Glycine Exerts Dual Roles in Ischemic Injury Through Distinct Mechanisms

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Background and Purpose—We characterized the differential effects of glycine at different levels in the induction of postischemic long-term potentiation, as well as in the neuronal damage induced by focal ischemia.

Methods—Whole-cell patch clamp recordings were obtained from rat hippocampal slice preparations. In vitro ischemia and postischemic long-term potentiation were induced by oxygen and glucose deprivation. In vivo ischemia was induced by transient middle cerebral artery occlusion.

Results—In both in vitro and in vivo ischemia models, glycine at low level exerts deleterious effects in postischemic long-term potentiation and ischemic neuronal injury by modulation of the N-methyl-D-aspartate receptor coagonist site; whereas glycine at high level exerts neuroprotective effects by activation of glycine receptor and subsequent differential regulation of N-methyl-D-aspartate receptor subunit components.

Conclusions—Our results provide a molecular basis for the dual roles of glycine in ischemic injury through distinct mechanisms, and they suggest that glycine receptors could be a potential target for clinical treatment of stroke. (Stroke. 2012;43:00-00.)

Key Words: glycine ■ ischemia ■ electrophysiology ■ middle cerebral artery occlusion ■ N-methyl-D ■ aspartate receptor
stroke, respectively.\textsuperscript{17,18} This NR2 subtype hypothesis highlights that the regulation of NR2 components may be an important mechanism by which neuroprotection is achieved.\textsuperscript{19}

Although numerous studies have reported the effect of glycine in ischemic cerebral stroke, the results remain quite controversial. Some studies suggest that glycine may contribute to the development of ischemic injury,\textsuperscript{20,21} whereas other studies propose the neuroprotective effect of glycine.\textsuperscript{17,22,23} Moreover, the mechanisms underlying glycine’s effects on ischemia are also quite controversial. NMDAR was initially suggested to take a critical role in this neuroprotective effect by glycine.\textsuperscript{17} Recently, more experimental evidence points to the involvement of strychnine-sensitive GlyR or NMDAR in neuroprotection of ischemic stroke by glycine.

In the present study, we aimed to elucidate the mechanisms underlying the inconsistencies on the role of glycine in ischemia.

**Materials and Methods**

**Hippocampal Slice Preparation**

Male Sprague Dawley rats, age 18 to 21 days old, were anesthetized with ethyl ether and decapitated. The entire brain was removed and coronal brain slices (350-μm thickness) were cut using a vibrating blade microtome in ice-cold artificial cerebrospinal fluid (ACSF) containing: (in mmol/L) 126 NaCl, 2.5 KCl, 1 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, 1.25 KH\textsubscript{2}PO\textsubscript{4}, 26 NaHCO\textsubscript{3}, and 20 glucose. pH was adjusted to 7.4. ACSF was bubbled continuously with Carbogen (95% O\textsubscript{2}/5% CO\textsubscript{2}). Fresh slices were incubated in a chamber with carbogenated ACSF and were recovered at 34°C for at least 1.5 hours before being transferred to a recording chamber.

**Electrophysiological Studies**

Conventional whole-cell recordings were made with patch pipettes containing (in mmol/L): 132.5 Cs-glucuronate, 17.5 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, 0.5 EGTA, 10 HEPES, 4 adenosine triphosphate (ATP), and 5 QX-314, with the pH adjusted to 7.2 by CsOH. Synaptic responses were evoked at 0.05 Hz. Excitatory postsynaptic currents (EPSC) were recorded at −70 mV holding potential in ACSF perfusion media containing bicuculline methiodide (10 μmol/L) to block α-aminobutyric acid-A-receptor-mediated inhibitory synaptic currents. CA1 neurons were viewed under upright microscopy (ECLIPSE E600-FN, Nikon, Inc.) and recorded with an Axopatch-200B amplifier (Molecular Devices). Changes in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated EPSC (AMPA EPSC) amplitude were examined during the last 5 minutes of recording. NMDA receptor-mediated EPSCs (NMDA EPSC) were recorded in Mg\textsuperscript{2+}-free medium when cells were held at −70 mV. Data were low-pass filtered at 2 kHz and acquired at 5 to 10 kHz. Recordings from each neuron lasted ≥40 to 80 minutes. The series resistance in these recordings varied between 4 to 6 Ω. The series resistance was always monitored during recording for fear of sealing the ruptured membrane, which will cause changes in both the kinetics and amplitude of the EPSCs. Cells in which the series resistance or capacitance deviated by >20% from initial values were excluded from analysis. Also, cells with series resistance >20 Ω at any time during the recording were excluded from analysis. Data were collected with pCLAMP9.2 software and analyzed using ClampFit 9.2 (Molecular Devices).

