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Original Paper

GlyT1 Inhibitor NFPS Exerts Neuroprotection via GlyR Alpha1 Subunit in the Rat Model of Transient Focal Cerebral Ischaemia and Reperfusion

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Key Words
NFPS • Glycine • Ischaemia • Salicylate • GlyR α1

Abstract
Background/Aims*:** Glycine is a strychnine-sensitive inhibitory neurotransmitter in the central nervous system (CNS), especially in the spinal cord, brainstem, and retina. The objective of the present study was to investigate the potential neuroprotective effects of GlyT1 inhibitor N [3-(4'-fluorophenyl)-3-(4'-phenylphenoxy) propyl] sarcosine (NFPS) in the rat model of experimental stroke. ***Methods: *In vivo* ischaemia was induced by transient middle cerebral artery occlusion (tMCAO). The methods of Western Blotting, Nissl Staining and Morris water maze methods were applied to analyze the anti-ischaemia mechanism. ***Results:*** The results showed that high dose of NFPS (H-NFPS) significantly reduced infarct volume, neuronal injury and the expression of cleaved caspase-3, enhanced Bcl-2/Bax, and improved spatial learning deficits which were administered three hours after transient middle cerebral artery occlusion (tMCAO) induction in rats, while, low dose of NFPS (L-NFPS) exacerbated the injury of ischaemia. These findings suggested that low and high dose of NFPS produced opposite effects. Importantly, it was demonstrated that H-NFPS-dependent neuronal protection was inverted by salicylate (Sal), a specific GlyR α1 antagonist. Such effects could probably be attributed to the enhanced glycine level in both synaptic and extrasynaptic clefts and the subsequently altered extrasynaptic GlyRs and their subtypes. ***Conclusions:*** These data imply that GlyT1 inhibitor NFPS may be a novel target for clinical treatment of transient focal cerebral ischaemia and reperfusion which are associated with altered GlyR alpha 1 subunits.

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Introduction

Sodium- and chloride- dependent GlyT1 is a membrane protein which recaptures glycine. Glycine is a strychnine-sensitive inhibitory neurotransmitter in the central nervous system (CNS), especially in the spinal cord, brainstem, and retina [1]. The extracellular glycine concentration is uniquely regulated by its cellular reuptake via two genes, glycine transporters type1 and type2 (GlyT1 and GlyT2). GlyT1 is widely distributed in neurons and glia throughout the brain, while GlyT2 is selectively expressed spinal cord and brainstem [2, 3].

Over the last decade, accumulating studies have implied that functional extrasynaptic glycine receptors (GlyRs) and their subtypes are highly expressed in hippocampus and cerebral cortex [4, 5]. In contrast to the role of glycinergic inhibition, glycine is a strychnine-insensitive co-agonist. Glycine and glutamate together co-activate NMDA receptors (NMDAR) and they are required for LTP or LTD induction or facilitation [6-9]. Thus, glycine is considered as a dual-faceted bioactive compound [5, 10], which plays an essential role in both physiological and pathological conditions including synaptic plasticity [9, 11] and postischaemic plasticity [12] in the CNS.

Although glycine is a well-documented cytoprotective agent, results are either restricted or controversial about whether glycine is neuroprotective for cerebral ischaemia. Early reports suggested that glycine may play a contributable role in neurotoxicity [13, 14]. Accumulating experimental results also proposed that glycine could play a neuroprotective role in stroke [12, 15-17]. Furthermore, it is not clear whether endogenous glycine exerts a similar anti-ischemic effect on neuronal injury as exodogenous glycine does. Evidences indicate that endogenous glycine protects against Oxygen-Glucose-Deprivation (OGD) – induced ischaemia through reliable blockade of GlyT1 by NFPS [12], though some data have showed that blocking GlyT1 is ineffective against ischaemia [14, 17]. Moreover, the mechanism underlying glycine effects on the neurogenesis of ischaemic damage is also highly debated. It is well established that NMDAR takes a crucial role in this neuroprotection by glycine [15, 16]. Interestingly, recent controversies give a new light on the involvement of anti-ischaemia by strychnine-sensitive GlyRs [12, 17].

In the hippocampus, blocking type 1 of glycine transporter (GlyT1) enhances the activation of NMDAR-dependent synaptic transmission [14] and reduces neuronal signaling via [18, 19]. These observations suggest that endogenous glycine is produced and activated effectively by GlyTl blockade in the hippocampus, and more importantly, endogenous glycine may mediate synaptic integration by dual activation of both GlyR and NMDAR [9]. NFPS is a non-competitive GlyT1 inhibitor [20, 21], and it has been proved to be more selective than another GlyT1 blocker sarcosine [17, 22]. Recent electrophysiological work has shown that 0.1 umol/L of NFPS during OGD facilitates i-LTP (ischaemic-LTP) [23, 24] of EPSCs, whereas, 2.0umol/l of NFPS completely suppresses i-LTP of EPSCs [12]. High dose of NFPS shows a potential for novel treatment targets of *in vitro* ischaemia model [12]. However, by using NFPS it is still unknown whether endogenous glycine is also neuroprotective in experimental stroke *invivo*, especially in the tMCAO model. Does NFPS show similar anti-ischaemic effects in tMCAO as it works in the OGD model? Are exogenous glycine-dependent neuroprotective mechanisms achieved? What is the mechanism of NFPS-intervened ischemia and reperfusion (I/R)? All these questions remain unknown.

