, tMCAO, neurovascular, BBB, VEGFR2, pSTAT3

Introduction

As a dual-acting neurotransmitter, glycine can induce both glycinergic inhibition via the GlyR site (site A) and NMDAR-dependent neuronal excitation via the NMDAR-glycine co-agonist site (site B) [1]. Furthermore, glycine plays critical roles in neuronal plasticity [2] and cerebral ischemia [3, 4]. Fundamental studies have revealed that glycine bidirectionally regulates post-ischemic injury in a dose-dependent manner by balancing the inhibition of GlyR and the excitation of NMDAR in the hippocampus after focal ischemia [3]; glycine also mediates ischemic tolerance [5]. In 2016, our laboratory reported that GlyRa1 may be a potential mediator of glycine transport inhibition-dependent neuroprotection in ischemia [6]. However, GlyRa2 is believed to be potentially more effective than GlyRa1 in various animal models of neuronal development [7], autism [8], schizophrenia [9], and ethanol-related behaviors [10, 11]. To the best of our knowledge, whether and how GlyRa2 is related to cerebral ischemia have not been previously explored.

Cerebrovascular remodeling after stroke, a multifactorial phenomenon that is involved in atherosclerosis [12, 13], hypertension [14, 15], diabetes [16, 17], cognitive deficits [18, 19], and cell-based therapies [17, 20], results in the breakdown of the blood-brain barrier (BBB) and

Table 1. Time scale of tMCAO surgery



in controlled hemorrhagic insults, thereby exacerbating post-stroke injury and leading to a high rate of morbidity [20, 21]. It was also shown that D-serine, a ligand of GlyR, is highly related to astrocyte-mediated cerebrovascular remodeling [22, 23]. Moreover, inhibitory neurotransmitters can be synthesized and released from, and act upon, the local endothelium [24, 25]. However, the role of GlyRa2 in vascular remodeling has not been investigated.

In light of these observations, we hypothesized that neurovascular protection against ischemic injury after glycine treatment is due to endothelium-derived GlyRa2. In the present study, we examined the roles of GlyRa2 in a model of ischemia and investigated how the GlyRa2/VEGF/pSTAT3 pathway is related to cerebrovascular remodeling after stroke.

Materials and methods

Animals and the tMCAO model

Sprague-Dawley rats (300 ± 20 g, male) were purchased from Shanghai SIPPR-BK Laboratory Animal Co., Ltd. (Shanghai, China). Transient middle cerebral artery occlusion (tMCAO) surgery was performed, as previously described [3, 5]. All animals were anesthetized with an intraperitoneal injection of chloral hydrate (0.3 mg/kg). The tMCAO surgery was initiated using a 4-0 silicone-coated nylon suture for 1 h Four hours after the completion of surgery, the rats were administered drugs via intraperitoneal injection and then sacrificed 25 h after the start of surgery (**Table 1**). The sham (n = 6) and Mod (n = 6) groups were treated with an intraperitoneal injection of normal saline. The Mod+Gly group (n = 6) was administered glycine intraperitoneally. All surgical procedures and experimental protocols were approved by the ethics committee of Wenzhou University and performed in accordance with approved guidelines and regulations.

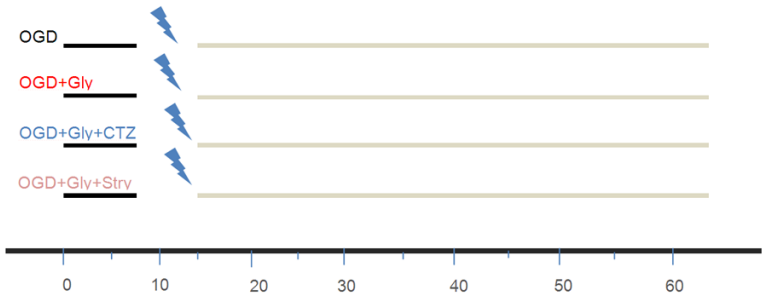
TTC staining

After surgery, the rats were anesthetized with chloral hydrate and perfused with ice-cold saline. The brains were immediately removed and then frozen at -20°C for 15 min. Whole brains were sectioned into coronal segments (2 mm thick). The sections were then incubated with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, MO, USA) for 30 min at 37°C. The focal infarct areas were clearly visible and were indicated by the pale regions in the TTC-stained sections.

Electrophysiology and OGD induction

Coronal slices (350 μm thick) were sectioned using a vibrating microtome. Rat coronal slices were cultured for 1 h in artificial cerebrospinal fluid (ACSF) (34°C, 2.5-3 mL/min, in 95% O_2/5% CO_2). The composition of the ACSF was as follows: 126 mM NaCl, 2.5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$, 11 mM glucose, and 25 mM $NaHCO_3$. Field excitatory postsynaptic potentials (fEPSPs) [5] were recorded using an Axopatch-200B amplifier (Molecular Devices, Palo Alto, CA, USA). Hippocampal slices were bathed in ACSF containing 0.3 mM Mg^{2+} and 10 μM bicuculline, which were added to enhance NMDAR-mediated responses and prevent seizures, and to inhibit $GABA_A$ receptors, respectively. A bipolar tungsten electrode was positioned in the Schaffer collaterals of the hippocampal CA1 region to deliver the stimuli. Test stimuli were evoked at 0.05 Hz. As previously reported, post-ischemic LTP (iLTP) was then induced in the hippocampus by brief oxygen-glucose deprivation (OGD; the glucose in the ACSF was replaced with sucrose, and the solution was bubbled with 95% N_2 and 5% CO_2 for 5 min) (**Table 2**) [3, 5]. All drugs were dissolved in saline. Quantitative data were collected and analyzed using Clampfit software, version 10.2; the fEPSP initial slope value was normalized to the control slope value [3, 5]. The mean values of the stimuli were acquired for 5-10 min. The data were recorded and analyzed by using pCLAMP 10.2

Table 2. The experimental grouping of Electrophysilogy and OGD induction



and Clampfit 10.2 (Molecular Devices, Palo Alto, CA, USA) softwares.

Quantitative real-time PCR

Brain tissues were rapidly obtained from anesthetized rats and placed into ice-cold saline. Control samples were taken from parallel regions of uninjured rats. The tissue segments were stored at -70°C. Total cellular RNA was extracted from frozen tissues using the RNX-Plus solution (GenScript Biotech Corp., Nanjing, China), according to the manufacturer's instructions. The purity and integrity of the extracted RNA were evaluated by optical density measurements (260/280 nm ratios). Reverse transcription was performed using 1 mg DNase (GenScript Biotech Corp., Nanjing, China) treated total RNA from each sample and the AccuPower RT Premix (GenScript Biotech Corp., Nanjing, China) with random hexamer priming in a 20 μL reaction volume.

Briefly, 25 ng cDNA and gene-specific primers were combined with SYBR Green PCR Master Mix (IQ SYBR Green I Dye, Bio-Rad) and subjected to PCR amplification (Tm = 56°C, extension time = 45 s, cycles = 40), as described previously [26]. All reactions were run in duplicate. The amplified transcripts were quantified using the comparative cycle threshold (CT) method. The primers and analysis methods used for real-time PCR were described previously [26].

The primers used for real-time PCR were GlyRa1: 5'-GCACCAAGCACTACAACAC-3' (forward) and 5'-AGGACAGGATGACGATAAGC-3' (reverse), GlyRa2: 5'-GAGACAGCAGTGGAACGATTC-3' (forward) and 5'-TCCGCAGCAACTTGTTATCAG-3' (reverse). The expression levels were normalized to those of constitutive 18S ribosomal RNA amplified with the primers 5'-GTAACCCGTTGAACCCCATT-3' (forward) and 5'-CCATCCAATCGGTAGTAGCG-3' (reverse). Relative expression data were quantified using the ^{2DD}CT method. All target genes were normalized to the 18S housekeeping gene.