**Oxygen-Glucose-Deprivation-Induced Ischemia Model**

Anoxia/hypoglycemia was induced by replacing 95% O\textsubscript{2}/5% CO\textsubscript{2} with 95% N\textsubscript{2}/5% CO\textsubscript{2} and switching to an ACSF containing 20 mmol/L sucrose instead of glucose for 5 to 10 minutes.\textsuperscript{24,25}

**In Vivo Middle Cerebral Artery Occlusion Ischemia Model**

Transient cerebral focal ischemia was produced by middle cerebral artery occlusion (MCAO) as reported previously.\textsuperscript{17,26–28} Briefly, male Sprague-Dawley rats (280–300 g) were anesthetized, and MCAO was produced by occluding the MCA with a monofilament suture. Rectal temperature, blood pressure, and blood gases were monitored during the experiments. Animals were killed 24 hours after MCAO onset. For posttreatment experiments, animals were subjected to a 1-hour MCAO, and drug treatments were then performed via intraperitoneal injection 3 hours after reperfusion (4 hours after the onset of MCAO). Volume of cerebral infarction was analyzed using brain sections stained with 2,3,5-triphenyltetrazolium chloride. Ten minutes before the animals were killed, neurological evaluation was performed on a 6-point scale to assess postischemic motor and behavioral deficits.\textsuperscript{26,29}

**Stereotactic Virus Infection**\textsuperscript{30,31}

Twelve-day rats were anesthetized with trichloroacetaldehyde hydrate and were placed in a stereotaxic frame containing a mouse/neonatal rat adaptor (RWD Life Science). The virus was injected into hippocampus region with a 10 μL syringe and the flow rate (5 μL at 0.5 μL/min) was controlled by a microsyringe pump controller (Word Precision Instruments). Rats were returned to home cage after injection and were used for whole-cell recording at 18 to 20 days.

**Drugs**

Blockers, including AP5 and NBQX, were purchased from Sigma-Aldrich. Bicuculline methiodide was purchased from Tocris.

**Data Analysis**

All population data were expressed as mean±SEM. Within-group comparisons were performed using paired-samples t tests, and differences between groups were compared using Independent-Samples t test and analysis of variance (ANOVA) post hoc comparisons. A 1-way ANOVA test was used when equal variances were assumed. Differences were considered significant when P<0.05, and the significance for homogeneity of variance test was set at 0.1.

**Results**

Glycine Exerts Dose-Dependent Bidirectional Effects in Pathological Plasticity

We carried out whole-cell patch clamp recordings of evoked AMPA receptor (AMPAR)-mediated EPSCs in CA1 neurons of hippocampal slices. AMPA EPSCs were recorded when cells were held at −70 mV. In vitro oxygen-glucose deprivation (OGD) treatment induces a persistent increase of EPSC amplitude (n=6, P<0.01, paired-samples t test; Figure 1A, 1B). This potentiation of EPSCs was completely abolished by perfusing slices with selective NMDAR antagonist DL-AP5 (50 μmol/L; Supplemental Figure I) during OGD, suggesting i-LTP requires NMDAR activation (n=6; P<0.01). During OGD treatment, a range of concentrations of glycine were applied for 5 minutes to activate briefly NMDARs under OGD condition in CA1 neurons. A brief 0.01 mmol/L glycine treatment significantly facilitated pathological potentiation of EPSCs by elevating potentiation magnitude (P<0.01, 1-way ANOVA LSD test). Increasing the glycine level to 0.2 mmol/L failed to display any effect on i-LTP (P>0.05). Interestingly, when the exogenous glycine was additionally increased to 0.6 mmol/L, a significant attenuation of the magnitude of i-LTP was observed (P<0.01; Supplemental Figure I). This pathological LTP was completely abolished when the exogenous glycine was in-
creased again to 1.0 mmol/L (n=6; P<0.01). These results demonstrated that exogenous glycine exerts dose-dependent bidirectional effect on pathological i-LTP of AMPAR-mediated EPSCs. Notably, the excitatory synaptic transmission recorded in the hippocampal CA1 area was not largely inhibited during OGD treatment. This resistance of EPSCs to OGD may be because the slices we used here were prepared from age 18- to 21-day-old rats.

