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Richard Bergeron (✉ rbergeron@ohri.ca)

University of Ottawa

Julia Cappelli

University of Ottawa

Pamela Khacho

University of Ottawa

Boyang Wang

University of Ottawa

Alexandra Sokolovski

University of Ottawa

Wafae Bakkar

Ottawa Hospital Research Institute

Sophie Raymond

University of Ottawa

Nina Ahlskog

University of Ottawa

Julian Pitney

University of Ottawa

Junzheng Wu

University of Ottawa

Prakash Chudalayandi

University of Ottawa

Adrian Wong

University of Ottawa

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Authors:

Julia Cappelli (jcapp082@uottawa.ca)^{1*}, Pamela Khacho (pkhac024@uottawa.ca)^{1*}, Boyang Wang (jwang149@uottawa.ca)¹, Alexandra Sokolovski (asoko047@uottawa.ca)¹, Wafae Bakkar (wafae.bakkar@hc-sc.gc.ca)², Sophie Raymond (sraym054@uottawa.ca)¹, Nina Ahlskog (nahlskog@gmail.com), Julian Pitney (julianpitney@gmail.com)¹, Junzheng Wu (Junzheng@gmail.com)¹, Prakash Chudalayandi (pchudala@uottawa.ca)¹, Adrian Y.C. Wong (awon2@uottawa.ca)¹, Richard Bergeron (rbergeron@ohri.ca)¹

*these authors contributed equally to this work

Affiliations:

¹Cellular and Molecular Medicine Department, University of Ottawa, 451 Smyth Road, Roger Guindon Building, Room 3501N, Ottawa, Ontario, Canada, K1H 8M5; ²Ottawa Hospital Research Institute, 451 Smyth Road, Roger Guindon Building, Room 3501N, Ottawa, Ontario, Canada, K1H 8M5.

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Figures and Supplemental Data

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33 **Abbreviations:**

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35	AAV:	Adeno associated virus;
36	ACSF:	Artificial cerebrospinal fluid;
37	CHO:	Chinese Hamster Ovary;
38	CPA:	Cyclopiazonic acid;
39	CV:	Cresyl violet;
40	DBP:	Dynamin blocking peptide;
41	ddH₂O:	Double distilled water;
42	EPSCs:	Excitatory postsynaptic currents;
43	ET-1:	Endothelin-1;
44	FJC:	FluoroJade C;
45	GBS:	Glycine binding site;
46	GINI:	Glycine-induced NMDAR internalization;
47	GluN2A^{-/-}:	NMDAR GluN2A subunit knockout;
48	GlyR:	Glycine receptor;
49	GlyT1:	Glycine transporter type 1;
50	GlyT1^{+/-}:	Heterozygous glycine transporter type 1;
51	GlyT1-A:	Glycine transporter type 1 antagonist;
52	GO:	Glycine oxidase;
53	i.p.:	Intraperitoneal;
54	LDF:	Laser Doppler flowmetry;
55	LSFM:	Light sheet fluorescence microscopy;
56	tMCAO	Transient middle cerebral artery occlusion;
57	MRI:	Magnetic resonance imaging;
58	NFPS:	<i>N</i> -[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine;
59	NMDAR:	<i>N</i> -methyl-D-aspartate receptor;
60	NMDG:	<i>N</i> -methyl-D-glucamine;
61	OGD:	Oxygen-glucose deprivation paradigm;
62	PBSG:	0.25% (w/v) gelatin in PBS;
63	PBSGT:	0.25% (w/v) gelatin and 0.2% Triton X-100 (v/v) in PBS;
64	PFA:	Paraformaldehyde;
65	P_{open}:	Open probability;
66	PT:	Photothrombosis;
67	RT:	Room temperature;
68	SR^{-/-}:	Serine racemase knockout;
69	TTC:	2,3,5-triphenyltetrazolium chloride;
70	VGAT:	Vesicular GABA transporter;
71	VGLUT:	Vesicular glutamate transporter;
72	WT:	Wild type.

Ischemic stroke is the second leading cause of death worldwide. Compelling evidence demonstrates that following an ischemic event, neuronal death is triggered by uncontrolled glutamate release leading to overactivation of glutamate sensitive *N*-methyl-D-aspartate receptor (NMDAR). For gating, NMDARs require not only the binding of glutamate, but also the binding of glycine or a glycine-like compound as a co-agonist. Glycine fulfills several roles in biology including protein synthesis, inhibitory transmission *via* glycine receptor activation and excitatory transmission through NMDARs. Low glycine doses enhance NMDAR function while high doses trigger glycine-induced NMDAR internalization (GINI) *in vitro*. The physiological relevance of GINI has been questioned given that the high-affinity glycine transporter type 1 (GlyT1), located on astrocytes and neurons, maintains synaptic glycine concentrations far below the level that would saturate the glycine binding site (GBS). Here, we report that following an ischemic event, *in vivo*, GINI also occurs and provides neuroprotection. Mice pre-treated with a GlyT1 antagonist (GlyT1-A), which increases glycine synaptic levels, exhibited smaller stroke volume, reduced cell death, and minimized behavioural deficits following stroke induction by either photothrombosis or endothelin-1. Therefore, increasing glycine levels in the synaptic cleft will enhance GBS occupancy and will reach the set point to trigger GINI. However, we report that GINI is triggered during ischemic stroke, *in vivo*, only in the presence of GlyT1-As. Moreover, we show evidence that in ischemic conditions, GlyT1-As preserve the vasculature in the peri-infarct area. Therefore, the clinical efficacy of GlyT1-As should be tested for the treatment of ischemic stroke.

Introduction

Ischemic stroke is a devastating health concern that often leaves victims with long-lasting disabilities, and induces substantial socioeconomic costs for the individual, their loved ones and society as a whole. Limited effective interventions are available to the at-risk population, highlighting the need to identify novel therapeutic targets that can prevent neuronal death following ischemic insults.

Activity-dependent changes in *N*-methyl-D-aspartate receptor (NMDAR)-mediated synaptic strength are of great importance, because they serve as the molecular trigger for synaptic responses in many physiological and pathological processes such as ischemic stroke. Neuronal death following an ischemic event is triggered by uncontrolled glutamate release leading to the NMDAR overactivation on surrounding neurons, inducing excessive Ca^{2+} -influx primarily through NMDARs¹. In physiological conditions NMDARs require glutamate binding on the GluN2 subunit and glycine binding on the glycine binding site (GBS) on the GluN1 subunit². Ascher's group showed that glycine³, or a glycine-like substance⁴, is a required co-agonist for NMDAR activation. Moreover, Salter and co-workers reported that high doses of glycine trigger GINI, *in vitro*, by promoting endocytosis of NMDAR through clathrin/dynamin-dependent machinery⁵. Unlike constitutive internalization, which requires no channel activation^{5,6}, NMDAR internalization following glycine "priming" requires both glutamate and glycine present in the synaptic cleft⁵.