In the present study, we aimed to use NFPS to examine the effects and mechanism of endogenous glycine in the *in vivo* ischaemic model (tMCAO).

Materials and Methods

Animals

Animal surgical procedures and experimental protocol were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) and the Bioethics Committee of Nanjing Medical University.

The tMCAO surgery

Transient cerebral focal ischemia and reperfusion were performed by transient middle cerebral artery occlusion (tMCAO) as in earlier studies [25, 26]. Seventy-two adult male Sprague Dawley (SD) rats (300 ± 20 g) (Sippr-BK, Shanghai, China) were used in the study. Briefly, male SD rats were anesthetized using 10% chloral hydrate (400 mg kg/1, i.p.), and tMCAO was completed by occluding the middle cerebral artery (MCA) with a monofilament suture (4-0 silicone-coated nylon suture). Rectal temperature of rats was maintained at 37°C throughout the anesthetic period. Blood pressure and heart rate of rats were monitored during the experiments. Animals were subjected to a 1 h tMCAO and then drugs were administered via intraperitoneal injection 4 h after tMCAO induction. Rats were sacrificed 25 h after tMCAO onset. Cerebral infarct size was calculated using brain sections stained with 2, 3, 5-triphenyltetrazolium chloride (TTC) [16, 25]. Neurological severity score (NSS) tests were performed at 4.5h and 24.5h after induction of ischemia and evaluated on a 6-point scale to measure postischaemic motor deficits [25, 27, 28].

Western blot analysis

24 h after experimental stroke, the infarct side of hemisphere (1 – 5 mm posterior to the bregma and 1–5 mm beside the sagittal suture) was harvested and assayed for the expression of cleaved caspase-3, Bcl-2 and Bax. The brain samples were homogenized and centrifuged. Proteins were electrophoresed by sodium dodecyl sulfate polyacrylamide gel and then transferred to nitrocellulose membrane. The membranes were incubated overnight at 4°C with primary antibodies (anti-Cleaved caspase 3, anti-Bcl-2 and anti-Bax, Cell Signaling Technology Inc, Boston, MA) in phosphate-buffered saline/0.1% Tween 20 (PBST) containing 5% milk. After washing with PBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The densitometry of the bands were scanned and analyzed with Scion Image (Scion, Frederick, Maryland).

Immunohistochemistry and Nissl staining

Rat brain sections were placed in 0.1 M sodium phosphate buffer, pH 7.4, mounted onto gelatin-coated slides and dried at 25°C. Washed with distilled water, sections were immersed for 5 min in 0.5% toluidine blue. Graded alcohol rinses were performed and the sections were then briefly dipped in 95% ethanol containing 1% glacial acetic acid. Finally, the slides were mounted and analyzed under a bright-field microscope (Nikon, Tokyo, Japan). The number of Nisslpositive cells were quantified in the DG subregion in each section of the dorsal hippocampus (at approximately -3 and -4.5 mm posterior to the bregma) using Image-Pro software 5.0.

The Morris water maze task

On the 4th day after the surgery, the Morris water maze task was performed. The Morris water maze consisted of a black-colored circular pool (1.2 m in diameter, 0.5m in height). Water was filled at a temperature of 24 - 26°C, and with black tempera paint (Shanghai Dyestuffs Research Institute Co. Ltd., China). The water surface was covered with floating black resin beads. Before training, a 60 s free swim trial without the platform was done Each rat was given 4 training trials with equally distributed starting positions in an acquisition session, and for three consecutive days, they were allowed to memorize the position of the submerged platform (1.5 cm below the surface of the water, invisible to the animal). On day 4, a single probe trial was carried out by removing the submerged platform and allowing each rat to swim freely for 60 s in the maze. The percentage of time spent in each quadrant and the number of crossings over the platform location were recorded. Data collection was automated by a computerized video system (Ethovision, Noldus Information Technology h.v., Netherlands).

Drugs

All the inhibitors were purchased from Sigma-Aldrich.

Data Analysis

All the data were expressed as mean ± SEM. Within-group comparisons were made by paired-samples t tests, and differences between groups were analyzed using independent-samples t test and ANOVA post hoc comparisons. A one-way ANOVA test was performed when equal variances were assumed. Differences were assessed significant when the value of P was below 0.05.

