Neuroprotection by a novel NMDAR functional glycine site partial agonist, GLYX-13
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GLYX-13 (threonine–proline–proline–threonine–amide) is an amidated di-pyrrolidine that acts as a functional partial agonist at the glycine site on N\textsuperscript{-}methyl-D-aspartate glutamate receptors (NMDARs). GLYX-13 can both increase NMDAR conductance at NR2B-containing receptors, and reduce conductance of non-NR2B-containing receptors. Here, we report that GLYX-13 potently reduces delayed (24 h) death of CA1 pyramidal neurons produced by bilateral carotid occlusion in Mongolian gerbils, when administered up to 5 h post-ischemia. GLYX-13 also reduced delayed (24 h) neuronal death of CA1, CA3, and dentate gyrus principal neurons elicited by oxygen/glucose deprivation in in-vitro hippocampal organotypic slice cultures, when applied up to 2 h post-oxygen/glucose deprivation. The glycine site full agonist \(\alpha\)-serine completely occluded neuroprotection, indicating that GLYX-13 acts by modulating activation of this site.

Introduction
Brain traumas from stroke to seizures, concussions and neurodegenerative disease, trigger cascades that result in delayed death of neurons. A region that is particularly prone to cumulative loss of neurons is the hippocampus, especially CA1 and CA3 pyramidal neurons [1–3]. A key trigger of delayed neuronal death is widespread pathological increase in intracellular \([Ca^{2+}]\), and one important route for \(Ca^{2+}\) entry into neurons is glutamate-activated N\textsuperscript{-}methyl-D-aspartate receptors (NMDARs) [4]. Both glutamate binding site and open-channel NMDAR blockers have been tested as potential neuroprotective therapies, but unacceptable cognitive side effects have limited their utility.

NMDARs have a number of binding sites that are targets of more subtle endogenous regulation. One of these is the obligatory coagonist glycine site, which must be bound by an endogenous agonist in order for glutamate binding at its distinct sites on the NMDAR to be able to trigger channel opening [5]. Studies indicate that the coagonist site is probably not saturated under baseline physiological conditions [6,7], and that neural activity releases endogenous agonists, and perhaps antagonists [8,9], that dynamically regulate NMDAR activity through this site.

GLYX-13 (threonine–proline–proline–threonine–amide) is an amidated di-pyrrolidine that readily crosses the blood–brain barrier and acts as an NMDAR functional glycine site partial agonist [10,11]. It has unique effects on learning, NMDAR-gated conductances and activity-dependent synaptic plasticity [12]. Behaviorally, GLYX-13 enhances learning in a hippocampus-dependent trace eyeblink conditioning paradigm, the Morris water maze and an alternating T-maze, and robust enhancement of learning has also been observed in learning-impaired aged rats [13]. At low concentrations (100 nM–1 \(\mu\)M), GLYX-13 acts as a glycine site agonist, and enhances both burst-driven extrasynaptic NMDAR conductances and long-term potentiation (LTP), whereas at higher concentrations (10–50 \(\mu\)M), it becomes an antagonist that reduces NMDAR conductance and LTP [12].

This led us to hypothesize that during stroke and brain trauma, GLYX-13 might serve to antagonize actions of endogenous full coagonists (e.g. \(\alpha\)-serine), which promote overactivation of NMDAR leading to neuronal damage. To test this hypothesis, we examined the effects of GLYX-13 on delayed neuronal death in two different stroke models, bilateral carotid occlusion in the Mongolian gerbil \textit{in vivo}, and oxygen/glucose deprivation (OGD) in organotypic hippocampal slice cultures \textit{in vitro}.

Materials and methods
In-vivo stroke model
Male Mongolian gerbils (70 g, 3 months old) were anesthetized with 5% isoflurane and rectal temperature...
was maintained at 37°C. In animals made ischemic, both carotid arteries were surgically isolated and clamped for 5 min, which produces widespread forebrain ischemia. Sham-operated animals had the carotid arteries isolated, but not clamped. GLX-13 was injected into the cerebral ventricle at coordinates (from Bregma): AP + 1.5 mm, L 2.0 mm, V 4.0 mm. (± 0.05 mm) in 5-μl injection volumes. Doses of 5 ng/5 μl, 10 ng/5 μl, or 50 ng/5 μl injection volume of GLX-13 were administered to determine the dose–response relationship. To determine the period of post-ischemia neuroprotection, GLX-13 was administered either immediately before, or 1, 5, or 8 h post-ischemia (for which animals were reanesthetized to administer the GLX-13 or vehicle control). In another set of animals, MK-801 (50 ng/5 μl) was given intraperitoneally to gerbils immediately before ischemia. Animals were killed 5 or 30 days later with an overdose of pentobarbital, to determine whether neuroprotection was maintained over time. At the time of killing, brains were removed and kept in 4% paraformaldehyde at 4°C for 24 h, then transferred to 15% sucrose at 4°C for 48 h. Brains were then placed into a zinc gelatin brain matrix (Zivic Instruments, Pittsburgh, Pennsylvania, USA) and a first cut was made at the level of the mammillary bodies. Thirty-micron serial sections were cut from that point, and stained with neutral red. Three sections were used for each data point and evaluated by a single-blind trained observer. Data were compared using a contingency table with Fisher’s exact test to determine whether the differences in number of animals showing either cell loss or no cell loss in each group were significantly different.