Using a multidisciplinary approach, we found that during an oxygen-glucose deprivation paradigm (OGD), *in vitro*, not only glutamate but also an excess of glycine is released in the extracellular space. However, this is not sufficient to trigger GINI since the level of extracellular glycine is buffered by the glycine transporter type 1 (GlyT1)⁷⁻¹⁰. Only when GlyT1s are antagonized, can glycine accumulate in the synaptic cleft and lead to robust NMDAR internalization. Photothrombosis (PT) and endothelin-1 (ET-1) are paradigms that mimic ischemic events, *in vivo*. We report that both PT and ET-1 induced a significantly smaller stroke volume, less cell death and less behavioural deficits in mice in the presence of a GlyT1 antagonist (GlyT1-A), which increases glycine concentrations, and hence the occupancy of the GBS. Moreover, the neuroprotective effect induced by high occupancy of the GBS was further supported by preservation of the vascularisation tree.

Overall, these data suggest that increased levels of synaptic glycine before an ischemic event may be a means of minimizing neuronal death. We report that GlyT1-A administration before or shortly after an ischemic event, *in vivo*, triggers GINI and provides neuroprotection.

Results

High concentrations of glycine induce NMDAR internalization

We first determined the effects of bath application of increasing glycine concentrations on stimulation-evoked NMDAR excitatory postsynaptic currents (NMDAR-EPSCs) recorded from CA1 pyramidal neurons from acute hippocampal brain slices (Fig. 1a). At glycine concentrations below 250μM, NMDAR-EPSC amplitudes were potentiated in a dose-dependent fashion^{3,10-12}. However, increasing the glycine concentration to 1mM resulted in a significant decrease in NMDAR-EPSC amplitude^{5,13} and this effect was reversible (Fig. 1b). To verify that this decrease in amplitude was due to GINI, we applied 1mM glycine in the presence of 100μM dynasore, a cell-permeable inhibitor of both dynamin-1 and dynamin-2, which blocks internalization^{6,14}. We found that the decrease in NMDAR-EPSC amplitudes by 1mM glycine was abolished in the presence of dynasore (Fig. 1c).

Calcium influx is required for GINI to occur

Previous studies have reported that the activity of dynamin is regulated by the Ca²⁺-sensitive phosphatase calcineurin^{15,16}. Therefore, we explored the role of extracellular and intracellular Ca²⁺ on NMDAR-EPSC amplitudes in the presence of 1mM glycine. We examined the effects of various external Ca²⁺ concentrations on the NMDAR response to 1mM glycine application. When 1mM glycine was applied with low Ca²⁺ (1mM), an increase in NMDAR-EPSC amplitude was observed, in contrast to what occurred with normal Ca²⁺ concentrations (3.5mM). To further confirm the role of Ca²⁺ in GINI, we included a Ca²⁺ chelator BAPTA (10mM), in the recording electrode. Here, we observed a significant attenuation in the decrease in NMDAR-EPSC amplitude induced by 1mM exogenous glycine. Moreover, extracellular application of 20μM nimodipine, an L-type Ca²⁺ channel blocker, also attenuated GINI compared to control, further corroborating the data acquired with BAPTA. In contrast, depleting intracellular Ca²⁺ stores by incubating hippocampal slices for 1hr in 30μM cyclopiazonic acid (CPA), an inhibitor of intracellular Ca²⁺

pumps, had no effect on the glycine-induced decrease in NMDAR-EPSC amplitude (Fig. 1d). Together these data suggest that external Ca^{2+} influx across the plasma membrane is required for GINI to occur.

Genetic elevation of extracellular glycine facilitates GINI

Heterozygous glycine transporter type 1 ($\text{GlyT1}^{+/-}$) mice exhibit a higher level of endogenous extracellular glycine^{17,18}. Therefore, we hypothesized that in these mice GINI could be triggered by lower doses of glycine. As illustrated in figure 1e, while there was no significant effect in the NMDAR-EPSC amplitude following bath application of 10 μM or 1mM glycine between wild type (WT) and $\text{GlyT1}^{+/-}$ mice, bath application of 250 μM glycine, which potentiated the NMDAR-EPSC amplitude in WT mice, significantly inhibited the NMDAR-EPSC amplitude in $\text{GlyT1}^{+/-}$ mice (Fig. 1f). The decrease of the NMDAR-EPSC amplitude induced by 250 μM glycine in $\text{GlyT1}^{+/-}$ mice was abolished in the presence of dynasore (Fig. 1g). Therefore, the high levels of endogenous glycine in the $\text{GlyT1}^{+/-}$ mice trigger GINI at lower exogenous glycine concentrations.

Role of glycine binding site occupancy

In addition to glycine, D-serine, also activates the GBS^{4,19}. As illustrated in figure 1h, the effects of increasing D-serine concentrations on NMDAR-EPSC amplitudes in acute slices from WT mice was also dose-dependent. Moreover, the dose-response curve of NMDAR-EPSC amplitudes to D-serine was left-shifted relative to that of glycine due to its higher affinity to the GBS²⁰ (Fig. 1i). The decrease in NMDAR-EPSC amplitude evoked following bath application of 1mM D-serine was also abolished in the presence of dynasore (Fig. 1j).

We next investigated whether GINI could be modulated by increasing levels of either glycine or D-serine in WT mice. Glycine levels were increased *via* bath application of the selective GlyT1-A, *N*-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine (NFPS; 300nM)²¹⁻²⁵. As expected, there was a significant increase in evoked NMDAR-EPSC amplitude in the presence of NFPS alone¹⁰. However, when NFPS was applied together with a potentiating concentration of D-serine (10 μM), a significant decrease in NMDAR-EPSC amplitude was observed (Fig. 1k). Interestingly, when NMDARs were first primed with high doses of glycine or D-serine, a subsequent application of a low dose of glycine or D-serine, also induced GINI (Fig. S1).

Next, we used a transgenic mouse model in which the serine racemase gene was knocked out (SR^{-/-} mice)²⁶⁻²⁸, as these mice exhibit low levels of D-serine. In both WT and SR^{-/-} mice, a low dose of D-serine (10μM) potentiated the evoked NMDAR-EPSC amplitude. However, SR^{-/-} mice required a higher dose of D-serine (2mM) than WT mice (1mM) to induce a decrease in NMDAR-EPSC amplitude (Fig. 11–m). These findings suggest that there is a common mechanism of action for glycine or D-serine to trigger GINI. Additionally, we found that GINI was not subunit-specific (Fig. S2a–e), nor attributed to AMPA receptor activity (Fig. S2f), and not limited to the hippocampal region (Fig. S2g).

Glycine is released during oxygen-glucose deprivation.

Immunohistochemical data illustrated in figure S3 suggest that glycine may be co-localized in glutamatergic neurons, we therefore hypothesised that depolarization of glutamatergic CA1 pyramidal neurons during the oxygen-glucose deprivation (OGD) paradigm could result in detectable local glycine release²⁹. In order to detect glycine release during an OGD paradigm, we used the sniffer-patch technique, wherein activation of glycine receptor α2 subunit indicated glycine release³⁰. When the OGD perfusate was applied to the slice, there was a marked increase in the frequency of channel opening in the patch (Fig. S4a) and a significant increase in open probability (P_{open}) compared to control (Fig. S4b). Overall, these results strongly suggest that during OGD conditions, glycine is released into the CA1 extracellular space. Given that multiple studies have demonstrated that glycine receptors (GlyRs) are only weakly expressed at CA1 hippocampal synapses³⁰⁻³², we speculated that the target for the glycine release following OGD could be NMDARs.