Western blot analysis

Twenty-five hours after MCAO onset, brain proteins were extracted from the infarcted hemisphere of the rats. The samples were homogenized, centrifuged, and separated on sodium dodecyl sulfate (SDS) polyacrylamide gels (10%). After transferring the proteins to polyvinylidene fluoride membranes, the membranes were incubated with anti-GlyRa2 antibody (1:100) (Santa Cruz Biotechnology, CA, USA) at 4°C overnight. Finally, the membranes were incubated with secondary antibodies (horseradish peroxidase-conjugated IgG), and images were obtained using a gel documentation system (Bio-Rad, GelDoc XR+) [3, 5]. The immunoreactive bands were scanned and quantified using Quantity One software (Bio-Rad, USA).

Immunohistochemistry and immunofluorescence

The brains were cut (10 μm thickness) using a paraffin slicer (CM1900, Leica, Germany). For immunohistochemistry, an ABC kit (Zhongshan Biotechnology Company, Beijing, China) was used for reactions with rabbit primary antibodies against GlyRa2 (1:400) (Santa Cruz Biotechnology, CA, USA), VEGF (1:300), VEGFR2 (1:300), occludin (1:300), α-SMA (1:300) (Bioss, Beijing, China), and pSTAT3 (1:400) (Cell Signaling Technology, MA, USA) [5, 6]. Then, a DAB kit (Zhongshan Biotechnology Company, Beijing, China) was used for visualization. Finally, the sections were mounted on slides and visualized using a digital microscope (Leica Microsystems, Germany). The immunopositive cells in the hippocampus and cortex were quantified (at approximately-3 and -4.5 mm posterior to the bregma) using light/fluorescence microscopy [5, 6].

BBB permeability

Rat BBB permeability was induced by intravenously injecting 1 mL extravasated Evans blue (EB, 1%, Tocris, MN, USA) dye after MCAO com-

pletion [5, 6] The animals were sacrificed 25 h after MCAO onset, and the cerebral hemispheres were removed from the skull. The fluorescence intensity of EB staining was measured at 630 nm using a spectrofluorophotometer.

Hemorrhage measurement

Cortical hemorrhages were measured by hematoxylin and eosin (HE) staining under light microscopy [5, 6]. The percentages and sizes of petechial and gross hemorrhages were assessed in each histological section.

Drugs

Glycine (Gly) and inhibitors were purchased from Sigma-Aldrich and Millipore Biotechnology Co., Ltd. All drugs were dissolved in normal saline.

Statistical analysis

Data were analyzed using SPSS software, version 16.0. All data are shown as the mean ± SEM of three independent trails. The data were analyzed using a one-way ANOVA, followed by the LSD test. *P* values less than 0.05 were considered statistically significant.

Results

GlyRa2 expression is altered after ischemic injury

How GlyR subunit expression is altered after cerebral ischemia has never been reported. To explore this phenomenon, we examined GlyRa2 in an MCAO model of stroke in rats by Q-PCR and western blot analysis. Real-time PCR revealed that *GLRA2* mRNA levels were significantly decreased after one day in the MCAO group ($P < 0.001$) (**Figure 1B** and **1C**), an effect that was markedly reversed after treatment with Gly ($P < 0.01$) (**Figure 1B** and **1C**). In line with these results, GlyRa2 protein levels were revealed to be significantly reduced by western blot analysis ($P < 0.01$) (**Figure 1D** and **1E**). In the MCAO+Gly group, the decreased GlyRa2 protein levels were slightly enhanced (**Figure 1D** and **1E**). Furthermore, to determine the localization and expression levels of GlyRa2 in the brain, we performed immunohistochemical staining of the rat brains using anti-GlyRa2 antibody. We found that GlyRa2 was widely expressed in the cortex and cerebral blood vessels. We observed that the immunoreactivity of GlyRa2 was markedly downregulated in the MCAO group compared to sham animals in both the cortex and hippocampus, and not surprisingly, glycine treatment after ischemia attenuated these effects (**Figure 1F** and **1G**). Taken together, these results suggest that GlyRa2 could be a clinical target in animal stroke research.

GlyRa2-dependent neuroprotection is induced in both the MCAO animal stroke model and the OGD cell ischemia model

Following the 1 h ischemia and 2 h reperfusion episodes of the I/R protocol (**Table 1**), animals were intraperitoneally injected with exogenous Gly (800 mg/kg) [3, 4], Gly+CTZ (cyclothiazide, a GlyRa2 inhibitor, 100 mg/kg) [27-29], or vehicle. HE staining was assessed 25 h after MCAO onset. It was found that the Gly+CTZ group did not have a reduction in the infarct volume, whereas treatment with Gly alone significantly attenuated the infarct volume ($P < 0.05$, **Figure 2A** and **2B**). In addition, OGD in vitro ischemia experiments were used to verify the results from the MCAO ischemia model. We carried out patch-clamp recordings of evoked fEPSPs related to ischemic injury in hippocampal CA1 neurons. After 5 min baseline and 8 min drug-bathing episodes, 8 min OGD (in vitro ischemia) episodes persistently increased the fEPSP slope ($n = 6$, $P < 0.001$, **Figure 2C** and **2D**). These pathological alterations were consistent with the results of previous studies revealing an enhanced fEPSP slope due to iLTP in hippocampal slices [3, 5]. The quantitative data showed that Gly (1.0 mM) [3, 5] treatment significantly decreased the fEPSP slope ($n = 6$, $P < 0.05$; **Figure 2C** and **2D**); however, iLTP was completely facilitated when CTZ (100 μM) [29] was co-applied with Gly ($n = 6$, $P < 0.01$; **Figure 2C** and **2D**). As a positive control, Stry (strychnine, a GlyR inhibitor, 5 μM) [3, 5] was used under the same conditions and resulted in similar effects, decreasing the fEPSP slope ($n = 6$, $P < 0.05$; **Figure 2C** and **2D**). Hence, our results revealed that GlyRa2 accounts for the anti-ischemic neuroprotective effect of Gly.

Gly reduces BBB damage and hemorrhage via GlyRa2

Having measured the role of GlyRa2 under ischemic conditions, we further investigated wheth-