Results

In-vivo ischemia

To examine the in-vivo efficacy of the functional glycine site partial agonist GLX-13 as a neuroprotectant, we used the bilateral carotid artery occlusion model in Mongolian gerbils, which have an incomplete Circle of Willis. All but one of the gerbils subjected to ischemia after intracerebroventricular injection of saline showed extensive cell loss in the CA1 region of the hippocampus 5 days post-ischemia. Initial experiments were designed to determine the dose of GLX-13 that would be neuroprotective in the majority of animals. Injection of 5 or 10 ng of intracerebroventricular GLX-13 (5 μl injection volume), followed by 5-min global ischemia, resulted in only a small, but statistically significant, neuroprotection, as assessed by neutral red stain intensity in the CA1 cell body layer. Neuroprotection was inconsistent at these doses, as most animals still showed some cell damage, with some exhibiting damage comparable with saline controls.

In contrast, all animals receiving 15–25 ng/5 μl doses of GLX-13 showed marked and significant neuroprotection. In all but one case in each group, CA1 pyramidal cell layer staining was equivalent to animals not subjected to ischemia (Fig. 1a). Animals treated with the NMDAR open-channel blocker MK-801 were used as positive controls for neuroprotection, and none of the animals in this group showed significant pyramidal neuron loss [Fig. 1a; MK-801 (50 ng)]. GLX-13 was as effective as MK-801 in preventing cell loss (Fig. 1b and c). In animals treated with GLX-13 after ischemic induction, both the 1 and 5 h post-ischemia GLX-13 treated groups showed significantly less cell loss than saline controls [Fig. 1c; P < 0.01 (contingency table and Fisher’s exact test)]. In contrast, there was no significant neuroprotective effect of GLX-13 when it was administered 8 h post-ischemia (Fig. 1b and c).

To determine whether the neuroprotective effect of GLX-13 was maintained as improved neuronal survival over time, some animals pretreated with GLX-13 were allowed to survive for 30 days. In three of four animals, the neuroprotection of CA1 pyramidal neurons afforded by GLX-13 administered 1 h post-ischemia was maintained for 30 days (data not shown). There was some cell loss in the fourth animal, but significantly less
than that observed in animals that received saline injections before ischemia.

**In-vitro oxygen/glucose deprivation in hippocampal organotypic slice cultures**

To determine whether the neuroprotective actions of GLYX-13 are because of direct actions on hippocampal neural tissue, we tested whether GLYX-13 is also protective when applied directly to hippocampal organotypic slice cultures in vitro. Stroke-induced ischemia was modeled in hippocampal slice cultures by a defined 45 min period of OGD, and delayed neuronal death assessed in all three principal cell layers (CA1, CA3, and dentate gyrus) by the intensity of staining with the vital fluorescent dead cell dye PI 24 h post-OGD.

Figure 2 illustrates the neuroprotective window for GLYX-13 in protecting CA1 pyramidal neurons from OGD-induced delayed death in slice cultures. GLYX-13 was bath applied to hippocampal slice cultures (5–10 days in vitro) either 30 min before, or 1–3 h after, a 45-min OGD insult, and PI fluorescence measured 24 h post-OGD. Typical PI-labeled fluorescence images are illustrated from slice cultures treated with 50 μM GLYX-13 30 min before ischemia, and GLYX-13 1 h post, 5 h post, and 8 h post refer to the administration of GLYX-13 at these times post-ischemia. Saline refers to hippocampi from animals treated with saline alone followed by ischemia. (Each bar mean ± SEM cell death staining intensity; *P<0.05, Student’s t-test compared with Saline neutral red stain intensity).
with significantly lower potency than earlier time points. When applied 3 h post-OGD, GLYX-13 was no longer significantly neuroprotective (data not shown).