Results

Biphasic effects of NFPS in in vivo ischaemia

It was investigated whether different levels of NFPS have neuroprotective or deteriorative influence *in vivo* by using a transient focal ischaemic model. It was found that NFPS induced a dose-dependent effect in tMCAO. NFPS at low level (L-NFPS: 0.2 mg/kg) worsened the condition of ischaemia, remarkably increasing the total infarct size ($n = 6$, $P < 0.05$; Fig. 1A and B) compared with the ischaemia and reperfusion subjects (I/R). However, high level of NFPS (H-NFPS: 6 mg/kg) [29] produced neuroprotection via reducing the infarct size ($n = 6$, $P < 0.01$; Fig. 1A and B). Neurological severity score test showed that L-NFPS displayed a trend toward poorer neurological function ($n = 6$, $P < 0.05$; Fig. 1C); whereas H-NFPS showed significant anti-ischaemic effects (1.25 ± 0.46, $P < 0.01$, $n = 8$). The findings support the hypothesis that endogenous glycine at different levels exert opposing effects on cerebral ischaemia.

GlyR α1-dependent neuroprotection by H-NFPS in the experimental stroke

Recent studies suggest that GlyR attenuates post ischaemic neuronal damage under endogenous glycine *in vitro*. So it is important to determine whether post ischaemic blockade of GlyR by H-NFPS could reduce ischaemic neuronal death *in vivo*. H-NFPS treatment was firstly carried out along with GlyR antagonist strychnine (Stry, 0.42 mg/kg [12, 27]. It was found that the infarct volume was decreased significantly by H-NFPS+stry ($n = 6$, $P < 0.01$; Fig. 2A and B), indicating that endogenous glycine acts via selective modulation of GlyRs.

Neurological functions were clearly reduced ($n = 6$, $P < 0.05$; Fig. 2C). In order to ascertain whether and how GlyR subtypes were involved, then GlyR α1 antagonist sodium salicylate (Sal) [30] was applied to examine NFPS-dependent anti-ischaemic effects. Interestingly, the infarct size and NSS by H-NFPS were dramatically elevated after co-injection of H-NFPS and salicylate (Sal) (100 mg/Kg) [31] ($n = 6$, $P < 0.05$; Fig. 2A,B,C).

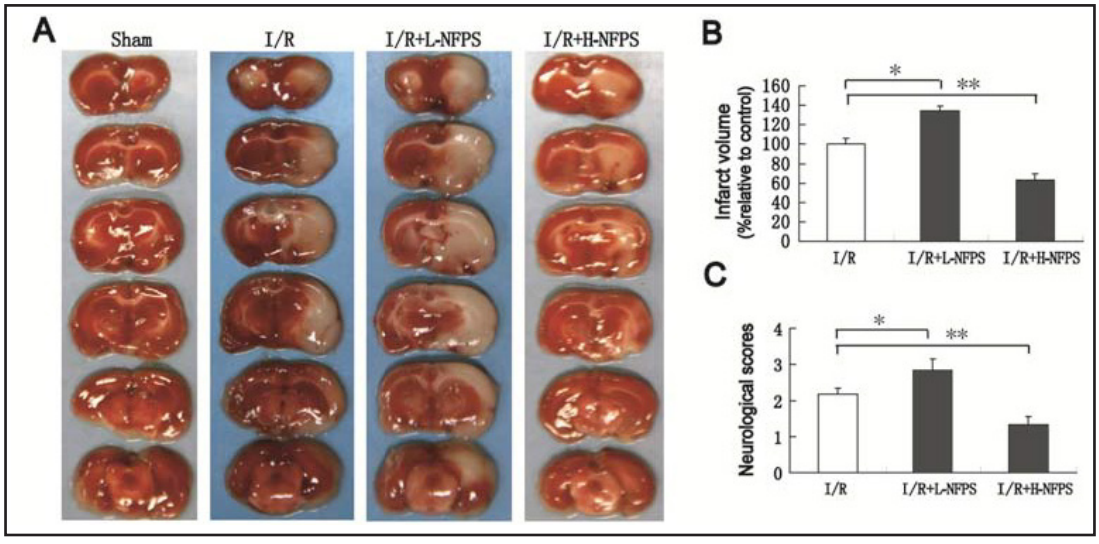

Fig. 1. Experimental protocol used to evaluate the role of NFPS in transient focal cerebral ischaemia. (A, B) The change of infarct volume after different treatments of ischemia and reperfusion. (C) The change in the 6-points of neurological severity scores (NSS) tests. L-NFPS increased the infarct size and NSS after tMCAO ($p < 0.05$), whereas, H-NFPS reduced the infarct size and NSS compared with I/R group ($p < 0.01$). D: The morphological changes of NFPS treatment in ischaemia. Sham: sham-operated rats; I/R: rats subjected to 60 mins of transient distal MCAs occlusion followed by reperfusion; I/R+L-NFPS: rats treated with an intraperitoneal injection of 0.3 mg/kg NFPS 3h after reperfusion; I/R+H-NFPS: rats received an intraperitoneal injection of 5 mg/kg NFPS 3h after reperfusion. Bar represents mean ± (SE)from 6 rats. $^{**}p < 0.01$, $^{*}p < 0.05$, vs the I/R group.