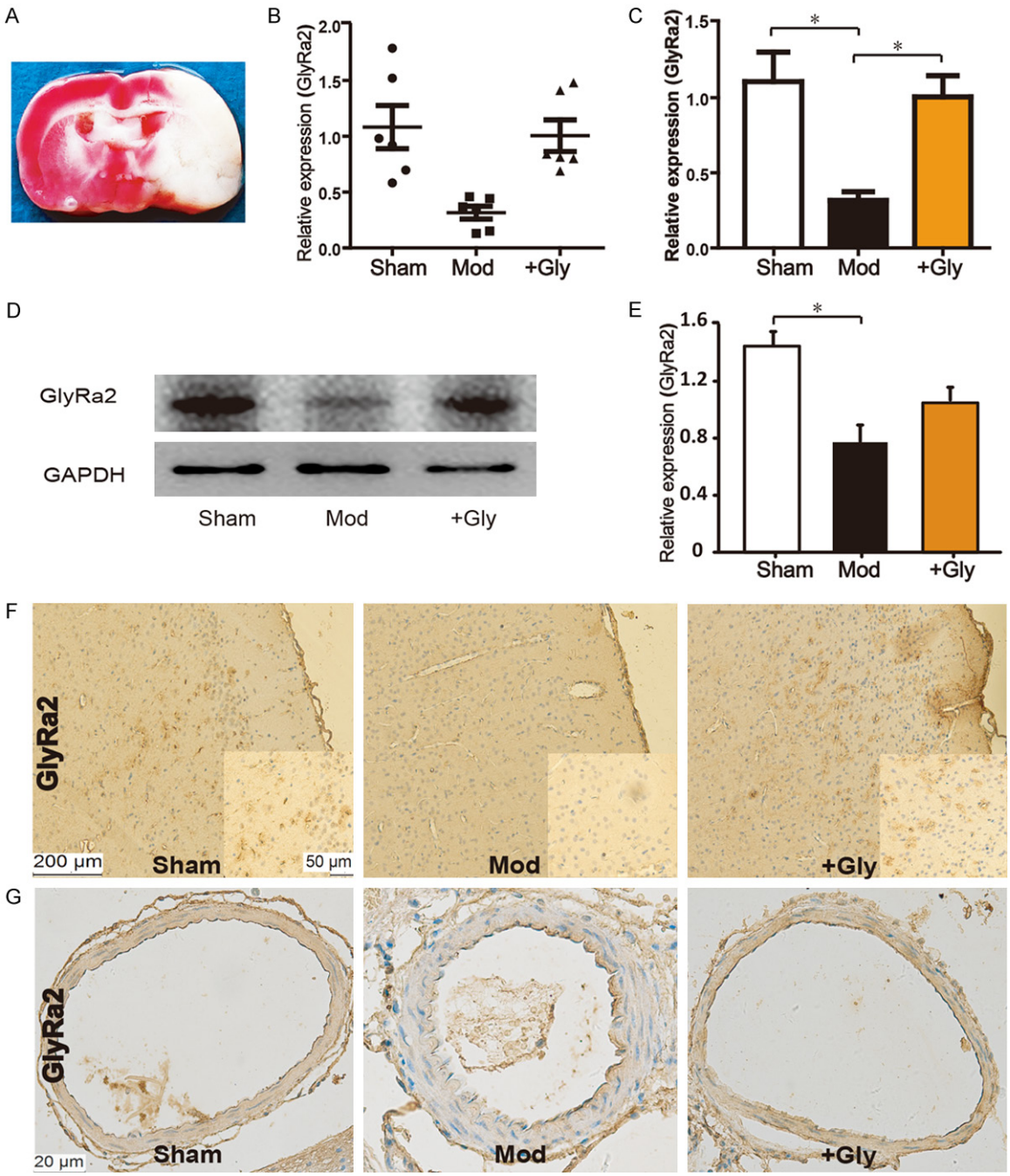


Figure 1. GlyRa2 expression is decreased after ischemia caused by tMCAO. A: Representative images are measured for infarcts by TTC staining. B and C: Relative expression levels of the GLAR2 gene are shown by Q-PCR. D and E: Relative levels of the GlyRa2 protein are shown by western blot analysis. F and G: Morphological expression of GlyRa2 is represented by immunohistochemical staining of the rat cortex and cerebral blood vessels, respectively. Sham: Sham-operated animals. Mod: Rats subjected to 60 min of transient distal MCA occlusion followed by reperfusion; +Gly: Rats treated with an intraperitoneal injection of 800 mg/kg Gly 3 h after tMCAO. Bar represents the mean ± SE. *P < 0.05, **P < 0.01 vs. the Mod group.

er and how GlyRa2 affects the likelihood of BBB damage and hemorrhage. Gly treatment significantly reduced extravasated EB leakage (n = 4, *P* < 0.05, **Figure 3A** and **3B**) in the ipsilateral cortex, but this reduction was attenuated when both Gly and a GlyRa2 inhibitor were co-administered (n = 4, *P* < 0.05, **Figure 3C**). Gly treatment also markedly reduced both the

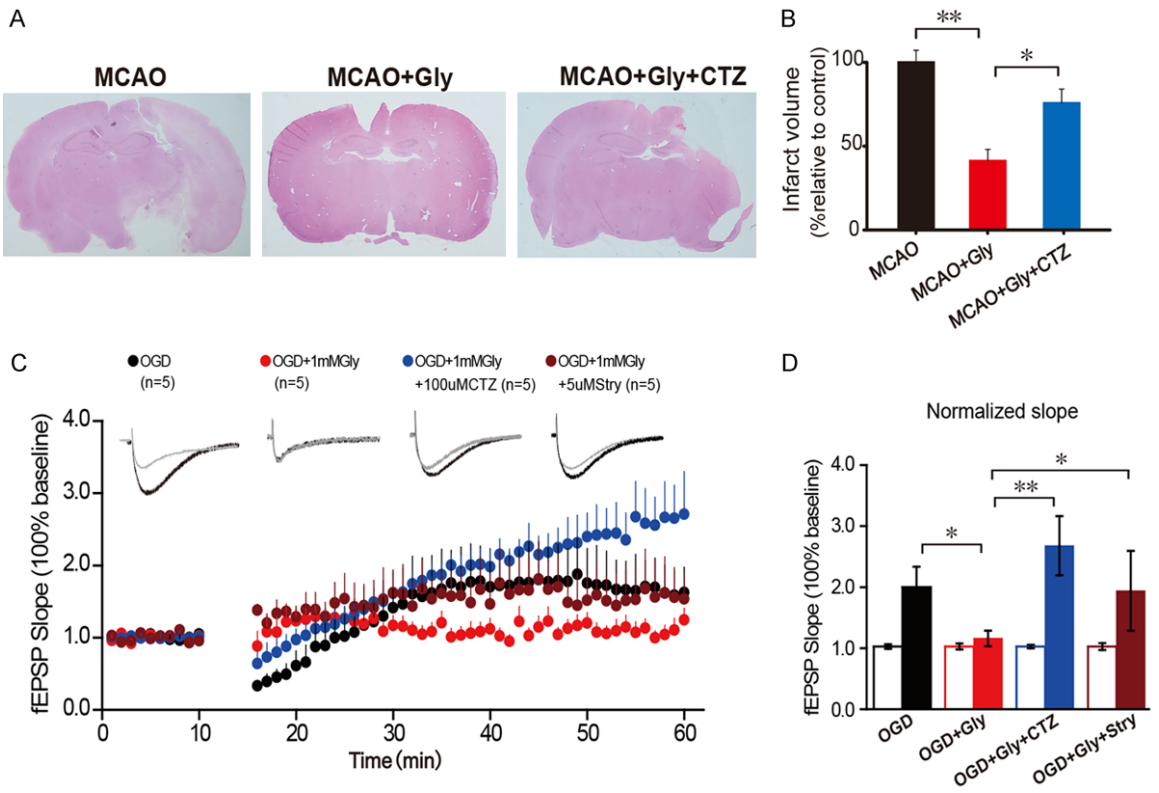


Figure 2. Gly reduces infarct volume and ischemic LTP via GlyRa2. A and B: Gly reduces infarct volume as shown by HE staining in brain tissues subjected to tMCAO. C and D: Gly decreases ischemic LTP via GlyRa2 in ischemic hippocampal slices. MCAO: Rats subjected to 60 min of transient distal MCA occlusion followed by reperfusion; MCAO+Gly: Rats treated with an intraperitoneal injection of 800 mg/kg Gly 3 h after tMCAO; MCAO+Gly+CTZ: Rats co-treated with an intraperitoneal injection of both Gly (800 mg/kg) and CTZ (100 mg/kg) 3 h after tMCAO. OGD: Hippocampal slices bathed under conditions of oxygen and glucose deprivation in the model of in vivo ischemia. OGD+Gly: OGD ischemia slices treated with Gly (1.0 mM). OGD+Gly+CTZ: cerebral ischemic OGD slices co-treated with both Gly (1.0 mM) and CTZ (100 μM). OGD+Gly+Stry: cerebral ischemic OGD slices co-administrated with both Gly (1.0 mM) and Stry (5 μM). Bar represents the mean ± SE. *P < 0.05, **P < 0.01 vs. the MCAO/OGD group.

hemorrhage rate (n = 4, $P < 0.05$, **Figure 3D** and **3E**) and volume (n = 4, $P < 0.05$, **Figure 3D** and **3F**), as shown by HE staining in the post-stroke brain. However, it was revealed that the hemorrhage rate and volume were not significantly increased (n = 4, $P < 0.05$, **Figure 3E** and **3F**) in the group receiving both Gly and CTZ. These results indicate that Gly treatment protects rats in the MCAO group against cerebrovascular remodeling via GlyRa2.