**NMDAR glycine site full agonist d-serine blocks GLYX-13 neuroprotection**

In earlier studies, we have shown that GLYX-13 is a functional partial agonist at the obligatory coagonist glycine-binding site on NMDAR [11]. As the endogenous full agonist at this site is probably d-serine [8], we tested whether d-serine could block the neuroprotective actions of GLYX-13. As shown in Fig. 3, coapplication of d-serine (100 μM) along with GLYX-13 (50 μM) more than doubled the neuronal death produced by 45 min OGD in all three principal cell regions (CA1, CA3 and dentate gyrus) compared with OGD in GLYX-13 alone, a complete occlusion of the neuroprotective properties of GLYX-13. These data indicate that the neuroprotective activity of GLYX-13 is also likely to be through interactions with the NMDAR glycine site that reduce NMDAR-gated conductance evoked during and after ischemia.

**Discussion**

The experiments presented here tested the neuroprotective potential of the NMDAR functional glycine site partial agonist GLYX-13 in two experimental models of stroke. In both in-vivo bilateral carotid occlusion and in-vitro OGD models of ischemia-induced delayed neuronal death, GLYX-13 was potently neuroprotective. The concentration range over which GLYX-13 was neuroprotective was the same as we have shown previously antagonizes NMDAR activation and reduces L TP, and much higher than the range that potentiates NMDAR and LTP in vitro [12], and is nootropic in vivo [11]. Surprisingly, GLYX-13 also exhibited significant neuroprotective activity up to 5 h post-ischemia in vivo, and up to 2 h post-OGD in in-vitro hippocampal slice cultures.

A number of NMDAR antagonists have reached clinical trials for the treatment of stroke and all have failed.
Explanations for these failures include poor pharmacokinetics, poorly designed clinical trials, high drug toxicity in humans not found in animal studies, short windows of efficacy and inappropriate receptor subtype specificity [15], Ikonomidou and Turski [16] suggested that NMDAR blockade inhibits normal neuronal function, which, either directly or indirectly, suppresses post-ischemic neuronal repair mechanisms.

Nevertheless, several recent studies suggest that the NMDAR may still be an excellent target for antistroke drug development. Liu et al. [17] reported that blocking NR2B-containing subtypes of NMDA receptors in an in-vivo rat model of focal ischemic stroke was an effective neuroprotection strategy, provided it was done at or near the time of the ischemia. These investigators also noted that activation of NR2A-containing NMDAR subtypes by high [glycine] administration, was robustly neuroprotective and, most importantly, neuroprotection could be effected up to 4.5 h after hypoxic insult. Nakashiba and colleagues [18] showed, using NR3A knockout mice, that expression of NMDARs containing this subunit confers significant neuroprotection when subjected to focal cerebral ischemia. Human NR3A NMDAR subunits contain a unique glycine-binding site that, when activated, causes a reduction in NMDAR Ca2+ permeability [19,20].

Recently, we [12] found that GLYX-13 has the ability to both enhance NMDAR conductance at NR2B-containing receptors, leading to enhanced LTP and to reduce NMDAR conductance at non-NR2B-containing receptors. Thus, both the partial agonist properties and subunit-specific actions of GLYX-13 are likely to be keys to its neuroprotective activity. As a functional partial agonist at NR2B-containing NMDARs, GLYX-13 would act as a neuroprotective competitive antagonist in the presence of high concentrations of D-serine, whereas similar actions at NMDARs that lack NR2B might stimulate neuroprotective mechanisms driven by either NR2A or NR3-containing NMDARs.

**Conclusion**

GLYX-13 is a novel NMDAR modulator that acts as a functional glycine site partial agonist. It displays low-dose stimulatory activity that enhances induction of LTP and learning, and high-dose antagonist properties that reduce NMDAR currents and LTP. Here, we show that GLYX-13 has unique neuroprotective properties in the antagonist range, reducing hippocampal pyramidal neuron death when administered up to 5 h post-ischemia in an in-vivo gerbil stroke model, and 2 h post-insult in in-vitro organotypic hippocampal slice cultures subjected to OGD. These findings indicate that GLYX-13 has significant potential as a novel therapeutic agent that can normalize NMDAR activity levels to simultaneously improve memory function and ameliorate delayed neuronal death in stroke and neurodegenerative disease.

**Acknowledgements**

Grant support: NIH Grant #R01-NS044421 to P.K.S and NIH Grant #R43-MH60572 to J.R.M.

**References**


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