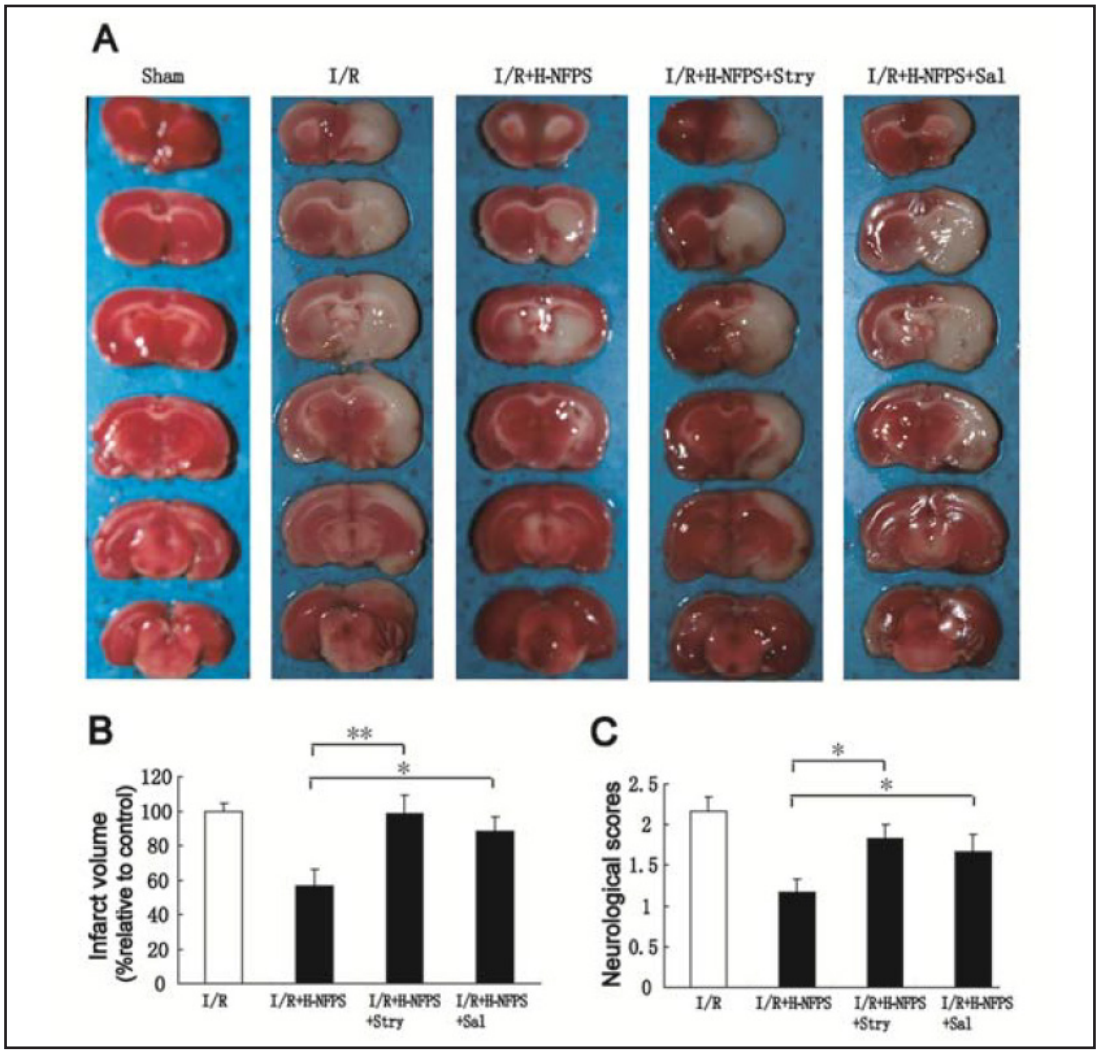

Fig. 2. Salicylate reversed the neuroprotection of H-NFPS after tMCAO. (A, B) The change of infarct volume after different treatments of ischaemia and reperfusion. (C) The change in the 6-points of neurological severity scores (NSS) tests. The previous neuroprotection achieved by H-NFPS treatment disappeared when rats were simultaneously co-treated with strychnine. Sham, I/R, I/R+H-NFPS (as showed above), I/R+H-NFPS+Stry: rats co-treated with an intraperitoneal injection of both 5 mg/kg NFPS and 0.42 mg/kg strychnine. I/R+ H-NFPS+Sal: co-injection of both 5 mg/kg NFPS and 15 mg/kg salicylate. n = 4 for each rats. **p < 0.01, *p < 0.05 vs the I/R group.