We additionally sought to determine whether endogenously released glycine can exert bidirectional effects in i-LTP as did exogenous glycine. To test this possibility, we inhibited type 1 glycine transporters to observe how accumulation of extracellular glycine with these treatments could affect i-LTP. It is reported that blockade of glycine transporter potentiates NMDAR currents, suggesting that suppressing the uptake of glycine causes accumulation of endogenous glycine in the synaptic cleft.32 We used specific GlyT1 blocker, N[3-(4-fluorophenil)-3-(4-phenilphenoxy)] propylsarcosine (NFPS), to examine whether endogenous accumulation of glycine following GlyT1 blockade could alter i-LTP. We found that perfusing slices with a subsaturating concentration of the NFPS (0.1 μmol/L) during OGD facilitated i-LTP of EPSCs (P<0.01; Figure 1C, 1D). In contrast, a saturating concentration of the NFPS (2.0 μmol/L) completely abolished i-LTP of EPSCs (n=6; P<0.01). These results were additionally confirmed by utilizing another GlyT1 blocker sarcosine (Supplemental Figure II) and they suggest that endogenous glycine at different levels can facilitate or suppress pathological i-LTP of EPSCs in CA1 hippocampal neurons.

**Modulation of Distinct Sites Underlies Glycine-Induced Dual Effects in i-LTP**

We also investigated the mechanisms by which glycine exerts differential roles on pathological i-LTP. Glycine mainly exerts its action on 2 sites: the NMDAR coagonist binding site (site B) and the GlyR site (site A), which mediate excitatory and inhibitory actions, respectively. We hypothesize that the potentiative effect by low level of glycine is achieved by its modulation of the NMDAR coagonist binding site (site B), whereas the suppressive effect produced by high-level glycine is mediated by activation of GlyRs (site A). We first examined whether modulation of the NMDAR coagonist site takes a role in potentiation of i-LTP. L689–560 is a highly specific glycine site B antagonist. Low-level L689–560 (0.5 μmol/L) applied during OGD partially blocked the glycine’s effect at site B and in turn antagonized enhancement of NMDA EPSCs induced by glycine (0.01 mmol/L) in the OGD model (Supplemental Figure IV). NMDA EPSCs were recorded in Mg2+-free medium when cells were held at −70 mV. Interestingly, the same L689–560...
(0.5 μmol/L) treatment totally abolished the potentiation of i-LTP \( (P<0.05; \text{Figure 3A, 3B}). \) In contrast, selective GlyR antagonist strychnine (5 μmol/L) failed to exert any additional effect on glycine-induced potentiation of i-LTP \( (P>0.05). \) These results suggest that modulation of the NMDAR coagonist binding site by glycine accounts for its potentiative effect on i-LTP.

We then investigated whether the suppressive effect produced by high-level glycine (1.0 mmol/L) is mediated by activation of GlyRs (site A). We found that GlyR antagonist strychnine (5 μmol/L) applied during OGD totally reversed glycine-induced suppression of i-LTP \( (n=6; P<0.01; \text{Figure 3C, 3D}). \) In contrast, antagonizing site B function with L689–560 (0.5 μmol/L) failed to affect the glycine-induced suppression of i-LTP \( (n=7; P>0.05), \) suggesting that suppression of i-LTP by glycine requires activation of GlyRs. These results demonstrate that modulation of distinct sites underlies glycine-induced dual effects on i-LTP, and thus points to the notion that distinct mechanisms underlie glycine-induced bidirectional effects on i-LTP.