Gly decreases arterial injury and arteriosclerosis via GlyRa2

To test whether GlyRa2 is involved in vascular injury and arteriosclerosis in the MCAO group, immunohistochemical analyses, HE staining, trichrome staining, and elastin staining were performed. Gly treatment of the MCAO group significantly reduced the arterial density (n = 4, $P < 0.05$, **Figure 4A** and **4B**) and thickness (n = 4, $P < 0.05$, **Figure 4C** and **4D**), whereas Gly co-administered with CTZ under the same conditions markedly weakened these reductions (n = 4, $P < 0.05$, **Figure 4C** and **4D**), as revealed by the quantification of both α-SMA-positive arteries and HE-stained arteries. Interestingly, the thickness of the arterial intima (n = 4, $P < 0.05$. **Figure 4E-H**) was significantly decreased after Gly was applied in both trichrome-stained and elastin-stained sections, whereas the thickness was modestly elevated when Gly was co-applied with CTZ. As expected, another arteriosclerosis-related factor, arterial diameter (n = 4, $P < 0.05$. **Figure 4E-H**) was significantly increased in Gly-treated rats but sharply decreased (n = 4, $P < 0.05$. **Figure 4E-H**) after CTZ was co-injected with Gly into rats in the MCAO group. Taken together, these results

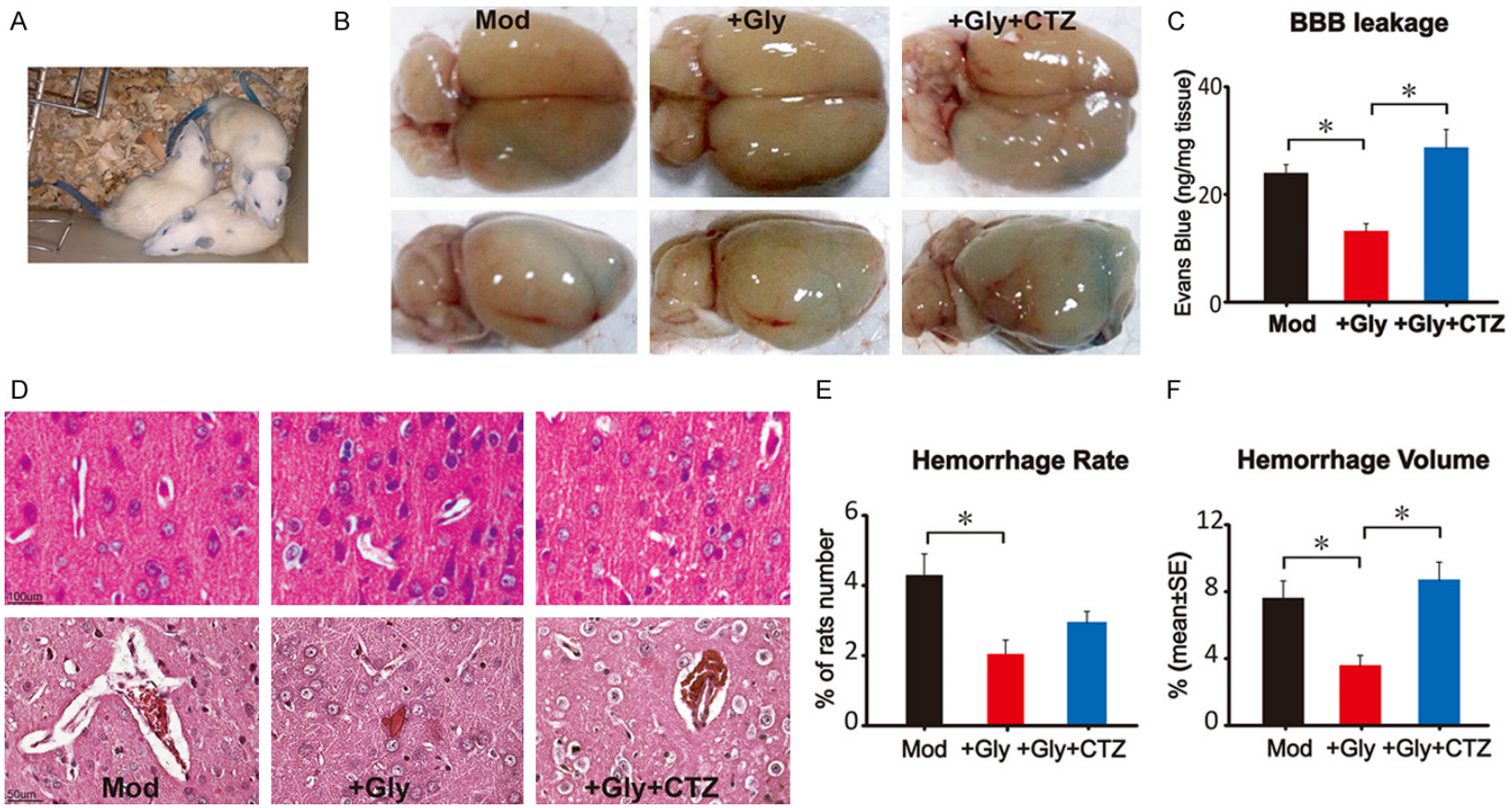


Figure 3. Gly reduces BBB damage and hemorrhage via GlyRa2. A-C: Leakage of EB (blue area) indicates BBB breakdown after tMCAO. D-F: Red area indicates hemorrhages from cerebral vessels. Mod: Rats subjected to 60 min of transient distal MCA occlusion followed by reperfusion; +Gly: Rats treated with an intraperitoneal injection of Gly (800 mg/kg) 3 h after tMCAO; +Gly+CTZ: Rats co-injected with both Gly (800 mg/kg) and CTZ (100 mg/kg) 3 h after tMCAO.