Reduced neuronal loss by H-NFPS after tMCAO

What should be investigated was whether neuronal death was reduced by H-NFPS in the Nissl staining of hippocampal DG neurons after ischaemia and reperfusion. Compared with I/R-operated rats, the results showed that the neuronal cell loss in Nissl staining was reduced significantly after H-NFPS administration in tMCAO models (n = 4, P < 0.05; Fig. 3A and B) in the hippocampal DG region. When animals were co-treated with H-NFPS and Sal, DG neurons in the hippocampus deteriorated markedly (n = 4, P < 0.05; Fig. 3A and B).

H-NFPS Inhibits cleaved caspase-3 expression after I/R

Large quantities of evidences indicate that the administration of GlyR-dependent taurine or L-serine apparently inhibits the activation of caspases including caspase-3 after experimental stroke [27, 28]. It raises the possibility that H-NFPS may be attributable to readjustment of the cleaved caspase-3 expression that was already altered in stroke.

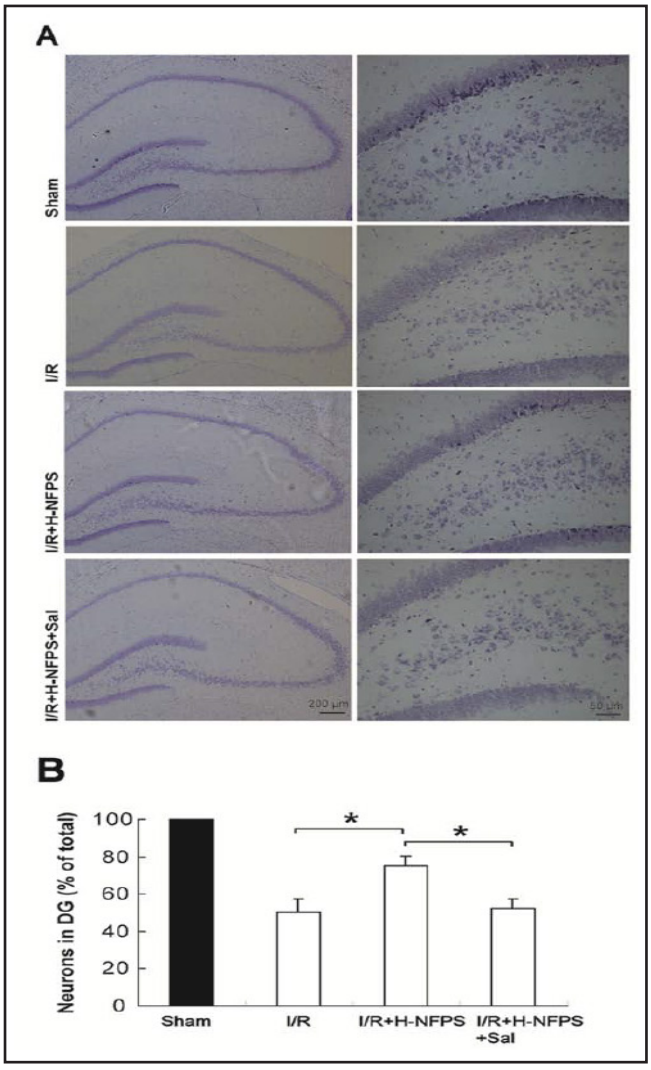

Fig. 3. Decreased neuronal loss by H-NFPS. (A, B) H-NFPS treatment reduced neuronal loss by Nissl staining ($p < 0.05$), however, this neuroprotection was reversed when co-treated with salicylate ($p < 0.05$). n = 4 for each rats. *$p < 0.05$ vs the I/R group.

Compared with I/R operation, it was found that the expression of cleaved caspase-3 decreased significantly in the cortex 24h after H-NFPS treatment with I/R (n = 4, $P < 0.01$; Fig. 4A and B). Nevertheless, this decrease was reversed sharply with the co-treatment of both H-NFPS and Sal (n = 4, $P < 0.05$; Fig. 4A and B).

Elevation of Bcl-2/Bax by H-NFPS after tMCAO

After having concluded that H-NFPS inhibits neuronal loss and capase-3 cleavage after I/R, it was further investigated whether H-NFPS exerts anti-apoptotic effects through Bcl-2/Bax pathway *in vivo*. Accordingly, western blotting was performed and it showed that H-NFPS treatment dramatically increased Bcl-2/Bax ratio (n = 4, $P < 0.05$; Fig. 5A and B) compared with the I/R group. This elevation, however, was significantly reversed when both H-NFPS and Sal were injected (n = 4, $P < 0.05$; Fig. 5A and B).