**GlyR Activation and Subsequent Regulation of NMDAR Subunit Components**

Recent studies suggested that NMDAR subunits have differential roles in mediating postischemic neuronal damage both in vitro and in vivo.\(^{17,25}\) It raises the possibility that the
suppression of i-LTP by glycine may be attributable to readjustment of NMDAR subunit components that were already altered by OGD treatment. To examine this possibility, we investigated whether and how the NMDAR NR2 subunits were affected by glycine treatment in the OGD in vitro model. We employed a set of experimental protocols and took advantage of the selective NR2B antagonist ifenprodil (3 μmol/L) to examine the functional NR2A or NR2B amplitude under various conditions (Figure 4A). We detected a marked increase of the amplitude of NR2B-containing, NMDAR-mediated EPSCs under brief OGD treatment accompanied by the potentiation of amplitude of total NMDA EPSCs (P<0.01; Figure 4B, 4C). In contrast, we only observed a slight increase in the NR2A-containing, NMDAR-mediated EPSCs under brief OGD treatment, and this change did not show any significance (P>0.05). Interestingly, simultaneous glycine (1.0 mmol/L) treatment suppressed the NR2B-containing, but not NR2A-containing, NMDAR-mediated EPSCs. As a result, the functional NR2B-containing NMDAR and total NMDAR-mediated EPSCs amplitude were readjusted, and thus restored to normal baseline level. The suppression of NR2B-mediated NMDAR function elicited by glycine was completely reversed by strychnine treatment (5 μmol/L; P<0.01). These results suggest that the neuroprotective effect of glycine through GlyR activation may be correlated with its suppression of the NR2B-containing NMDAR component.

**Glycine Exerts Neuroprotective Effect via Suppressing the NR2B Component in In Vitro Stroke Model**

To examine further whether change in the NR2B component accounts for the glycine’s neuroprotection mediated by GlyRs activation, we used a low concentration of the NR2B-specific antagonist ifenprodil to block partially the elevated NR2B-mediated function caused by brief OGD treatment, and thus increased and restored NMDAR NR2 components. Then we observed how this change in the NR2 component through pharmacological manipulation could affect i-LTP of both AMPAR- and NMDAR-mediated EPSCs. However, it is possible that partial NR2B block could decrease total NMDAR function, which might in turn lead to change in i-LTP. To rule out this possibility, we first investigated the dose-response relationship of a competitive NMDAR antagonist AP-5 and chose a concentration (1.2 μmol/L) that did not display any marked effect synaptic plasticity. Then we adjusted the concentration of ifenprodil to a level that matched the NMDAR inhibition by 1.2 μmol/L AP-5 (Figure 5B). We determined that AP-5 at concentration of 1.2 μmol/L failed to exert any obvious effect on OGD-induced i-LTP (P>0.05, Independent-Samples t test; Figure 5D). Ifenprodil in 1.0 μmol/L could inhibit NMDAR-mediated EPSCs to the same level as did 1.2 μmol/L AP-5 (P>0.05; Independent-Samples t test; Figure 5B). However, ifenprodil at 1.0 μmol/L concentration applied before and during i-LTP induction markedly decreased NR2B-containing NMDAR to baseline level (n=8, P<0.01; Figure 5A). In sister slices obtained from the same hippocampus with the same concentration ifenprodil perfusion, brief OGD treatment failed to produce i-LTP (Figure 5C). No additional change was observed when ifenprodil (1.0 μmol/L) was coapplied with glycine application (1.0 mmol/L), suggesting regulation of NR2A/NR2B by ifenprodil excluded the effect caused by GlyR activation. Thus, restoration of NR2B-containing NMDAR achieved by partial NR2B blockade completely suppressed the induction of i-LTP. Glycine exerts neuroprotective effect via suppressing the NR2B component in the in vitro stroke model.

We used knockdown animals to examine whether above results that are based on pharmacological treatments are

![Figure 4](http://stroke.ahajournals.org/)