OGD paradigm on acute slices, *in vitro*, decreases NMDAR current amplitude

In brief, we found that an OGD paradigm applied to acute slices during a train stimulation induced NMDAR internalization (Fig. S4c–g). To further confirm that glycine is responsible, we purified glycine oxidase (GO), an enzyme that catalyzes the breakdown of glycine. After demonstrating the effectiveness of purified GO on exogenous glycine levels (Fig. S4h), NMDAR-EPSC trains (20Hz) were recorded with GO and the decrease of the NMDAR-EPSC amplitude was abolished following OGD (Fig. S4i–j). Altogether, these *in vitro* data demonstrate that glycine levels increase

during ischemia, however, GINI is only triggered when we further elevate glycine using the train stimulation paradigm. Therefore, we speculated that GINI could also be triggered *in vivo* during stroke in mice with elevated glycine levels.

Genetic elevation of brain glycine reduces infarct size following photothrombosis

Glycine has been shown to be neuroprotective in both *in vitro*³¹ and *in vivo* models of stroke³²⁻³⁵, yet proposed mechanisms have never been expanded into feasible pharmacotherapies. To determine if high glycine levels could result in a decrease in neuronal death following ischemia, we used a well-established focal ischemic paradigm, photothrombosis (PT). Since our *in vitro* data demonstrate that high glycine/D-serine levels are required to trigger GINI, one would expect that the stroke volume in GlyT1^{+/-} mice should be smaller than that observed in WT mice. Indeed, there was a statistically significant decrease in stroke volume in the GlyT1^{+/-} mice compared to WT. In contrast, stroke volumes were larger in SR^{-/-} mice, compared to WT (Fig. 2a).

Pharmacological elevation of brain glycine reduces infarct size following photothrombosis

To acutely increase the levels of endogenous glycine, WT mice were treated with NFPS 24hrs pre-stroke²¹⁻²⁴. Forty-eight hours following PT stroke in both the saline- and NFPS-treated cohorts, stroke volume was quantified using 2,3,5-triphenyltetrazolium chloride (TTC) or *via* magnetic resonance imaging (MRI). The box-and-whisker plot shows a statistically significant decrease in median stroke volume in the NFPS-treated mice compared to the saline-treated mice (Fig. 2b). This decrease in infarct volume following NFPS treatment is consistent with what has been previously observed in the transient middle cerebral artery occlusion (tMCAO) model of ischemic stroke^{36,37}. In addition, FluoroJade C (FJC) staining demonstrated that the NFPS-treated mice also have significantly decreased levels of cell death compared with the saline-treated mice (Fig. S5a). Therefore, these data demonstrate that the blockade of GlyT1 is required for the reduction of stroke volume. Interestingly, this decrease in stroke volume was maintained when NFPS was administered up to 10mins post-stroke (Fig. 2d).

Pharmacological elevation of brain glycine minimizes motor behavioural deficits following photothrombosis

Although encouraging, a decrease in stroke volume does not necessarily correlate with a decrease in post-stroke behavioural deficits³⁸. To determine if pre-treatment with NFPS could minimize post-stroke behavioural deficits, we used a well-established behavioural test of motor function, the adhesive removal test³⁹. Following PT, a significant attenuation of post-stroke motor behavioural deficits was observed in the cohort of mice treated with NFPS in both time to contact and time to remove, with no significant stroke or drug effect on the unimpaired paw (Fig. 2c).

Pre-stroke administration of NFPS decreases stroke volume and improves motor behavioural deficits following endothelin-1 stroke

The PT stroke model does not recapitulate all of the clinical aspects of ischemia, particularly with respect to reperfusion of the infarct⁴⁰. Therefore, to ensure that the observed decrease in stroke volume and attenuation of behavioural deficits was not an artefact of the PT stroke model, we repeated the experiments using a second known model of focal stroke, the endothelin-1 (ET-1) model³⁷. Mice pre-treated with NFPS had significantly smaller ET-1 stroke volumes compared with their saline-treated counterparts (Fig. 2e).

In the adhesive removal task, NFPS-treated mice showed significantly less post-stroke impairments in the impaired paw than the saline-treated mice, in both time to contact and time to remove (Fig. 2f). There was no significant stroke or drug effect on the unimpaired paw (Supplementary adhesive and cylinder task⁴¹ data for both stroke models in figure S5b-e and validation of ET-1 model in figure S6a-c). The horizontal ladder test was an additional assessment of motor function⁴². Following ET-1 stroke, there was a significant increase in impaired paw misses in the saline-treated group, however, in the NFPS-treated group, no significant increase in misses was observed (Fig. S5f-g). These data demonstrate that the blockade of GlyT1 ameliorated post-stroke outcomes in two models of stroke. Furthermore, this effect was not due to hypothermia (Fig. S6e). Therefore, these data emphasize the crucial role of GlyT1-A in the observed neuroprotection, and this is likely occurring due to GINI.

Blocking NMDAR internalization abolishes the neuroprotective effect of NFPS on stroke volume and behaviour

GINI is driven by the recruitment of AP-2 and is mediated by A714 on the C-terminal domain of

GluN1. Glycine priming for internalization is specific to A714, therefore this residue is necessary for priming of NMDARs containing either GluN2A or GluN2B in recombinant systems¹³. To confirm that our *in vivo* observations are due to GINI, we introduced a point mutation into the NMDAR GluN1 subunit (A714L), which abolishes glycine-mediated NMDAR internalization *in vitro*¹³. We first assessed the functionality of the mutation *via* transient transfection of GluN1-WT or GluN1-A714L together with WT GluN2A subunit into HEK293 cells resulting in a functional NMDAR. Application of 1mM glycine in cells expressing GluN1-WT induced a significant decrease in the amplitude of the NMDAR-EPSC, while in cells expressing GluN1-A714L this concentration significantly increased the NMDAR amplitude (Fig. S7a–b). To visually confirm that GINI was occurring, the movements of NMDARs were tracked over time by live-cell imaging following application of 1mM glycine (Fig. 3a; Fig. S7c; Video S1 and S2).

This mutation was then packaged into an adeno-associated virus (AAV) 2/9 and injected into the sensory-motor cortex of mice. The functionality of the mutation was re-assessed in acute slices. A dose of 1mM glycine did not decrease NMDAR-EPSC amplitudes in cells infected with the AAV-GluN1-A714L constructs (Fig. S7d). The spread of the virus occupied a volume that was comparable to the PT stroke (Fig. 3b; Fig. S8a), and there were no significant differences in the PT-induced stroke volume between the mice infected with either the AAV-GluN1-WT or the AAV-GluN1-A714L constructs (Fig. 3c). However, there was a significant decrease in stroke volume following pre-treatment with NFPS in mice infected with AAV-GluN1-WT. NFPS administration had no effect on stroke volume in the mice infected with AAV-GluN1-A714L.

