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

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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
Glycine attenuates neurovascular reconstruction via glycine receptor alpha 2 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% O₂/5% CO₂). The composition of the ACSF was as follows: 126 mM NaCl, 2.5 mM KCl, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 2.4 mM CaCl₂, 11 mM glucose, and 25 mM NaHCO₃. 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 Mg²⁺ 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% N₂ and 5% CO₂ 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.
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'-GCACCAAGCACTACAACC-3' (forward) and 5'-AGGACAGGATGACGATAAGC-3' (reverse), GlyRa2: 5'-GACACAGCAGTGGAACGATTC-3' (forward) and 5'-TCCCGAGCACTTGTTACAG-3' (reverse). The expression levels were normalized to those of constitutive 18S ribosomal RNA amplified with the primers 5'-GTAACC-CGTTGAACCCATT-3' (forward) and 5'-CCATC-CATCGGTAGTAGCG-3' (reverse). Relative expression data were quantified using the \(2^{-\Delta\Delta CT}\) 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-
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-
Glycine attenuates neurovascular reconstruction via glycine receptor alpha 2 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 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.
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Glycine attenuates 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.
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
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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
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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.

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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.

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**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.