**Figure 4.** Glycine, through activating GlyRs, suppressed OGD-induced elevation of NR2B-containing NMDAR components. A, Schematic paradigms showing experimental protocols used to detect the NR2 subunit components under various conditions including control, OGD, OGD with 1.0 mmol/L glycine (+Gly), and coapplication of glycine with strychnine (+Gly&stry). Selective NR2B antagonist ifenprodil (3 μmol/L) was used to examine the amplitude of NR2A- or NR2B-containing NMDA EPSCs in recorded neurons. The vertical arrows in the diagram indicate the time points to record NMDA EPSCs. B, Normalized NMDA EPSCs showing magnitude of total NMDA EPSCs, NR2A- and NR2B-containing NMDAR subunit component under various conditions. Sample traces of evoked NMDAR-mediated synaptic responses (held at +40 mV) were displayed at top to illustrate the changes in amplitude under various treatments. C, Comparison of NR2A- and NR2B-containing NMDAR components among different experimental groups subjected to different treatments. Only a slight increase in NR2A-containing NMDA EPSCs was detected, but this change did not show significance. Sample traces were rescaled and overlaid at top to show changes in the decay time of NMDA EPSCs under various conditions. **P<0.01, compared with control.**
valid. We performed in vivo transfection by microinjecting lentivirus-packed construct containing small hairpin ribonucleic acid into hippocampus under stereotaxic guidance. Next, we cut hippocampal slices and performed whole-cell patch recording in transfected green fluorescent protein-tagged Hippocampal CA1 cells in OGD model (Supplemental Figure V). We found that OGD-induced i-LTP was not observed in NR2B knockdown rats ($n = 6; P < 0.05$), suggesting that attenuating NR2B-containing NMDAR can suppress i-LTP. Moreover, 1.0 mmol/L glycine treatment failed to exert additional effect ($n = 6; P < 0.05$; Figure 6A, 6B). In contrast, treatment with NR2A-antagonist NVP-AAM077 (NVP, 2.4 mg/kg; from YP Auberson, Novartis Pharma AG$^{17,34}$) reversed the attenuation of infarct volume produced by glycine ($n = 6$), indicating that glycine acted via selective enhancement of NR2A-containing NMDAR function. Additional strychnine treatment (0.42 mg/kg) did not display additional effect ($P > 0.05$), suggesting that NR2A inhibition excludes the effect of GlyR activation and acts downstream from it. These results demonstrated that in contrast to the dominant role of NR2B-containing NMDAR in acute in vitro stroke model, NR2A-containing NMDAR seems to play a more critical role in the in vivo postischemic model.

**Discussion**

Mounting studies have reported the effect of glycine in ischemic cerebral stroke. However, the results remain quite controversial. Some studies favored the facilitation of ischemic cerebral stroke, while others suggested a neuroprotective role. Our findings support the hypothesis that glycine exerts a neuroprotective effect via elevating NR2A-containing NMDAR function in vivo.

**Figure 5.** Selective pharmacological inhibition of NR2B-containing NMDAR components excludes glycine’s neuroprotective effect on i-LTP. **A.** Changes in both total NMDAR and NR2-containing NMDAR mediated EPSCs when subjected to various treatments. Selective inhibition of NR2B-containing NMDAR components with 1.0 μmol/L NR2B specific blocker ifenprodil restored NR2B-mediated EPSCs to control levels. Additional glycine treatment (1.0 mmol/L) failed to display additive effect. Sample traces of evoked NMDAR-mediated synaptic responses (held at +40 mV) were rescaled and overlaid at top to illustrate the difference in decay time under various treatments. **B.** Similar extent of inhibition of NMDA EPSCs by 1.0 μmol/L ifenprodil and 1.2 μmol/L AP5. Selective inhibition by ifenprodil shortened the decay time of NMDA EPSCs, whereas the nonselective antagonist AP5 did not, as shown by sample traces at top. **C.** Partial NMDAR blockade by 1.0 μmol/L ifenprodil totally abolished the i-LTP of AMPAR-mediated EPSCs. Additional glycine treatment (1.0 mmol/L) failed to display additive effect. **D.** Partial NMDAR blockade by 1.2 μmol/L AP5 failed to exert significant effect on i-LTP.
mic brain damage by glycine, \textsuperscript{20,21} whereas others proposed the neuroprotective effect of glycine. \textsuperscript{17,22,23} We report here that the level of glycine, either exogenously applied or endogenously released, could be a major determinant in setting the polarity of glycine’s role in the development of brain ischemia and may account for this discrepancy in previous investigations. We demonstrated that distinct mechanisms underlie glycine’s bifurcated actions at different levels. Glycine at a low level facilitates i-LTP, whereas it suppresses i-LTP at a relatively high level through activation of GlyRs. This observation may be attributable to glycine’s characteristic features. Glycine is a 2-faceted, bioactive molecule in the central nervous system. It can exert its action via both coagonist sites in NMDARs and in GlyRs at extrasynaptic sites in Hippocampal CA1 neurons. Because glycine has a higher affinity to NMDARs than to GlyRs, it displays a preference to combine with NMDARs at a relatively low level. When the concentration of glycine increases, it may activate extrasynaptic GlyRs and display an inhibitory effect that is helpful to restoring the excitation-inhibition balance. Therefore, our results match well with the sequential activation of these 2 sites by glycine at different levels.