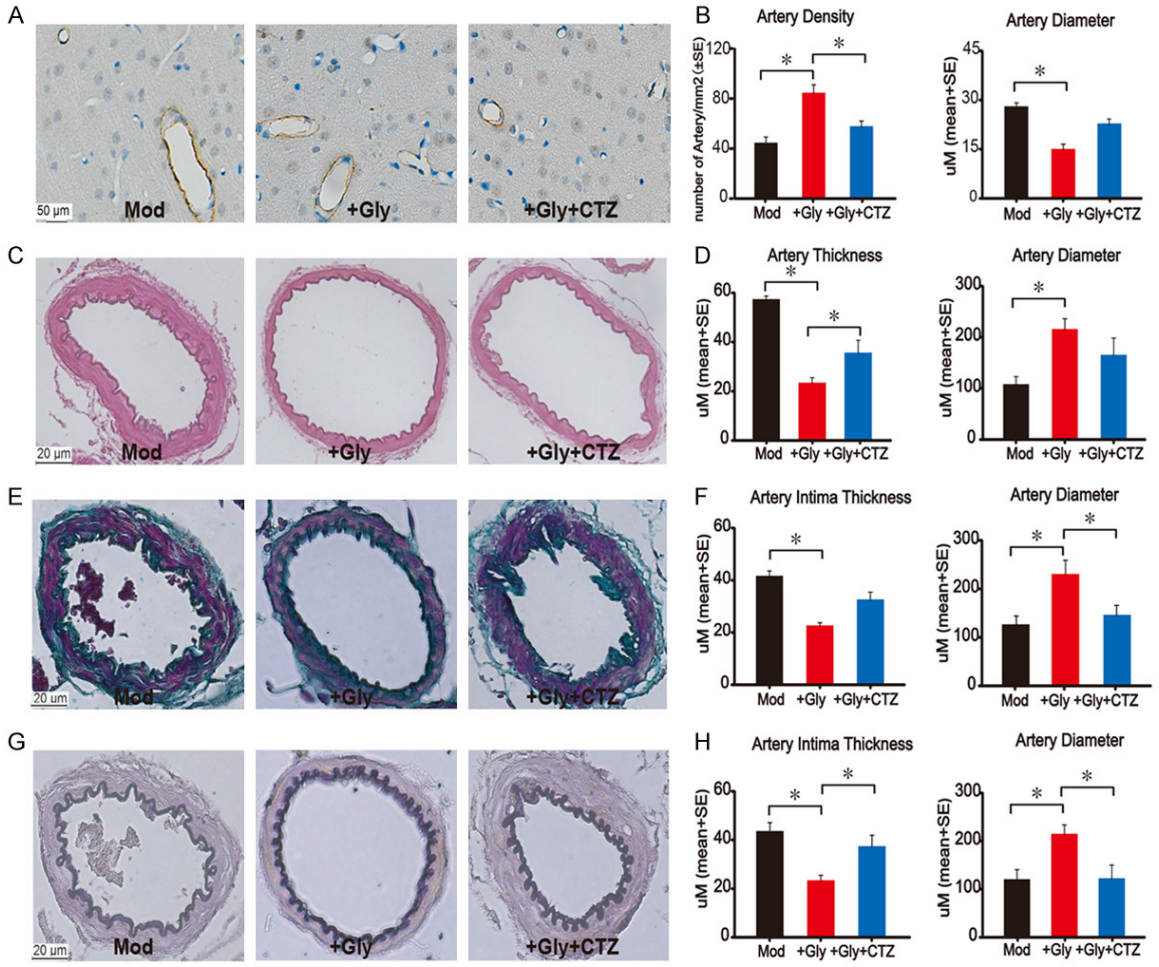


Figure 4. Gly attenuates arterial injury and arteriosclerosis via GlyRa2. A and B: Arterial density and diameter were measured in α-SMA immunopositive vessels. C and D: Arterial thickness and diameter are represented by HE staining. E-H: Thickness of the arterial intima and arterial diameter are shown by trichrome and elastin staining, respectively. Mod: Rats subjected to 60 min of transient distal MCA occlusion followed by reperfusion; +Gly: Rats treated with an intraperitoneal injection of Gly (800 mg/kg) 3 h after tMCAO; +Gly+CTZ: Rats co-injected with both Gly (800 mg/kg) and CTZ (100 mg/kg) 3 h after tMCAO.

show that GlyRa2 may play an important role in preventing vascular injury and arteriosclerosis under conditions of post-stroke Gly treatment.

The VEGFR2/pSTAT3 pathway is correlated with GlyRa2-dependent neurovascular remodeling

To explore the mechanism by which GlyRa2 confers neurovascular protection, we performed both immunohistochemical and immunofluorescence techniques to analyze the potential pathways involved. We demonstrated that both VEGFR2 and VEGF expression were upregulated by Gly treatment in animals with ischemia (**Figure 5A**) and showed that these enhancements were reduced when Gly was co-administered with CTZ (**Figure 5A**). Furthermore, the expression of VEGFR2 was found to co-localize with pSTAT3 expression, following a similar trend among all groups with ischemia (**Figure 5B**). Therefore, altered VEGFR2 correlated with pSTAT3 may serve as a key foundation for GlyRa2-dependent neurovascular remodeling.

Discussion

The four major findings of our investigation are provided below. First, unlike *GLRA1*, both the *GLRA2* gene and GlyRa2 protein were shown to be altered significantly after stroke. Second, it was demonstrated for the first time that GlyRa2 can be of vascular origin and play a protective

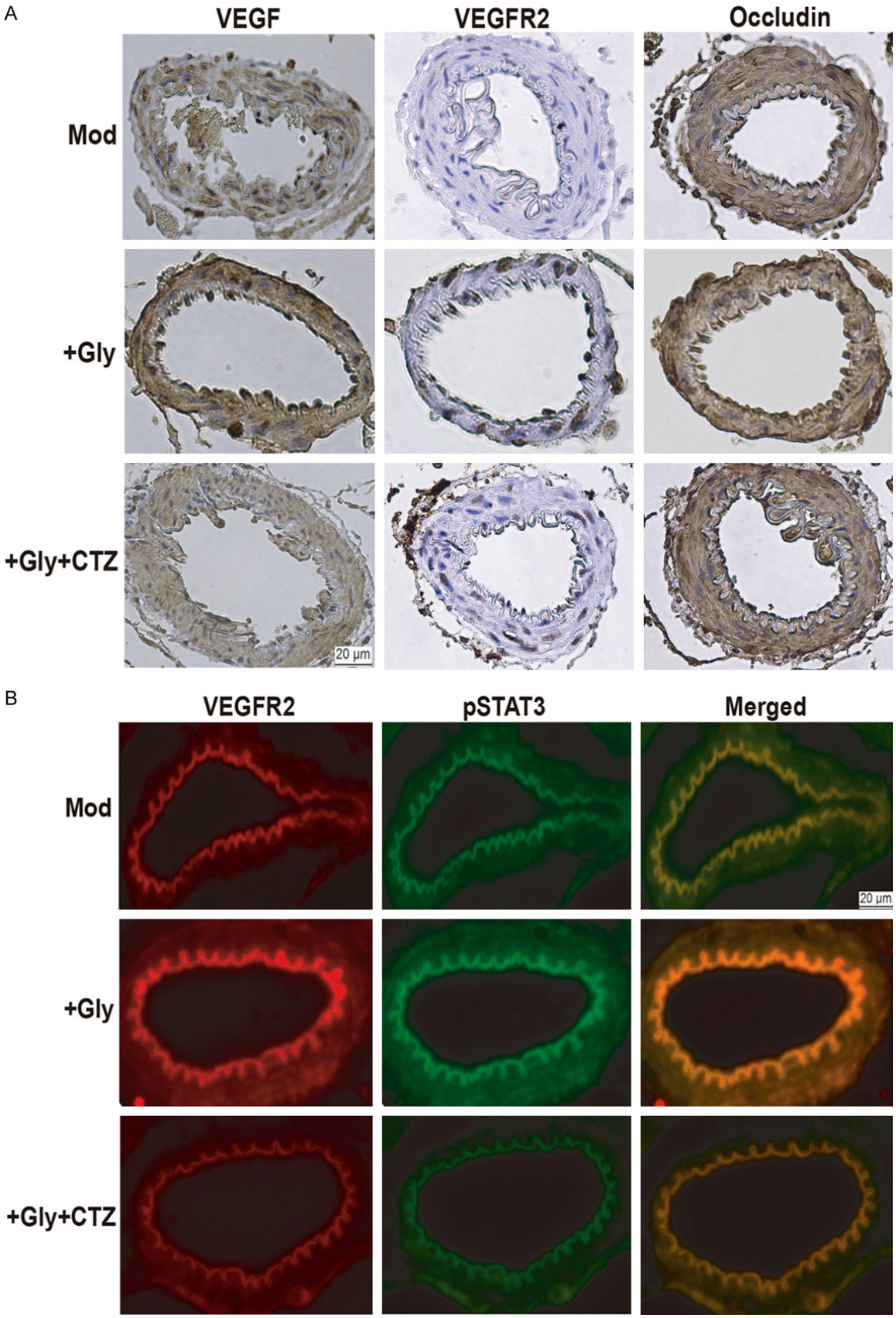

A
VEGF
VEGFR2
Occludin
Mod
+Gly
+Gly+CTZ
20 μm
B
VEGFR2
pSTAT3
Merged
Mod
+Gly
+Gly+CTZ
20 μm