The adhesive removal test was repeated on mice infected with either the AAV-GluN1-A714L mutation or the AAV-GluN1-WT. Administration of NFPS to the mice infected with AAV-GluN1-WT resulted in a significant decrease in post-stroke time to contact and time to remove in the impaired paw (Fig. 3d). Interestingly, in mice infected with the AAV-GluN1-A714L (Fig. 3e), there was no significant change in time to contact and time to remove following stroke in the NFPS-treated mice. Data illustrated in figure S6d confirm that the injections of the AAV-GluN1-WT or -GluN1-A714L alone had no effect on behaviour. Taken together, these data confirm that GlyT1-A administration induces neuroprotection *in vivo*, *via* GINI.

Pre-stroke administration of NFPS attenuates vascular dysfunction

Stroke is primarily characterized as a vascular disease; therefore, we evaluated the impact of NFPS on vascular function and morphology following PT stroke. Using Laser Doppler flowmetry (LDF), we found that PT stroke induced a significant decrease in blood flow and this effect was rescued with NFPS pre-treatment (Fig. 4a). In GlyT1^{+/-} mice there was no significant change in blood flow following stroke. Interestingly, in SR^{-/-} mice, PT stroke induced highly significant decrease in blood flow (Fig. 4b).

We next assessed if NFPS could modify vascular morphology by pairing transcatheter perfusions of a fluorescent dye with tissue clearing and light sheet fluorescence microscopy (LSFM). This strategy allowed for complete labeling of the cerebral vasculature (Fig. 4c; Video S3 and S4). We first used a deep learning segmentation model to automatically calculate stroke volume from our cleared tissue (Fig. 4d–e; Fig. S8b; Video S5). We observed a decrease in stroke volume in NFPS-treated mice compared to saline-treated mice (Fig. 4e). These results consistent with data illustrated in figure 2b. We further explored the effect of NFPS following PT on vascular density. The PT-induced decrease in vascular density was attenuated with NFPS treatment compared to saline-treated mice, in the peri-infarct region (Fig. 4f–g). Furthermore, NFPS pre-treatment decreased the PT-induced loss in vessels of smaller diameter and length (Fig. 4h–i) compared to saline-treated mice, in the peri-infarct region. Taken together, treatment with NFPS before an ischemic event protects the function and morphology of the cerebral vasculature.

Discussion

Our results demonstrate that during an ischemic event, not only glutamate but also glycine is released in the extracellular space. In such ischemic conditions, when GlyT1s are antagonized, glycine accumulates in the synaptic cleft, reaches the “set point”, and triggers GINI. This is the first report demonstrating that GINI occurs *in vivo*, provides neuroprotection, and preserves brain vasculature.

Using whole-cell patch-clamp recordings, we generated dose-response curves and we measured the effects of glycine on NMDAR current amplitudes. We observed that application of low concentrations of glycine ($\leq 250\mu\text{M}$) increased the NMDAR-EPSC amplitudes^{3,43}. Paradoxically,

we found that application of high concentrations of glycine (> 1mM) reduced significantly the NMDAR-EPSC amplitudes. This internalization of NMDARs has been reported to be triggered by an increase in NMDAR binding to intracellular clathrin/dynamin-dependent endocytotic machinery^{5,13}. Since the role of GlyT1s is to keep glycine concentrations below the saturating level of the GBS on NMDARs⁴⁴ the relevance of the pivotal work from Salter and co-workers was questioned by several groups. This low synaptic concentration of endogenous glycine is far from the concentration required to trigger GINI⁷⁻¹⁰. As the effect of different doses of glycine on NMDAR-EPSCs appears to match that of the “inverted-U” shaped curve, we investigated the relationship between glycine levels and NMDAR internalization using *in vitro* ischemic paradigms. We report evidence that synaptic NMDARs internalize following elevation of glycine during a train of stimuli during OGD, an *in vitro* model of ischemia⁴⁵. Moreover, we show that application of a high concentration of glycine or D-serine not only triggers GINI but also primes NMDARs for GINI. When a high dose is applied and washed off prior to application of a low dose of one of the co-agonists, GINI is induced.

Interestingly, we demonstrate, *in vivo*, that elevation of extracellular glycine by pharmacological blockade or genetic deletion of GlyT1 resulted in a decreased stroke volume and an attenuation of motor deficits in mice following ischemic stroke induced by PT or ET-1. This was observed when NFPS was administered 24 hours pre-stroke, or up to 10 minutes post-stroke. We also show evidence that GINI, *in vivo*, is directly modulating the GluN1 subunit of NMDAR channel function during ischemic stroke as the effect of NFPS on both stroke volume and behaviour is completely abolished when mice are focally infected with a viral vector expressing a non-internalizing GluN1 receptor subunit (AAV-GluN1-A714L)¹³.

The NMDAR co-agonist, glycine, has been previously shown to be neuroprotective in both *in vitro*³¹ and *in vivo* models of stroke^{32-35,46}. However, the mechanism by which glycine affords neuroprotection during stroke remains elusive. Recent work suggests that it is *via* modulation of intracellular pathways, including the Phosphatase and tensin homolog (PTEN)/protein kinase B (AKT) signaling pathway^{34,35}, or vascular endothelial growth factor receptor 2⁴⁶. Glycine is also thought to exert its neuroprotective effects *via* mediation of non-ionotropic NMDAR function³¹⁻³³, or by promoting microglial polarization⁴⁷. Partial agonists at the GBS on NMDARs also afford

neuroprotection following an OGD challenge⁴⁸ and during MCAO paradigm⁴⁹. Pharmacological elevation of brain glycine following NFPS administration potentiates ischemic preconditioning²⁵ and confers neuroprotection *via* activation global activation of ionotropic glycine receptors during transient MCAO³⁶. Here, we report for the first time the important role of GlyTs as the blockade of these transporters minimized cell death following an ischemic stroke in an *in vivo* model.

A recent review suggests that glycine and d-serine may be therapeutically beneficial by down regulating NMDARs, such as rodent models of traumatic brain injury and lipopolysaccharide-induced neuroinflammation⁵⁰. There is also a growing body of evidence to suggest that extracellular glycine is neuroprotective in several rodent ischemic stroke models^{34,36,46,47,49,51}. Moreover, the level of extracellular glycine appears to be important in stroke outcome. A low level of glycine, corresponding to increased NMDAR activation, appears to be deleterious. In contrast, an elevated level of glycine appears to be neuroprotective⁵². These latest findings are in agreement with our data. Indeed, the transgenic GlyT1^{+/-} mice, which have high endogenous levels of glycine and consequently a high occupancy of the GBS, are more resistant to PT, while the SR^{-/-} mice, which have a low occupancy of the GBS, are more sensitive to PT challenge.

Despite an overwhelming body of evidence from animal studies that implicate NMDARs in neuronal loss⁵³⁻⁵⁶, all clinical trials of drugs targeting one of the numerous binding sites on NMDARs have failed due to poor tolerance or lack of efficacy⁵⁷⁻⁵⁹. One reason for this may be the difficulty in obtaining a therapeutic degree of NMDAR-blockade that does not interfere with critical NMDAR-dependent functions in neuronal circuits⁶⁰. The widespread inhibition of NMDAR function is not compatible with baseline synaptic transmission. As such, our data suggest that attention should turn to modulation of NMDAR function during stroke. In this study, we show that GINI is not a direct antagonism of NMDARs but rather a dynamic and reversible phenomenon which dampens NMDAR-mediated excitotoxicity during ischemia while maintaining basal synaptic activity of NMDARs.