H-NFPS Contributes to Spatial Memory after ischaemia

It is widely accepted that synaptic plasticity serves as a cellular mechanism underlying learning and memory [32, 33]. Furthermore, recent studies indicate that endogenous glycine obtained by NFPS blocking GlyT1 plays bidirectional roles in both physiological plasticity [9] and pathological plasticity [12] and it may be helpful to the memory deficits in neurobiology diseases. Thus, the effects of H-NFPS on spatial memory after ischaemia by Morris water maze tests were further examined. The results showed that intraperitoneal injection of

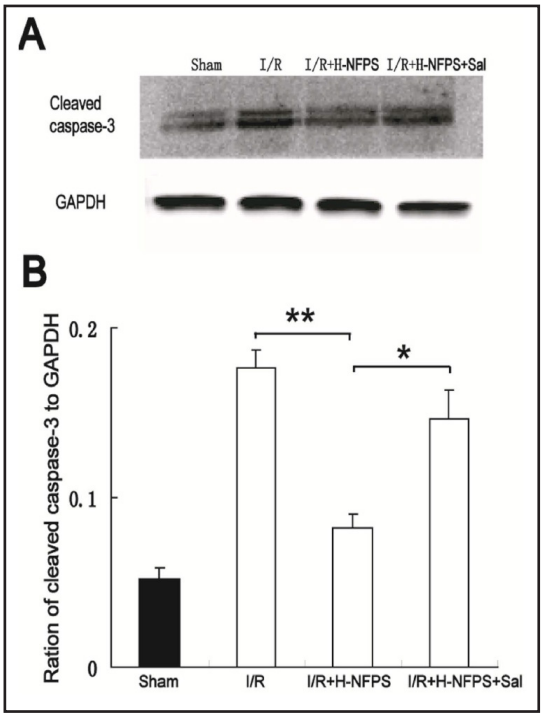

Fig. 4. Inhibited cleaved Caspase-3 expression by H-NFPS. (A, B) Compared with I/R group, the expression of cleaved Caspase-3 was reduced by H-NFPS administration ($p < 0.01$). After co-treated with H-NFPS and salicylate, the expression was increased ($p < 0.05$). n = 4 for each rat. **$p < 0.01$, *$p < 0.05$ vs the I/R group.

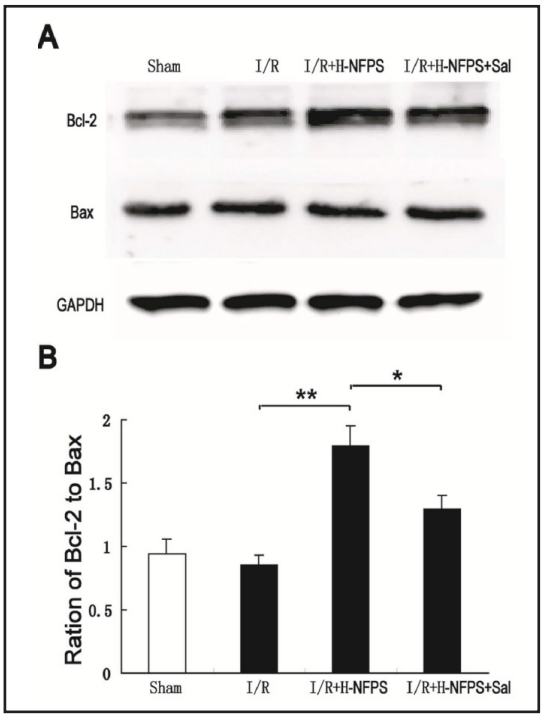

Fig. 5. Elevated Bcl-2/Bax by H-NFPS. (A, B) Compared with I/R group, the expression of Bcl-2/Bax was elevated by H-NFPS administration ($p < 0.01$). In contrast, this elevation was dropped by co-applying H-NFPS and salicylate ($p < 0.05$). n = 4 for each rat. **$p < 0.01$, *$p < 0.05$ vs the I/R group.

H-NFPS significantly improved spatial memory, when more time was spent in the target quadrant (n = 10, $P < 0.05$; Fig. 6A and B). This effect was completely reversed by co-injection of Sal (3 mg/kg)(n = 10, $P < 0.05$; Fig. 6A and B), suggesting that spatial memory deficits may be reversible, at least partly, by GlyRα1-dependent high level of endogenous glycine treatment after cerebral ischaemia and reperfusion.

Discussion

A number of anti-ischaemic strategies were used to overcome postischaemic injury-induced NMDAR-dependent neuronal hyperexcitability via enhancing inhibitory transmission. Although the expression of functional GlyR in the frontal cortex and hippocampus remains controversial, recent reports indicate that glycine, a major inhibitory neurotransmitter, may be involved in the cerebral stroke.