Figure 5. The VEGFR2/pSTAT3 pathway is correlated with GlyRa2 after stroke. A: VEGF, VEGFR2, and occludin expression are shown by immunohistochemical staining of the cerebral blood vessels. B: VEGF2 and pSTAT3 were measured by double-immunofluorescence. Mod: Rats subjected to 60 min of transient distal MCA occlusion followed by reperfusion; +Gly: Rats treated with an intraperitoneal injection of Gly (800 mg/kg) 3 h after tMCAO; +Gly+CTZ: Rats co-injected with both Gly (800 mg/kg) and CTZ (100 mg/kg) 3 h after tMCAO.

role upon glycine treatment for ischemia. Third, the blockade of GlyRa2 via CTZ was found to abolish previous improvements in cerebrovascular survival by glycine in the MCAO group. Fourth, GlyRa2-dependent neurovascular remodeling was found to be correlated with the VEGFR2/pSTAT3 pathway.

Some studies have shown that *GLRA1* is involved in the hereditary hyperekplexia phenotype [30-32], whereas *GLRA2* has been shown to play important roles in neuronal development [7], autism [8], schizophrenia [9], and ethanol-related behaviors [10, 11]. To the best of our knowledge, whether *GLRA1* or *GLRA2* is the primary modulator of cerebral ischemia has never been investigated. We discovered that although *GLRA2* is significantly downregulated in ischemia, this effect is partially reversed by glycine treatment. These findings are in accordance with results regarding *GLRA2* at injured regions within the hypothalamus [33] and the spinal cord [26]. Although there was evidence that GlyRa1 may be neuroprotective in our previous report [34] and in other studies [33], other reports have suggested that GlyRa2 mediates stronger effects than GlyRa1 [26, 35-37].

In the central nervous system, glycine, as a dual-acting co-agonist, can induce both neuronal excitation by binding to the NMDAR-glycine-binding site and neuronal inhibition by binding to GlyRs [38]. Our findings indicated that the ligand-gated chloride channel GlyR plays a neuroprotective role in animal models of stroke [3, 5] and in vitro [3, 5, 34]. However, the source of these receptors themselves, especially GlyRa2, has yet to be investigated. Earlier studies revealed that GlyRa2 is mainly expressed in the spinal cord, hypothalamus, and cerebellum, and demonstrated that GlyRa2 can be functional in the cortex [39, 40] and hippocampus [41-43]. Moreover, the GlyR subunit was shown to potentiate ligand-gated Cl^- currents in human pancreatic β-cells [44] and confer hepatoprotection [45, 46]. It is evident, therefore, that the expression of functional GlyRa2 is ubiquitous, although this possibility has not yet been deeply studied. Based on these observations, the current study proves for the first time that GlyRa2 can be expressed and activated by the vascular endothelium (Figure S2). Our results are consistent with the peripheral origination hypothesis regarding the GABAergic system [24, 25].

A recent study showed that glycine is involved in vascular remodeling via the activation of VEGF-induced angiogenesis [22]. Although the authors argued for a mitochondrial role in the vascular effects of glycine, an alternative interpretation is that GlyR subtypes might be involved in the direct mechanism of glycine-mediated vascular reconstruction. In fact, our previous data suggested this possibility through the use of EB staining in a model of BBB protection via glycine treatment [5]. In the current study, our findings elucidate a role for GlyRa2 in cerebrovascular remodeling. Of course, we cannot exclude a similar effect of GlyRa1, given that functional GlyRa1 has been discovered in the pancreas [44] and liver [45, 46]. We therefore conducted additional experiments to further examine the different GlyR subtypes. The results showed more positive staining for GlyRa2 than for GlyRa1 in cortical arteries (Figure S1). Thus, we conclude that the cerebrovascular protective effect of glycine after stroke is mediated by GlyRa2.

VEGFR1 and 2 are key factors that modulate various conditions, including physiological and pathological angiogenesis [41]. Among many downstream signaling pathways, the involvement of the STAT3 pathway in the induction of angiogenesis is highly debated. Some evidence suggests that VEGFR1/pSTAT3 is the dominant pathway [47, 48]; however, other studies argue that VEGFR2/pSTAT3 is the main signaling pathway in vascular disease models [49-52]. In our model, GlyRa2-dependent cerebrovascular remodeling was apparently due to VEGFR2/pSTAT3 activation. Many factors may contribute to the effect of GlyRa2; nevertheless, it is clear that VEGFR2/pSTAT3 signaling is a potential major pathway.

In conclusion, our data provide evidence that vascular-derived GlyRa2 mediates protective

effects in cerebrovascular reconstruction in post-ischemic injury. We infer that the VEGFR2/pSTAT3 pathway that confers vascular protection via GlyRa2 may represent a novel mechanism connecting modulation of the peripheral and central circulation, which may be beneficial for ischemic treatment strategies and therapeutic approaches. The findings in this study significantly provide new mechanistic insights into cerebrovascular protection and provide a better understanding of the function of glycine in cerebral ischemia.

Acknowledgements

This work was supported by grants from the Science Foundation of Zhejiang Province (LY-19H090007, LQ19H090008, LY15H190007); the National Natural Science Foundation of China Young Scientists (31672295); the Key Laboratory of Vector Biology and Pathogen Control of Zhejiang Province, Huzhou University (HUZUL201908).

Disclosure of conflict of interest

None.

Abbreviations

Gly, Glycine; GlyR, Glycine receptor; NMDAR, N-methyl-D-aspartic acid receptor; GlyRa2, Glycine receptor alpha 2; GlyRa1, Glycine receptor alpha 1; tMCAO, transient middle cerebral artery occlusion; BBB, Blood-brain barrier; CTZ, Cyclothiazide; VEGFR2, Vascular endothelial growth factor receptor 2; pSTAT3, Phosphorylated signal transducers, and activators of transcription 3.

Address correspondence to: Dr. Hua Yao, Guangxi Key Laboratory of Brain and Cognitive Neuroscience, Guilin Medical University, 109 North 2nd Huan Cheng Road, Guilin 541004, PR China. E-mail: hua.yao@wzu.edu.cn

References

[1] Xu TL and Gong N. Glycine and glycine receptor signaling in hippocampal neurons: diversity, function and regulation. Prog Neurobiol 2010; 91: 349-361.

[2] Sheng M, Cummings J, Roldan LA, Jan YN and Jan LY. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature 1994; 368: 144-147.

[3] Yao W, Ji F, Chen Z, Zhang N, Ren SQ, Zhang XY, Liu SY and Lu W. Glycine exerts dual roles in ischemic injury through distinct mechanisms. Stroke 2012; 43: 2212-2220.