Although numerous studies support the neuroprotective effect by glycine on brain ischemia, the current knowledge on its molecular basis is still paradoxical. NMDAR has been proven to have a pathological role in various neurological diseases, including in ischemic stroke, \textsuperscript{35} and glycine can affect NMDAR function through priming NMDAR internalization. \textsuperscript{36} These studies point to the possibility that glycine is involved in the neuroprotective effect via regulating NMDAR function. In the present study, however, we demonstrated that the GlyR-selective blocker strychnine completely reversed glycine’s neuroprotective effect in both in vitro and in vivo stroke models, thus providing solid evidence to the notion that GlyR is an important mediator for glycine’s effect. These observations receive support from some other recent studies that point to the possible involvement of GlyR in neuroprotection. \textsuperscript{22,23}

All these studies, including our present one, however, raise new questions that need to be addressed. First, can glycine concentrations after systemic administration of 80 and 800 mg/kg of glycine reach around 0.01 and 1 mmol/L, respectively, at which the bifurcated effects of glycine are observed in the in vitro model? To this purpose, we performed in vivo microdialysis at different time points after focal ischemia to examine the time-dependent profile of extracellular glycine concentration in the hippocampus (Supplemental Figure III). We found that the peak glycine concentrations in microdialysates in the MCAO-model rats after administration of 80 and 800 mg/kg of glycine were 237 ± 50 and 697 ± 84 pmol/40 μL microdialysate, respectively. Extracellular glycine concentrations were therefore estimated as 21.21 ± 4.46 and 65.25 ± 7.50 μmol/L, respectively. It seems that these results are inconsistent with the results under OGD treatment in Figure 1, which states that exogenous glycine exerts dose-dependent bidirectional effect on pathological i-LTP at 0.1 and 1.0 mmol/L, respectively. This discrepancy is attrib-
utable to, at least in part, the difference between the OGD-induced in vitro model and in vivo MCAO models, including different mechanisms underlying glycine’s effects and different experimental protocols of the glycine intervention. First, the mechanisms underlying glycine’s neuroprotective effect in these 2 models are different. Although activation of the GlyR was required for the neuroprotection in both in vitro and in vivo models, different NR2 subunit-containing NMDARs were involved in glycine’s neuroprotective effects under the 2 conditions. Inhibition of NR2B-containing NMDARs mediated glycine’s neuroprotective effects in the OGD-induced in vitro model, whereas activation of NR2A-containing NMDARs mediated glycine’s neuroprotective in the in vivo MCAO model. It has been proposed that different downstream signaling pathways coupled with NR2A- and NR2B-containing NMDAR.15

Synaptic (predominantly NR2A-containing) and extrasynaptic (predominantly NR2B-containing) NMDARs have differential functions, promoting survival and death, respectively.37 It is possible that the dosage of glycine required to affect downstream signaling cascade coupled with these 2 different subunits are different. In the in vivo MCAO model, a low concentration of glycine would be able to activate extrasynaptic NR2B to produce damaging effects. Because of the relatively low extrasynaptic glutamate concentration during perfusion, an additional increase in glycine concentration may not be able to increase these NR2B-mediated damaging effects; however, a slight increase in glycine concentration may have quickly surpassed glycine uptake activity and thereby reached the threshold to activate synaptic NR2A receptors, triggering NR2A-mediated survival signaling. Therefore, compared with in vitro experimental conditions, glycine may have a much narrower window between damaging and protective effects under our in vivo conditions. Second, the time points of glycine application and, as a result, the stage of intervention by glycine, were different. In the in vitro OGD-induced stroke model, glycine was applied during OGD treatment, which was supposed to exert its role on i-LTP induction (onset of ischemia). After the 5-minute application, glycine was quickly washed out. Therefore, the actual effective time of glycine was transient, not to mention it also took some time before glycine penetrated the surface of brain slices to the region surrounding the recorded cells to exert its action. In contrast, glycine intervention in the in vivo MCAO model occurred 3 hours after termination of MCAO and start of reperfusion (4 hours after the onset of MCAO). This mimicked real situations in most clinical settings. Because transporting a patient to the hospital and obtaining a definitive diagnosis usually take time, treatment is not usually possible until several hours after stroke onset. According to our in vivo microdialysis data, the level of glycine after in vivo glycine intervention was steady and could last for 2 hours. This would favor glycine to exert its effect on infarct volume. Therefore, compared with the OGD model, the concentration of glycine required to display neuroprotective effect in the MCAO model could be lower.