The complex vascular network of the brain and its integrity are essential for normal brain function. Following an ischemic event, the delivery of oxygen and nutrients to neurons and glia cells are impaired. Since the brain is highly vulnerable to compromises in blood supply, we investigated

the potential impact of NFPS in preserving brain vasculature. It has been previously reported that changes in microvasculature, such as density and diameter, correlate with disease states⁶¹. PT stroke induced a decrease in vascular density in the peri-infarct region. We found that this decrease was attenuated by a pre-treatment with NFPS. Our histogram analysis shows that vessels of 2-3µm in diameter were the most affected post-stroke. Application of NFPS decreased the size of vessels occluded, suggesting that the peri-infarct region could undergo enhanced vascular remodelling during the recovery period. We cannot conclude that the mechanism underlying this observation is directly linked to GINI. However, increasing the level of endogenous glycine with NFPS protects the vascular network following stroke, and ultimately lead to improved behaviour outcomes.

Overall, our data demonstrate that elevation of glycine *via* blockade of GlyT1s before or shortly after an ischemic event may provide a rationale for the repurposing of currently approved pharmaceuticals with a similar mechanism of action as potential stroke treatments. For example, the glycine re-uptake blocker sarcosine is authorized for clinical use in the treatment of schizophrenia at daily doses of 1-2g per day and is well tolerated in these patients⁶²⁻⁶⁶. As our *in vivo* data demonstrate the pre-clinical efficacy of this class of drugs in minimizing the deficits induced by PT and ET-1 paradigms, and considering that several GlyT1-As have been tested and proven to be safe and well tolerated in human clinical trials^{67,68}, GlyT1 should be tested for a new therapeutic for ischemic stroke.

Methods

Animals

All procedures in this study were carried out on female and/or male 8–10-week-old mice in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Ottawa Animal Care Committee. The following transgenic mouse lines were utilized: heterozygous glycine transporter type 1 (GlyT1^{+/-}), serine racemase knockout (SR^{-/-}), and *N*-methyl-D-aspartate receptor (NMDAR) GluN2A subunit knockout (GluN2A^{-/-}) mice⁶⁹, along with their wild type (WT) litter mates (on C57Bl/6;S129, C57Bl/6;S129 and C57Bl/6 backgrounds respectively). *In vivo* behavioural experiments were performed on C57Bl/6 WT mice from Charles River ®. The animals were housed under standard conditions and had access to chow and water *ad libitum*.

Whole-cell electrophysiology on hippocampal slices and HEK293 cells

Whole-cell voltage-clamp recordings were obtained from visually identified CA1 pyramidal cells from acute hippocampal brain slices (300µm thick) in oxygenated artificial cerebrospinal fluid (ACSF) as previously described^{70,71}. The cells were voltage-clamped at -65mV using cesium methane sulfonate based internal solution and postsynaptic currents were evoked by electrical stimulation of the Schaffer collaterals with a bipolar stimulating electrode positioned in the *stratum radiatum*. The intensity of the stimulation was adjusted to obtain evoked excitatory postsynaptic currents (EPSCs). The stimulation protocol consisted of a single 100µs current pulse (10–200µA) evoked every 12s. For the train stimulation protocol, 10 current pulses (100µs long) were evoked at 50Hz for 200ms and then repeated once every 20s. To isolate the NMDAR-EPSC, a low concentration of MgCl₂ (0.13mM) ACSF was used wherein the CaCl₂ concentration was increased to 3.5mM to maintain cation balance.

HEK293 cells were used for electrophysiology recordings 48–72hrs following transfection with either the GluN1-WT or GluN1-A714L cDNA along with an equimolar ratio of the fluorescent marker cDNA, GFP-GluN2A. NMDAR currents were evoked using pressure ejection (10psi) from a picospritzer micropipette filled with 10µM glycine and 1mM glutamate (Sigma-Aldrich) for a duration of 25–50ms every 20s at a membrane potential of -60mV. HEK293 cells were recorded

in HEPES-buffered saline external solution with low MgCl_2 using a potassium gluconate recording solution.

When required, additional drugs were applied including various concentrations of D-serine and glycine (Millipore Sigma), as well as 300nM *N*-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine (NFPS; Tocris Bioscience), 10mM BAPTA (Thermo Fisher), 30 μM cyclopiazonic acid (CPA) (Tocris Bioscience), and 20 μM nimodipine (Millipore Sigma). The inhibitors of clathrin-mediated endocytosis, 100 μM dynasore (Millipore Sigma), 100 μM dynamin blocking peptide (DBP; Tocris Bioscience), and 30 μM Dyno4a (Abcam) were included in the internal solution.

Sniffer-patch technique

To detect glycine release, we used the “sniffer patch” technique⁷²⁻⁷⁵. A Chinese Hamster Ovary (CHO) cell line were generated and stably transfected with the $\alpha 2$ subunit of the glycine receptor (GlyR)⁷⁶. Outside-out membrane patches were excised from the CHO cells using thick-walled borosilicate glass pipettes filled with a cesium chloride internal solution. Following patch excision, the electrode was placed in the *stratum radiatum* of the CA1 region of the hippocampus to detect glycine release and allow channel activation. Channel open probability (P_{open}) was derived by measuring the mean open time of all the single channel events during the recording window, then dividing by the sum of the mean open and shut times. Multiple channel openings were set as a $P_{\text{open}} = 1$ for that particular time period.

Oxygen-glucose deprivation paradigm

To mimic ischemia, the acute slices were challenged by an oxygen-glucose deprivation paradigm (OGD) modified from Rossi *et al.*⁴⁵. In this paradigm, external glucose was replaced with 7mM sucrose, and the external solution was saturated with 95% N_2 / 5% CO_2 instead of 95% O_2 / 5% CO_2 . Iodoacetate and cyanide were also added to the OGD external solution to block glycolysis and oxidative phosphorylation.

Purification of glycine oxidase

The plasmid containing His-tagged glycine oxidase (GO) was generated from *Bacillus subtilis*. This plasmid was a gift from Dr. Steven Ealick (Cornell University, NY, USA). The protein was expressed in *E. coli* and purified as previously described⁷⁷⁻⁸¹.

NMDAR internalization imaging in HEK293 cells

HEK293 cells were transiently transfected with either GluN1-WT or GluN1-A714L cDNAs together with pHluorin-GluN2A cDNA. HEK293 cells were then grown for 24-48hrs in the presence of D-APV (Tocris Bioscience). Images were acquired with an LSM880 Confocal Microscope (Zeiss), with cells in a modified HEPES buffer (add 1mM Glutamate, omit 0.13mM MgCl₂). A 647nm-tagged FluoTag®-X4 anti-GFP (1:250-1:500; NanoTag Biotechnologies) was added to tag extracellular NMDARs prior to the acute application of an internalizing dose of glycine. The nanotags (anti-GFP nanobody) are cell impermeable and tag NMDARs on the cell surface. Therefore, the nanotags were observed within the cell only when NMDARS had been internalized. Images were acquired every 3mins over 10-12mins to visualize internalization. Internalization was deemed to have occurred when the cell-impermeable NanoTag (647) was observed within the cell.