[4] Liu Y, Wong TP, Aarts M, Rooyakkers A, Liu L, Lai TW, Wu DC, Lu J, Tymianski M and Craig AM. NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. J Neurosci 2007; 27: 2846-2857.

[5] Chen Z, Hu B, Wang F, Du L, Huang B, Li L, Qi J and Wang X. Glycine bidirectionally regulates ischemic tolerance via different mechanisms including NR2A-dependent CREB phosphorylation. J Neurochem 2015; 133: 397-408.

[6] Huang B, Chen P, Huang L, Li S, Zhu R, Sheng T, Yu W, Chen Z and Wang T. Geniposide attenuates post-ischaemic neurovascular damage via GluN2A/AKT/ERK-dependent mechanism. Cell Physiol Biochem 2017; 43: 705-716.

[7] Young-Pearse TL, Ivic L, Kriegstein AR and Cepko CL. Characterization of mice with targeted deletion of glycine receptor alpha 2. Mol Cell Biol 2006; 26: 5728-5734.

[8] Pilorge M, Fassier C, Corronc HL, Potey A, Bai J, De Gois S, Delaby E, Assouline B, Guinchat V and Devillard F. Genetic and functional analyses demonstrate a role for abnormal glycinergic signaling in autism. Mol Psychiatry 2015; 21: 936.

[9] Feng J, Craddock N, Jones IR, Cook EH, Goldman D, Heston LL, Peltonen L, Delisi LE and Sommer SS. Systematic screening for mutations in the glycine receptor α2 subunit gene (GLRA2) in patients with schizophrenia and other psychiatric diseases. Psychiat Genet 2001; 11: 45-48.

[10] Blednov YA, Benavidez JM, Black M, Leiter CR, Osterndorff-Kahanek E and Harris RA. Glycine receptors containing alpha 2 or alpha 3 subunits regulate specific ethanol-mediated behaviors. J Pharmacol Exp Ther 2015; 353: 181-191.

[11] Mccracken LM, Lowes DC, Salling MC, Carreau VC, Odean NN, Blednov YA, Betz H, Harris RA and Harrison NL. Glycine receptor α3 and α2 subunits mediate tonic and exogenous agonist-induced currents in forebrain. Proc Natl Acad Sci U S A 2017; 114: E7179.

[12] Pruthi D, Mccurley A, Aronovitz M, Galayda C, Karumanchi SA and Jaffe IZ. Aldosterone promotes vascular remodeling by direct effects on smooth muscle cell mineralocorticoid receptors. Arterioscler Thromb Vasc Biol 2014; 34: 355-364.

[13] Shi GP and Dolganov GM. Comprehensive transcriptome of proteases and protease inhibitors in vascular cells. Stroke 2006; 37: 537.

[14] Pires PW, Girgla SS, Moreno G, Mcclain JL and Dorrance AM. Tumor necrosis factor-α inhibition attenuates middle cerebral artery remodeling but increases cerebral ischemic damage in hypertensive rats. Am J Physiol Heart Circ Physiol 2014; 307: H658-H669.
[15] Rudic R, Brinster D, Cheng Y, Fries S, Song W, Austin S, Coffman T and FitzGerald G. COX-2-derived prostacyclin modulates vascular remodeling. Circul Res 2005; 96: 1240-1247.
[16] Yan T, Venkat P, Chopp M, Zacharek A, Ning R, Cui Y, Roberts C, Kuzmin-Nichols N, Sanberg CD and Chen J. Neurorestorative therapy of stroke in type 2 diabetes mellitus rats treated with human umbilical cord blood cells. Stroke 2015; 46: 2599-2606.
[17] Tao Y, Venkat P, Chopp M, Zacharek A and Chen J. Neurorestorative responses to delayed human mesenchymal stromal cells treatment of stroke in type 2 diabetic rats. Stroke 2016; 47: 2850.
[18] Boehm-Sturm P, Fuchtemeier M, Foddis M, Mueller S, Trueman RC, Zille M, Rinnenthal JL, Kypraios T, Shaw L, Dirnagl U and Farr TD. Neuroimaging biomarkers predict brain structural connectivity change in a mouse model of vascular cognitive impairment. Stroke 2017; 48: 468-475.
[19] Ong LK, Chow WZ, Tebay C, Kluge M, Pietrogrande G, Zalewska K, Crock P, Åberg ND, Bivard A and Johnson SJ. Growth hormone improves cognitive function after experimental stroke. Stroke 2018; 49: 1257.
[20] Zhang Z and Chopp M. Vascular endothelial growth factor and angiopoietins in focal cerebral ischemia. Trends Cardiovasc Med 2002; 12: 62-66.
[21] Leblanc GG, Golanov E, Awad IA and Young WL. Biology of vascular malformations of the brain. Stroke 2009; 40: e694-e702.
[22] Guo D, Murdoch CE, Xu H, Shi H, Duan DD, Ahmed A and Gu Y. Vascular endothelial growth factor signaling requires glycine to promote angiogenesis. Sci Rep 2017; 7: 1-10.
[23] Stobart JL, Lu L, Anderson HD, Mori H and Anderson CM. Astrocyte-induced cortical vasodilation is mediated by D-serine and endothelial nitric oxide synthase. Proc Natl Acad Sci U S A 2013; 110: 3149-3154.
[24] Li S, Szabó G, Jain RK, Kohling R, Vasudevan A, Peeyush KT, Joshee S, Kirschstein T, Subburaju S and Khalili JS. Endothelial cell-derived GABA signaling modulates neuronal migration and postnatal behavior. Cell Res 2018; 28: 221-248.
[25] Sen S, Roy S, Bandyopadhyay G, Scott B, Xiao D, Ramadoss S, Mahata SK and Chaudhuri G. γ-Aminobutyric acid is synthesized and released by the endothelium novelty and significance. Circul Res 2016; 119: 621-634.
[26] Esmaeili A and Zaker SR. Differential expression of glycine receptor subunit messenger RNA in the rat following spinal cord injury. Spinal Cord 2011; 49: 280-284.
[27] Addae JI, Ali N and Stone TW. Effects of AMPA and clomethiazole on spreading depression cycles in the rat neocortex in vivo. Eur J Pharmacol 2011; 653: 41-46.
[28] Addae JI, Ali N, Youssef FF and Stone TW. AMPA receptor activation reduces epileptiform activity in the rat neocortex. Brain Res 2007; 1158: 151-157.
[29] Zhang XB, Sun GC, Liu LY, Yu F and Xu TL. Alpha 2 subunit specificity of cyclothiazide inhibition on glycine receptors. Mol Pharmacol 2008; 73: 1195-1202.
[30] Brune W, Weber RG, Saul B, von Knebel Doeberitz M, Grond-Ginsbach C, Kellerman K, Meinck HM and Becker CM. A GLRA1 null mutation in recessive hyperekplexia challenges the functional role of glycine receptors. Am J Hum Genet 1996; 58: 989-997.
[31] Thomas RH, Chung SK, Wood SE, Cushion TD, Drew CJ, Hammond CL, Vanbellinghen JF, Mullins JG and Rees MI. Genotype-phenotype correlations in hyperekplexia: apnoeas, learning difficulties and speech delay. Brain 2013; 136: 3085-3095.
[32] Hirzel K, Müller U, Latal AT, Hülsmann S, Grudzinska J, Seeliger MW, Betz H and Laube B. Hyperekplexia phenotype of glycine receptor α1 subunit mutant mice identifies Zn2+ as an essential endogenous modulator of glycinergic neurotransmission. Neuron 2006; 52: 679-690.
[33] Szabolcsi V, Albisetti GW and Celio MR. Parvalbumin-neurons of the ventrolateral hypothalamic parvafox nucleus receive a glycinergic input: a gene-microarray study. Front Mol Neurosci 2017; 10: 8.
[34] Huang B, Xie Q, Lu X, Qian T, Li S, Zhu R, Yu W, Chen G, Chen Z, Xu X, Wang T and Li L. GlyT1 inhibitor NFPS exerts neuroprotection via GlyR alpha1 subunit in the rat model of transient focal cerebral ischaemia and reperfusion. Cell Physiol Biochem 2016; 38: 1952-1962.
[35] García-Alcocer G, Mejía C, Berumen LC, Miledi R and Martínez TA. Developmental expression of glycine receptor subunits in rat cerebellum. Int J Dev Neurosci 2008; 26: 319-322.
[36] Liu Q and Wong-Riley MT. Postnatal development of glycine receptor subunits α1, α2, α3, and β immunoreactivity in multiple brain stem respiratory-related nuclear groups of the rat. Brain Res 2013; 1538: 1-16.
[37] Jonsson S, Kerekes N, Hyytiä P, Ericson M and Söderpalm B. Glycine receptor expression in the forebrain of male AA/ANA rats. Brain Res 2009; 1305: S27-S36.