Some studies propose that endogenous glycine produced by using GlyT1 inhibitor NFPS, which is more potent than another blocker sarcosine [17, 22], does not elicit effective neuroprotection [14, 17, 34]. This can be attributed to the increased glycine level after ischaemic stroke [13, 35], whereas a novel evidence supports the neuroprotective effect via increased glycine level after NFPS treatment in OGD conditions [12].

In the present study, it was reported for the first time that endogenously applied glycine (by NFPS) could be an effective neuroprotective agent against *in vivo* ischaemia (tMCAO model). With the TTC staining method, it was clearly demonstrated that treatment of high

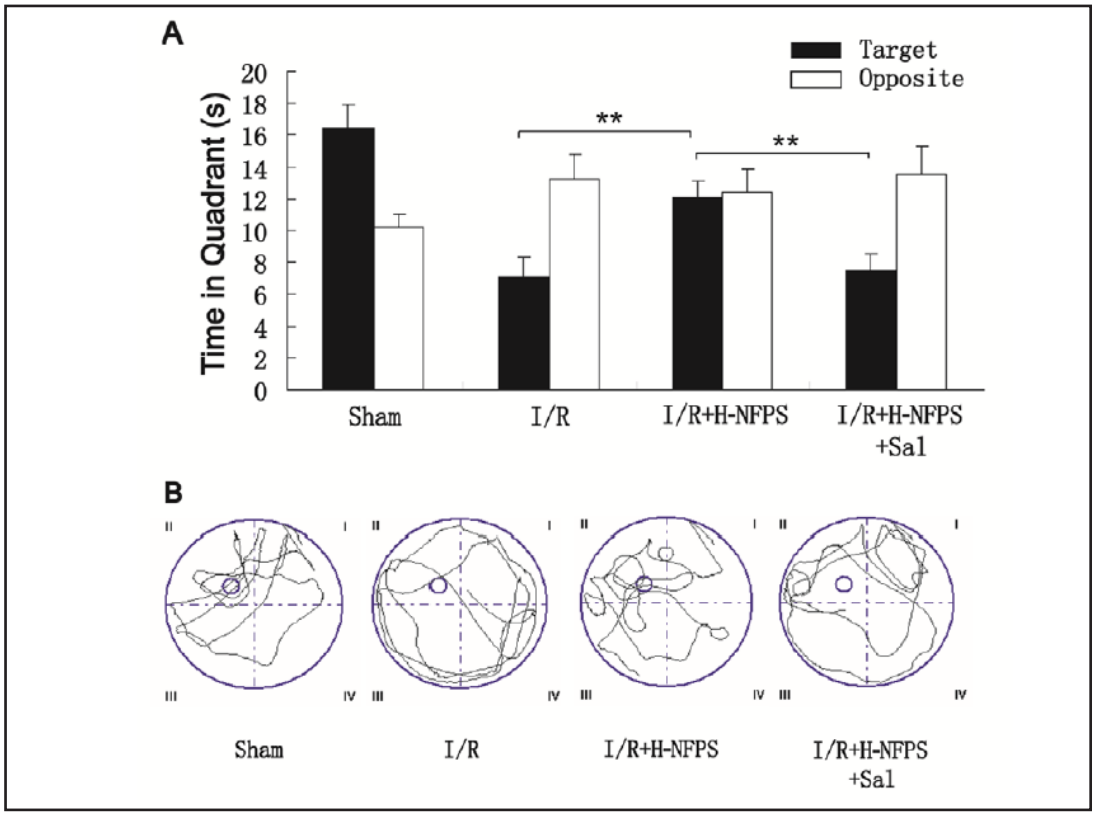

Fig. 6. Improvement of spatial memory by H-NFPS. (A, B) H-NFPS improved spatial memory deficits after tMCAO in water maze task ($p < 0.01$). This improvement was reversible after co-injection with both H-NFPS and salicylate ($p < 0.01$). n = 10 for each rats. **$p < 0.01$ vs the I/R+H-NFPS group.

dose of NFPS (H-NFPS) after tMCAO could reduce the cerebral infarct size and prevent the severe neurological behaviour deficits of rats. With an effective dose at 6mg/kg, it was further found that H-NFPS significantly increased the neuronal survival as shown by Nissil staining and cleaved caspase-3, Bcl-2/Bax measurement. In addition, H-NFPS treatment attenuated the ischemia-induced spatial memory deficits in the Morris water maze task. Our observation has the potential of giving fundamental insights and prospects for neuroprotective target on NFPS, and the results match well with the previous results obtained in the OGD model [12]. However, NFPS produces biphasic effects. In contrast to H-NFPS, a low dose of NFPS (0.2 mg/kg) elicits deterioration in the infarct volume and NSS. These results may be attributable to the NMDAR overexcitation under low level of endogenous glycine after ischaemia [12, 14]. Interestingly, it was found that co-applying GlyR α1 inhibitor sal with H-NFPS can significantly reverse H-NFPS's neuroprotection after tMCAO. These results demonstrated that in contrast to its controversial role in acute *in vitro* stroke model, GlyR seems to play a more critical role in NFPS-induced anti-ischaemia in ischaemia *in vivo*. It was suggested, nevertheless, that GlyR subtype α1 may be a dominant factor in the GlyR-dependent neuroprotection. Thus, GlyR α1 may contribute to the H-NFPS-dependent effect and shall be considered as an anti-ischaemic mechanism which is supported by recent studies on the high concentration of glycine-dependent neuroprotection [12].