Immunohistochemistry

Slices were washed then permeabilized with 0.25% (w/v) gelatin and 0.2% Triton X-100 (v/v) in PBS (PBS-GT). Slices were incubated in primary antibodies in PBS-GT overnight, then washed three times with PBS-GT before the addition of fluorescent secondary antibodies at room temperature (RT). Slices were rinsed, air-dried, and mounted onto slides (Table S1)³⁰.

The channels representing glycine (650nm), vesicular glutamate transporter (VGLUTs 1-2; 488nm), and vesicular GABA transporter (VGAT;550nm) were merged into a single image using Adobe Photoshop 7.01 and all quantifications were done manually by a researcher blinded to the treatment groups. Platelet endothelial cell adhesion molecule-1 (CD-31; 647nm) and Collagen IV (CollIV;647nm) were depicted in purple and visually colocalized to filled, perfused vessels (488nm).

Generation of WT and A714L viral constructs

The GluN1 constructs were made by cloning the GluN1 coding region of a SuperEcliptic Phluorin (SEP)-tagged GluN1 construct (Addgene #23999) into pDrive cloning vector (pDrive cloning vector, Qiagen). We then used this as a template to create the A714L mutant clone by site-directed mutagenesis. These cDNAs were used for transfection of HEK293 cells. For generation of GluN1-WT and GluN1-A714L adeno-associated virus (AAV), the coding fragments of these constructs were sub-cloned into an adeno-associated viral vector, and viral constructs were then packaged with plasmid AAV2/9 at the University of Laval.

Photothrombosis and Endothelin-1 Stroke

NFPS or a vehicle control solution was injected intraperitoneally (*i.p.*) into C57Bl/6 mice either 24hrs prior to stroke or 10mins/60mins/120mins post-stroke, at a dose of 5mg/kg. Photothrombotic (PT)⁸² or cortical endothelin-1 (ET-1)⁸³ strokes were induced as previously described. Mice were anesthetized with 2.5% isoflurane in O₂ and mounted onto a stereotaxic frame. For PT strokes, a dose of 10mg/mL of Rose Bengal (Tocris) was injected *i.p.*. Immediately following the injection of the dye, the skull was exposed to visualize bregma. Using the stereotaxic device, a 520nm laser (~20mW; Beta Electronics) was positioned above the sensorimotor cortex (AP+0.7, ML+2.0) and turned on for 10mins to induce a permanent occlusion. For ET-1 strokes, once the skull was exposed and a craniotomy performed for each injection site (1. AP +0.0, ML +2.0, DV -1.6; 2. AP +0.2, ML +2.0, DV -1.4; 3. AP +0.4, ML +2.0, DV -1.3), 1μL of 2μg/μL human, porcine ET-1 (Abcam), dissolved in 2.7μg/μL L-NAME (Abcam), was injected over 5mins with a 28G 10μL Hamilton syringe to induce a transient ischemic stroke.

Cortical infection with AAVs

Mice were anesthetized with 2.5% isoflurane in O₂ and mounted onto a stereotaxic apparatus. The intact skull was exposed to visualize bregma. The following injection sites were measured from bregma: 1. AP +1.2, ML +2.0, DV -0.5; 2. AP +0.2, ML +2.0, DV -0.5. A craniotomy was performed at each site prior to injecting 0.5μL of 10⁻¹² PFU/mL (plaque forming units) of either a AAV-WT-GluN1 or the mutant AAV-GluN1-A714L construct, over 5mins with a 28G 10μL Hamilton syringe.

Laser Doppler Flowmetry

Laser Doppler Flowmetry (LDF) recordings following PT were performed as previously described⁸⁴. Mice were anesthetized with an *i.p.* injection of a 0.01ml/g cocktail consisting of 120mg/kg ketamine and 10mg/kg xylazine, and then mounted onto a stereotaxic apparatus. Following exposure and thinning of the skull, the laser probe (Transonic Systems) was positioned over the sensory motor cortex (AP +0.7, ML +2.0) and baseline activity was recorded for 5mins. The laser probe was replaced with a 520nm laser (~20mW; Beta Electronics) to induce PT stroke, as described above. Following PT, LDF recordings were performed for an additional 30mins.

Quantification of stroke volume — Magnetic resonance imaging:

Magnetic resonance imaging (MRI) was performed at the University of Ottawa pre-clinical imaging core using a 7 Tesla GE/Agilent MR 901. Mice were anaesthetized for the MRI procedure using isoflurane in O₂: induction at 3%, maintenance at 1.5% . A 2D fast spin echo sequence (FSE) pulse sequence was used for the imaging, with the following parameters: slice thickness = 0.5mm, spacing = 0mm, field of view = 2.5cm, matrix = 256 x 256, echo time = 41ms, repetition time = 7000ms, echo train length = 8, bandwidth = 16 kHz, fat saturation. Stroke lesions demonstrated hyperintensity.

Quantification of stroke volume — Triphenyltetrazolium chloride:

Stroke volume quantification was performed using 2,3,5-triphenyltetrazolium chloride (TTC; Sigma)^{85,86}. Forty-eight hours post-stroke, mice were deeply anesthetized with 5% isoflurane in O₂ before decapitation for slicing on a vibratome (Leica) in cold ACSF at 0.5mm. Slices were incubated in 2% TTC at 37°C for 10mins, then transferred to 4% paraformaldehyde (PFA) at 4°C. Brain slices were imaged from both sides and the surface area of the infarct regions were measured on Fiji (ImageJ.com) and multiplied by the thickness of the slice to obtain a final volume.

Quantification of stroke volume — Cresyl violet:

Forty-eight hours post-stroke, mice were transcardially perfused with 1X PBS, followed by 4% PFA. Brains were collected and post-fixed in 4% PFA overnight and then incubated in sucrose until saturation. Serial 25µm thick coronal sections were cut on a cryostat (Microm HM500), and collected onto positively charged Superfrost Plus Microscope Slides (Fisher Scientific). The slides were immersed in xylene and then rehydrated in decreasing concentrations of ethanol before being

placed in double distilled water (ddH₂O). Once rehydrated, slides were stained with cresyl violet (CV) (Electron Microscopy Sciences) and placed in ddH₂O. Slides were then dehydrated in increasing concentrations of ethanol before being immersed in xylene. Once removed, the slides were mounted with DPX mounting media (Sigma). Images of CV-stained slices were acquired with the *EVOS FLAuto2* inverted epifluorescence microscope under brightfield. The surface area of the infarct regions was multiplied by the distance between each collected slice (500µm) to obtain a volume. The sum of all slices was used to obtain a final stroke volume per brain.