Second, how does glycine exert its effect through both GlyRs and NMDARs? How do we reconcile these conflicting findings? Our results provide a hint by demonstrating that activation of GlyR by glycine could elicit subsequent functional regulation of NMDAR NR2 subunit components. This finding is complementary to those of a recent study that revealed the critical role of regulating NMDAR in treatment of ischemic injury.17 Moreover, we found that glycine can exert differential regulation of NMDAR NR2 subunits at different developmental stages of ischemia. In the in vitro stroke model achieved by brief OGD, which simulates acute brain ischemia, pathological i-LTP was found to contribute to abnormal enhancement of NR2B-containing NMDARs. At this stage, glycine exerts neuroprotective effect on i-LTP mainly through resetting the NR2B-containing NMDAR to control levels by suppressing the NR2B component. Under some pathological conditions, such as stroke and brain trauma, there is usually a transient and rapid increase in extracellular glutamate concentrations and consequent activation of extrasynaptic receptors, which are predominantly NR1/NR2B receptors38,39; this results in a predominant activation of the NR1/NR2B receptor-coupled apoptosis pathway. Thus, it is understandable that blocking these receptors can be neuroprotective under these pathological conditions. However, considering the fast restoration of the extracellular glutamate concentration to prestroke levels (as quickly as 30 minutes after stroke onset)7,40 and that extrasynaptic NR1/NR2B receptors may not be activated thereafter, the down-regulation of NR2B by glycine may have a narrow window for practical treatment.17 Interestingly, even in the rat in vivo MCAO model of focal ischemia, glycine administration 3 hours after a 1-hour MCAO challenge displayed obvious neuroprotection on infarct volume when compared with MCAO alone. Under this condition, NR2A activation, but not NR2B antagonism, displayed obvious neuroprotection. The reversal of neuroprotection by NR2A antagonist was not additionally affected by blockade of GlyRs, suggesting both kinds of neuroprotection exerted by glycine or NR2A share a signaling pathway, and that NR2A regulation acts downstream of GlyR activation. Thus, differential regulation of NR2A and NR2B subunits is attributable to the neuroprotective effect by glycine at different postischemic stages.

Third, how does the activation of strychnine-sensitive glycine receptors affect NR2 subunits? We are still uncertain about this. One possibility is that there is crosstalk between strychnine-sensitive GlyRs and NMDARs. We have found in an ongoing study that glycine at a high level can induce NMDAR internalization via activation of GlyRs (unpublished data). This NMDAR internalization could be subunit-specific and partially contribute to the attenuation of NMDAR-mediated EPSCs following GlyR activation.

**Conclusion**

We have shown that the glycine at different levels exerts differential roles on pathological i-LTP and neuronal damage through distinct mechanisms. The deleterious effect exerted by low-level glycine was mediated by its modulation on the NMDAR coagonist site, whereas the neuroprotective effect of high-level glycine was mediated by GlyR activation and subsequent differential regulation of NMDAR NR2 subunit components in both the in vitro and in vivo stroke models.
Our results may reconcile previous conflicting findings on the role of glycine in ischemia.

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Disclosures

None.

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Supplemental Methods

In Vivo Microdialysis

Microdialysis probe implantation. Rats were anesthetized with chloral hydrate and placed in a stereotaxic apparatus (RWD Life Science, China). The head skin was shaved, disinfected, then cut with a sterile scalpel to expose the skull. A hole was drilled in order to allow the implantation of the probe into the brain parenchyma. The probe (MAB, Sweden) was coordinately implanted into the CA1/CA2 regions of the hippocampus (AP -5.8 mm, ML ±5.0 mm from bregma and DV -8.0 mm from dura according to the Paxinos and Watson atlas (1986). Then, the probe secured to the skull with two stainless steel screw and dental cement. All the probes performed in vivo experiments were with a nominal active length of 4 mm. In the end, the skin was sutured, and the rats were allowed to recover from surgery for at least 4 days before the MCAO.