[38] Chen RQ, Wang SH, Wen Y, Wang JJ, Fang J, Yan JZ, Ren SQ, Zheng C, Liu SY and Lu W. Role of glycine receptors in glycine-induced LTD in hippocampal CA1 pyramidal neurons. Neuropsychopharmacology 2011; 36: 1948-1958.
[39] Avila A, Vidal PM, Dear TN, Harvey RJ, Rigo JM and Nguyen L. Glycine receptor α2 subunit activation promotes cortical interneuron migration. Cell Rep 2013; 4: 738-750.
[40] Avila A, Vidal PM, Tielens S, Morelli G, Laguesse S, Harvey RJ, Rigo JM and Nguyen L. Glycine receptors control the generation of projection neurons in the developing cerebral cortex. Cell Death Differ 2014; 21: 1696-1708.
[41] Aroeira RI, Ribeiro J, Sebastião AM and Valente CA. Age-related changes of glycine receptor at the rat hippocampus: from the embryo to the adult. J Neurochem 2011; 118: 339-353.
[42] Jiang P, Kong Y, Zhang XB, Wang W, Liu CF and Xu TL. Glycine receptor in rat hippocampal and spinal cord neurons as a molecular target for rapid actions of 17-β-estradiol. Mol Pain 2009; 5: 2.
[43] Lin MS, Xiong WC, Li SJ, Gong Z, Cao X, Kuang XJ, Zhang Y, Gao TM, Mechawar N, Liu C and Zhu XO. α2-glycine receptors modulate adult hippocampal neurogenesis and spatial memory. Dev Neurobiol 2017; 77: 1430.
[44] Yan-Do R, Duong E, Manning Fox JE, Dai X, Suzuki K, Khan S, Bautista A, Ferdaoussi M, Lyon J, Wu X, Cheley S, MacDonald PE and Braun M. A Glycine-insulin autocrine feedback loop enhances insulin secretion from human β-Cells and is impaired in type 2 diabetes. Diabetes 2016; 65: 2311.
[45] Senthilkumar R, Viswanathan P and Nalini N. Glycine modulates hepatic lipid accumulation in alcohol-induced liver injury. Pol J Pharmacol 2013; 55: 603.
[46] Senthilkumar R and Nalini N. Glycine prevents hepatic fibrosis by preventing the accumulation of collagen in rats with alcoholic liver injury. Pol J Pharmacol 2004; 56: 121-128.
[47] Ganta V, Choi M, Kutateladze A and Annex B. VEGF165b modulates endothelial VEGFR1-STAT3 signaling pathway and angiogenesis in human and experimental peripheral arterial disease. Circul Res 2017; 120: 282-295.
[48] Thirunavukkarasu M, Juhasz B, Zhan L, Menon V, Tosaki A, Otani H and Maulik N. VEGFR1 (Flt-1+/-) gene knockout leads to the disruption of VEGF-mediated signaling through the nitric oxide/heme oxygenase pathway in ischemic preconditioned myocardium. Free Radical Bio Med 2007; 42: 1487-1495.
[49] Bartoli M, Gu X, Tsai NF, Venema RC, Brooks S, Marrero M and Caldwell RB. Vascular endothelial growth factor activates STAT proteins in aortic endothelial cells. J Biol Chem 2000; 275: 33189-33192.
[50] Bartoli M, Platt D, Lemtalsi T, Gu XI, Brooks S, Marrero M and Caldwell RB. VEGF differentially activates STAT3 in microvascular endothelial cells. FASEB J 2003; 17: 1562-1564.
[51] Liao XH, Xiang Y, Li H, Zheng DL, Xu Y, Xi YC, Li JP, Zhang XY, Xing WB, Cao DS, Bao LY and Zhang TC. VEGF-A stimulates STAT3 activity via nitrosylation of myocardin to regulate the expression of vascular smooth muscle cell differentiation markers. Sci Rep 2017; 7: 2660.
[52] Yang Z, Wang H, Jiang Y and Hartnett ME. VEGFA activates erythropoietin receptor and enhances VEGFR2-mediated pathological angiogenesis. AM J Pathol 2014; 184: 1230-1239.

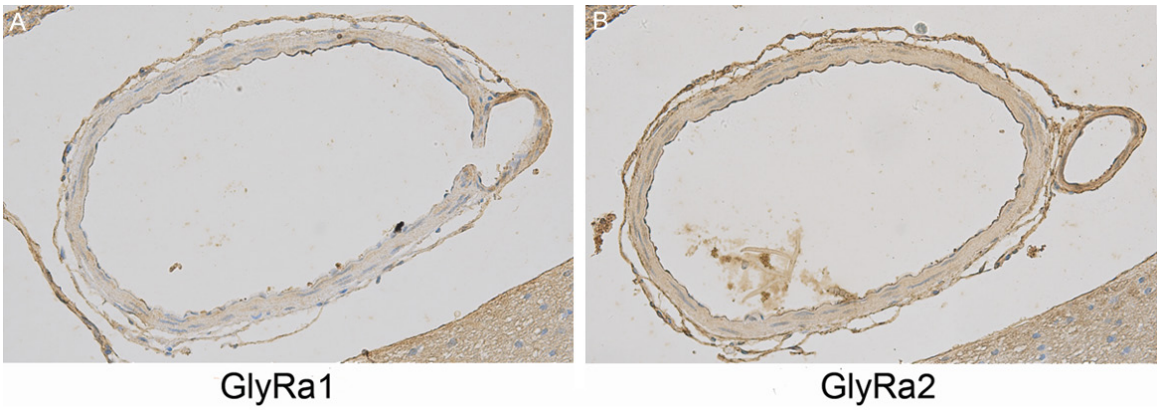


Figure S1. Expression levels of vascular GlyRa2 higher than GlyRa1. They were measured by immunohistochemical staining of whole-brain, cortical slices after cerebral ischemia. A: GlyRa1 expression levels are shown by immunohistochemical staining of the cerebral blood vessels. B: GlyRa2 expression levels are shown by immunohistochemical staining of the cerebral blood vessels.

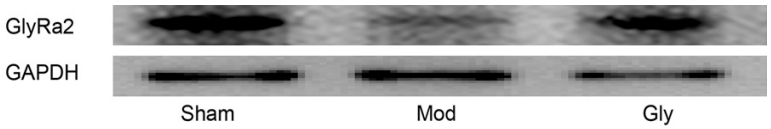


Figure S2. Additional original western images for relative levels of the GlyRa2 protein.