Quantification of neuronal loss — FluoroJade C:

The brain tissue preparation for FluoroJade C (FJC; EMD Millipore) was treated exactly as that of the CV brain tissue. Slides were first immersed in 1% sodium hydroxide in 80% ethanol, 70% ethanol and finally in ddH₂O before being incubated in a 0.06% potassium permanganate (Sigma-Aldrich) solution. This was followed by an incubation in a 0.0001% FJC solution dissolved in 0.1% aqueous acetic acid and combined with 0.0001% DAPI (Santa Cruz Biotechnology), and slides were once again rinsed with ddH₂O and left to air dry. Slides were then immersed in xylene and mounted with FluoroMountG (Sigma-Aldrich)⁸⁷. Imaging was completed with the *Zeiss AxioObserver Z1* inverted epifluorescence microscope using GFP (488/509nm) and DAPI (359/461nm) filters. Analysis of the total number of degenerating neurons was performed using IMARIS 9.2 (Bitplane). IMARIS 9.2 was set to detect and count all green (representing degenerating neurons) and blue (representing nuclear DNA) spots on each image and then calculate colocalization. The cells having been tagged by both DAPI and FJC were counted as FJC positive neurons.

Tissue clearing

CUBIC tissue clearing was completed as previously described⁸⁸. Following perfusion, the tissue was post-fixed overnight in 4% PFA, then washed in 1X PBS the following day. Following washes, tissue was submerged in half diluted CUBIC-L (1:1, CUBIC-L:Water) at 37°C overnight. Tissue was submerged in CUBIC-L at 37°C with gentle shaking over 10 days, changing the solution every 48hrs. Tissue was then washed in 1X PBS before being submerged in half diluted CUBIC-R⁺(M) (1:1, CUBIC-R⁺(M):Water) overnight at RT with gentle shaking. Tissue was submerged in CUBIC-R⁺(M) the following day, then replaced with fresh CUBIC-R⁺(M) 24hrs

later. Tissue was imaged with a light sheet microscope in a refractive index matched imaging solution consisting of a mixture of HIVAC-4 with mineral oil.

Quantification of stroke volume:

For CUBIC-cleared brains imaged by light sheet fluorescence microscopy (LSFM), infarct volume was measured using a deep learning segmentation model. For more information please refer to the “Light sheet fluorescence microscopy” section below.

Post-stroke vascular morphology quantification:

For analysis of vascular morphology in the peri-infarct region, transcardial FITC-BSA staining was paired with CUBIC brain clearing to allow for LSFM imaging. Forty-eight hours post PT stroke, mice were transcardially perfused with 20mL 1X PBS, then 20mL 4% PFA. Mice were then submerged in a 37°C water bath, facing down at an angle of 30° before being perfused with 10mL of 0.5% FITC-BSA (Sigma-Aldrich), in 2% gelatin (Sigma-Aldrich). Subsequently, mice were submerged in an ice bath for 30mins before the brain was dissected out⁸⁹. Brains were collected and post-fixed in 4% PFA overnight, then cleared following the CUBIC tissue clearing protocol. Following tissue clearing, brains were imaged by LSFM. A 1.125mm³ region of interest lateral to the stroke site was manually selected, then analyzed using AIVIA 9 (DRVISION Technologies).

Viral spread quantification:

Three weeks following cortical infection, mice were transcardially perfused with 1X PBS, followed by 4% PFA. Brains were sliced into 0.5mm coronal slices and cleared following the SeeDB tissue clearing protocol described by Ke *et al.*⁹⁰. Following tissue clearing, brains were imaged with the Zeiss LSM800 confocal microscope. All viral spread analysis was performed manually using Fiji.

Light sheet fluorescence microscopy

Imaging and segmentation:

Imaging was performed using our custom-built light sheet microscope. CUBIC-cleared brains were imaged using a 2.5X objective (NA0.07), 488nm excitation laser line and 5µm steps. Each

sample was scanned as a series of tiles, then stitched into a single image using TeraStitcher. The stitched scans were then run through AIVIA 9 to segment and create 3D reconstructions of the vascular network. Properties of each vessel (diameter and length) were automatically calculated by AIVIA 9, and then exported for analysis.

Automated stroke volume measurement (stroke volume prediction):

Stroke volume in cleared tissue was calculated as follows: the areas representing stroke in each slice were identified, multiplied by their z-depth (thickness), then summed to obtain a total volume. The stroke regions were identified in each slice using a deep convolutional neural network⁹¹⁻⁹³ which was deemed 98% accurate. The network was first pre-trained on a large data-set⁹⁴, then further trained using 906 experimental scans.

Behavioural tests

Adhesive removal test:

The adhesive removal test was performed as described³⁹. Mice were trained pre-stroke daily over 5 days and tested post-stroke over 2 days. Trials began with 1min of habituation to an empty home cage, before strips of adhesive were placed onto both forepaws. The mouse was then placed back into the cage and the times to contact and remove the adhesive strips were recorded by two experimenters. Mice were allotted a maximum of 2mins to complete the task. The times to contact and remove the pieces of adhesive tapes were compared per paw as well as pre- and post-stroke.

Horizontal ladder test:

The horizontal ladder test was performed based on protocols described previously^{42,95} with slight modifications. Mice underwent one day of training prior to stroke. In the pre-stroke trials, mice crossed the ladder up to seven times or until they had performed two acceptable runs. In turn, during the post-stroke trials, mice had three attempts to cross the ladder, two of which were scored. Each trial was recorded with a video camera. Scoring and analysis were performed by an experimenter blind to the conditions. The video recordings of the best two trials from each mouse were analyzed frame-by-frame with Noldus Observer XT program. Each limb's step was scored as either "correct", "partial" or "miss". The percentage of missed steps pre- and post-stroke were compared.

Cylinder test:

The cylinder test was performed based on protocols described^{41,96}, with slight modifications. Mice were placed in a transparent cylinder and filmed with an overhead camera until they reared 22 times. With each rear, three types of behaviours were recorded: (A) right paw is exclusively weight bearing; (B) left paw is exclusively weight bearing; (C) both paws are weight bearing at the same time. The video recordings of each trial were analyzed frame-by-frame with Noldus Observer XT program by a single experimenter blind to the conditions. The length and frequency of forelimb contacts to the wall of the cylinder were scored. The behaviours were expressed per paw as an average time in relation to the sum of the independent left and right weight bearing. The average time spent on the impaired paw (right) was compared in pre-stroke and post-stroke trials.

Quantification and Statistical analysis

All data are presented as means $\pm$ S.E.M.; n represents the number of mice or cells in each group, as indicated in each figure. In most cases, statistical significance was determined by a paired, two-tailed Student's t-test or a two-way repeated measure ANOVA followed by Bonferroni post hoc comparisons, for multiple group comparisons. In cases in which datasets had multiple missing values, a mixed-model (ANOVA) was implemented by GraphPad Prism. For analyses of groups across multiple time points, a one-way ANOVA was utilized. Statistical analyses and data presentation were completed using both OriginPro 8.5 (OriginLab Software) and GraphPad Prism 8 (GraphPad Software).

For electrophysiological data, decay kinetic and amplitude analysis were performed on averaged traces. Decay time constants were best fit with a double exponential function and expressed as a weighted mean. Due to summation, EPSC amplitudes in the train were measured from the end of the previous EPSC rather than from the initial baseline.

Please refer to Supplementary Methods for more detailed description of all methods.