Microdialysis samples collection. Microdialysis experiments were performed on conscious rats in CMA/120 system for freely-moving Animals. Throughout ischemia, dialysis probes were perfused with Krebs-Ringer's solution containing (in mmol/L): NaCl 145, KCl 0.6, MgCl2 1.0, CaCl2 1.2, Ascorbic acid 0.1, KH2PO4 2.0, K2HPO4 2.0 at a rate of 2 μl/min using CMA 2.5 ml Exmire microsyringe MS-GAN250 and CMA 402 syringe pump. The microdialysate (40 μl) was collected every 20 minutes. In all experiments, the microdialysis membrane was stabilized for 1 h without collecting samples. The samples were collected from 1 hour before MCA occlusion to 6 hours after MCAO. Drug treatments were performed via intraperitoneal 4 h after the onset of MCAO. In vitro recovery of the probe for glycine was about 28%. Glycine concentrations in the samples were measured by high performance
liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

**Supplemental References**


**Figure S1.** Change of pathological i-LTP under various treatments. A, OGD-induced i-LTP was completely abolished by selective NMDAR antagonist, D,L-AP5 (50 µmol/L; n = 6, *P* < 0.01). B, Glycine at 0.6 mmol/L markedly suppressed the i-LTP (n = 6, *P* < 0.01).
Figure S2. Bidirectional regulations of i-LTP by endogenous glycine. A, Differential regulations of OGD-induced i-LTP by endogenous glycine at different levels, achieved by blockade of GlyT1 with sarcosine at different concentrations. Sarcosine, a GlyT1 blocker, when was applied at subsaturating concentration (0.5 mmol/L), significantly facilitated OGD-induced i-LTP (n = 6), whereas completely abolished i-LTP when applied at saturating concentration (5.0 mmol/L, n = 6). Sarcosine at an intermediate concentration failed to display any noticeable effect on i-LTP (n = 6). B, Histogram summarizes data from experiments shown in A. ** p < 0.01, compared with OGD group.
Figure S3. Time-dependent profile of extracellular concentrations of glycine in focal ischemia. In vivo microdialysis at different time-points was performed in the MCAO model to examine the time-dependent profile of extracellular glycine concentration in hippocampus after intraperitoneal administration of 80 mg/kg (L-G, n = 4), 400 mg/kg (M-G, n = 5) and 800 mg/kg (H-G, n = 6) of glycine. Because the glycine was applied at 3 hours after MCAO (marked by the vertical arrow), we skip the intermediate timepoints and only monitored glycine concentration before and 140 minutes after MCAO. In contrast, glycine level of MCAO control (Con, n = 5) was monitored throughout the whole experimental process. Consistent with previous study,1 MCAO caused increase of glycine level. The peak glycine concentrations in microdialysates in the MCAO model rats after administration of 80 mg/kg, 400 mg/kg and 800 mg/kg of glycine were 237 ± 50 pmol/40 µL, 456 ± 62 pmol/40 µL and 697 ± 84 pmol/40 µL microdialysate, respectively. Extracellular glycine concentrations were therefore estimated as 21.21 ± 4.46 µmol/L, 40.73 ± 5.33 µmol/L and 65.25 ± 7.50 µmol/L, respectively. The horizontal bar stands for the MCAO period (60 minutes). **P < 0.01, compared with baseline before MCAO.
Figure S4. Samples displaying changes in amplitude of NMDA EPSCs under various conditions including OGD treatment (A), co-treatment of OGD and 0.01 mM glycine (OGD + Gly; B), co-treatment of OGD, glycine and L689 (OGD + Gly + L689; C).
**Figure S5** Knockdown of NR2B-containing NMDAR components excludes glycine’s neuroprotective effect on i-LTP. A and B, Photomicrographs of EGFP expression in hippocampal slices prepared from injected hemispheres following unilateral injections (5 µl) of a lentivirus coexpressing EGFP and NR2B-shRNA (A) or NR2A-shRNA (B). Images were collected from fixed slices following electrophysiological recordings. Scale bar: 15 µm. C, Sample traces showing the changes of NR2B component in NMDAR-mediated currents in NR2B-shRNA and NR2B-shRNA animals. As expected, compared with wildtype control (WT), NR2A-shRNA caused increase of Ifen-sensitive NR2B component (middle panel), whereas NR2B-shRNA largely decreased NR2B component (right panel). D, OGD treatment failed to induce i-LTP in NR2B knockdown rats (n = 3, P > 0.05). Further glycine (1.0 mmol/L) application did not display any additive effect (n = 4, P > 0.05). E, In NR2A knockdown animals (n = 4, P < 0.01), OGD-induced i-LTP was abolished by glycine at 1.0 mmol/L (n = 4, P < 0.01). The OGD controls (OGD WT) in D and E are the same as in Figure 5C.