Endogenous glycine concentration in in vivo ischaemia

According to the findings and previous results, a fundamental question is raised and needs to be addressed. That is, can endogenous glycine concentrations, after systemic

administration of 0.2 and 6 mg/kg of NFPS, reach the similar level of exogenous ligand production in the tMCAO model? GlyT1, mainly expressed in astroglial, is a member of the glycine transporter family [2, 3]. It has biphasic effects on NMDAR at both excitatory and inhibitory synapses, controls extracellular glycine concentrations in the submicromolar range for modulation of excitatory NMDAR [18], and activates inhibitory glycinergic synapses [19]. Previous studies suggest that GlyT1 inhibition by NFPS (100nM) is unlikely to sufficiently enhance the extracellular glycine level, a failed neuroprotection found in the OGD condition [17, 36]. Systematical use of glycine was regarded as an unsuccessful method for increasing glycine concentration in the CNS under ischaemic (OGD cell) conditions [13]. However, a recent report strongly argued that the inadequate neuroprotective effect of endogenous glycine can be compensated by using a saturating concentration of NFPS (2.0μM) in a very similar condition (OGD patch-clamp) [12]. This research also found that extracellular glycine concentration can be significantly or slightly enhanced by systemic treatment of glycine at a high or low level, respectively, under *in vivo* microdialysis combined with tMCAO conditions [12]. Thus, the level of NFPS applied may be a key factor influencing the anti-ischaemic effects of endogenous glycine. Indeed, NFPS can be elevated in a dose-dependent manner *in vivo* (0.1–10 mg/kg) and dosing at a level below 10mg/kg is safe without causing motor behavioural deficits [29].

Endogenous glycine neuroprotection via GlyR and its subtypes

As a coagonist of NMDARs, glycine has been reported to induce neuronal damage through co-activation of the glycine site of NMDARs, resulting in neuronal overexcitation *in vivo*, even in high concentration [13]. It was highly debated whether NMDAR [16] and GlyR-dependent [17] neuroprotection can be achieved by high-level extracellular glycine in the frontal cortex and hippocampus. Glycine has been proved to be a dual-faceted, neurotransmitter in the CNS. It can be activated via both synaptic NMDARs glycine site and extrasynatic GlyR in hippocampal CA1 neurons [5, 9]. At a relatively low concentration, glycine as an excitatory transmitter is combined with NMDARs rather than GlyRs. Therefore, it is not surprising that when the concentration of glycine increases under certain circumstances it will also display inhibition by activating GlyRs. A series of researches [9, 12] have demonstrated that not only exogenous glycine, but also endogenous glycine can mediate bidirectional plasticity and restore the excitation-inhibition balance through activating GlyRs-induced inhibition *in vitro* in the hippocampal CA1 region. Chen [9] and Yao [12] observed that high dose of endogenous glycine can induce LTD in both physiological and pathological conditions. Thus, high dose of NFPS may produce neuroprotection via reducing iLTP overexpression induced by GlyR- dependent LTD. Futhermore, by administering both GlyR antagonist strychnine and GlyR α1 blocker salicylate [30], glycine-induced neuroprotection can be terminated after ischaemia. These findings suggested that endogenous glycine obtained by GlyT1 blockade, in high concentration, can sufficiently activate extrasynaptic GlyRs. Moreover, GlyRs and their subtypes were proposed to be activated when strychinine reversed the cGMP reduction obtained by systemic treatment NFPS [29]. Importantly, it was concluded that GlyR α1 may represent a novel target toward which anti-ischaemic and anti-apoptotic drugs might be developed. According to our data and previous results [12, 29], H-NFPS is deemed as a possible *in vivo* agent and a potential treatment for neurological diseases especially cerebral ischemia.

In conclusion, this study shows that systematical application of H-NFPS in the tMCAO models can generate endogenous glycine-dependent neuroprotection. Although low level of NFPS induces neurotoxicity due to the NMDAR overexcitation, what should be proposed is that high dose of NFPS can decrease infarct volume, reduce neuronal death and the expression of cleaved caspase-3, and enhance Bcl-2/Bax and spatial memory in a GlyR α1-dependent mechanism, thus providing a very promising strategy for preclinical research and clinical therapy.

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Disclosure Statement

The authors declare no conflict of interest.

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