Author Contributions:

Performed and analyzed electrophysiological experiments: P.K., B.W., W.B., A.Y.C.W., S.R. Purified and determined activity of glycine oxidase: N.A. Carried out behavioural testing and surgical procedures: J.C., A.Y.C.W., A.S., P.K., B.W. Performed immunohistochemistry experiments: J.C., A.S., A.Y.C.W. Generated viral constructs: P.C. Prepared cleared brain samples: J.C. Constructed and operated the light sheet microscope: B.W., and J.P. Analyzed data imaged with the light sheet microscope: B.W., J.P., J.W. Performed and analyzed laser doppler flowmetry recordings: J.C., and A.Y.C.W. Provided and facilitated access to essential infrastructure: R.B. Supervised the study, performed experimental design and provided scientific direction: R.B. All authors provided advice on data interpretation and critical edits to the text and approved the final manuscript.

We have no competing interest to declare

Additional information:

Supplementary information is available for this paper

Correspondence and requests for materials should be addressed to:

Richard BERGERON

Ottawa Hospital Research Institute,

451 Smyth Road, Roger Guindon Building, Room 3210D,

Ottawa, Ontario, Canada, K1H 8M5

Email: rbergeron@ohri.ca

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Figure 1

Figure 1: Increasing exogenous glycine and D-serine concentrations results in NMDAR internalization, which is dependent on Ca²⁺ influx.

a) Normalized raw traces showing the effect of increasing concentrations of exogenous glycine on Schaffer Collateral NMDAR-EPSCs and mean time-course data showing the effect of a 15mins application of various glycine concentrations. **b)** A dose-response curve of glycine and NMDAR-EPSC amplitudes. **c)** The effect of 250μM and 1mM glycine on NMDAR-EPSC amplitudes in the presence of dynasore. **d)** The role of various concentrations of extracellular Ca²⁺ on NMDAR-EPSC amplitudes in the presence of 1mM glycine, as well as changing intracellular Ca²⁺ levels using BAPTA, nimodipine, or CPA. **e)** The effect of various concentrations of glycine on

1012 NMDAR-EPSC amplitudes in GlyT1^{+/-} mice, compared to WT. **f)** The dose-response curve of the
1013 effects of glycine on NMDAR amplitudes in GlyT1^{+/-} mice. **g)** The effect of 250μM glycine on
1014 NMDAR-EPSC amplitudes in GlyT1^{+/-} mice in the presence of dynasore. **h)** The effect of low
1015 (10μM) and high (1mM) concentrations of glycine or D-serine concentrations on NMDAR-EPSC
1016 amplitudes. **i)** The dose-response curve of NMDAR-EPSC amplitudes to D-serine compared to
1017 glycine. **j)** The effect of 1mM D-serine in the presence of dynasore. **k)** The effect of 10μM D-serine
1018 while elevating endogenous glycine levels with NFPS. **l)** A dose-response curve showing the effect
1019 of exogenous D-serine levels on SR^{-/-} mice. **m)** The effect of a higher dose of D-serine (1mM vs.
1020 2mM) on NMDAR-EPSC amplitudes in SR^{-/-} mice compared to their WT littermates. Data is mean
1021 ± SEM; statistical significance $p < 0.05$ *.

Figure 2

Figure 2: Elevation of extracellular glycine results in a smaller infarct volume and decreased motor behavioural deficits following photothrombotic and endothelin-1 stroke.

a) Representative serial coronal sections of TTC-stained mouse forebrain (slice thickness 500µM) and their corresponding box and whisker plots showing the infarct volume when assessed 48hrs after the induction of a unilateral photothrombotic stroke in GlyT1^{+/-} and SR^{-/-} mice relative to WT mice. **b)** Representative TTC-stained (top) or magnetic resonance imaging (bottom) sections showing representative stroke regions observed 48hrs following the induction of photothrombotic (PT) stroke, and a box and whisker plot showing stroke volume in saline-treated or NFPS-treated mice 24hrs prior to stroke induction. **c)** The effect of 24hrs pre-stroke NFPS administration on

post-stroke time to contact and time to remove in the adhesive removal task compared with saline treatment, when evaluated 48hrs following PT stroke. **d)** Effect of various post-stroke administration time-points of NFPS treatment on stroke volume with their corresponding box and whisker plots following PT stroke. **e)** Representative cresyl violet sections (25µm thick) 48hrs following endothelin-1 (ET-1) stroke obtained from saline-treated and NFPS-treated mice 24hrs prior, in which the extent of the infarct is shown within the yellow border and box and whisker plot depicting infarct volume. **f)** The effect of 24hrs pre-stroke NFPS administration on post-stroke time to contact and time to remove in the adhesive removal task compared with saline treatment following ET-1 stroke. Data is mean $\pm$ SEM; statistical significance $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and $p < 0.0001$ ****.

Figure 3

Figure 3: Infection of the stroke site with the non-internalizing GluN1-A714L mutation abolishes the protective effect of elevating extracellular glycine on stroke volume and during a behavioural task.

a) Visual representation of NMDAR internalization in GluN1-WT or GluN1-A714L transfected HEK293 cells following application of 1mM glycine. Transfected NMDARs are labeled in green, while extracellular NMDARs are additionally labeled with red cell impermeable nanobody staining. **b)** Representative images showing the extent of viral spread in the mouse forebrain following infection between mice infected with AAV-GluN1-WT or AAV-GluN1-A714L. **c)** Box and whisker plot showing the effect of NFPS administration 24hrs prior to PT stroke induction in mice infected with AAV-GluN1-WT or AAV-GluN1-A714L. **d-e)** The effect of NFPS on post-

1056 stroke time to contact and time to remove in the adhesive removal task compared with saline
1057 treatment, in mice infected with AAV-GluN1-WT **(d)**, and in mice infected with AAV-GluN1-
1058 A714L **(e)** 48hrs following PT stroke. Data is mean $\pm$ SEM; statistical significance $p < 0.05$ *, $p <$
1059 0.01 **, and $p < 0.001$ ***.

Figure 4

Figure 4: Laser doppler flowmetry blood flow and automatic segmentation of vascular data with AIVIA 9.

a) Diagram depicting laser doppler flowmetry set-up and the measured effect of NFPS on cerebral blood flow following photothrombotic (PT) stroke. **b)** Time-course of post-stroke cerebral blood flow following PT in mice with varying levels of glycine or D-serine. **c)** 50µm coronal section of brain perfused with FITC-BSA. Magnified images from the sensorimotor cortex demonstrating exact colocalization of FITC-BSA perfusion (green) with CD31 and Coll/V vascular immunostaining (purple). **d)** Colorized max projection of stroked hemisphere, and single section of raw images depicting vasculature at the stroke and below the stroke, acquired with a light sheet microscope 48hrs following PT stroke. **e)** Stroke volume bar graph in saline- or NFPS-treated mice, calculated by an automated deep learning prediction model. **f)** Merged image demonstrating exact colocalization of AIVIA's automatic segmentation to raw data. **g)** Density of vessels in peri-infarct region in saline- or NFPS-treated mice. **h–i)** Number of vessels in the stroke area according to diameter and to length. Data is mean $\pm$ SEM; statistical significance $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and $p < 0.0001$ ****.

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