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Glycine transporter-1 antagonist provides neuroprotection *in vivo*

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Figures and Extended Data

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33 **Abbreviations:**

34

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.

Glycine fulfills several roles in biology including protein synthesis, inhibitory transmission *via* glycine receptor activation and excitatory transmission through glutamate-sensitive *N*-methyl-D-aspartate receptors (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) on NMDARs. Here, we report evidence that GINI occurs also *in vivo* and is neuroprotective following ischemic insult. Mice pre-treated with a GlyT1 antagonist (GlyT1-A), which increased glycine levels, exhibited smaller stroke volume, reduced cell death, and minimized behavioural deficits following stroke induction by either photothrombosis or endothelin-1. We demonstrate that in a modified *in vitro* ischemic paradigm, glycine is released at levels surpassing what occurs during ischemia alone. Therefore, glycine accumulates in the synaptic cleft, enhances occupancy of GBS and reaches the set point to trigger GINI. We report that GINI is observed during stroke, *in vivo*, only in the presence of a GlyT1-A. Moreover, we show evidence of a protective effect on the vasculature in the peri-infarct area. Therefore, these data strongly suggest that GlyT1 is a therapeutic target to prevent cell death following an ischemic event.

Introduction

Neuronal death following ischemic stroke is triggered by uncontrolled glutamate release leading to the overactivation of *N*-methyl-D-aspartate receptors (NMDARs) on surrounding neurons, and in turn induces 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. We found that during an oxygen/glucose deprivation paradigm, *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 1 (GlyT1)⁷⁻¹⁰. We report that GlyT1 antagonist (GlyT1-A) administration before 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 250 μM , 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 100 μM 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 Ca^{2+} -sensitive phosphatase calcineurin^{15,16}. Therefore, we explored the role of extracellular and intracellular Ca^{2+} on NMDAR-EPSC amplitudes in the presence of 1mM glycine. We examined the effects of various external Ca^{2+} concentrations on the NMDAR response to 1mM glycine application. When 1mM glycine was applied with low Ca^{2+} (1mM), an increase in NMDAR-EPSC amplitude was observed, in contrast to what occurred with normal Ca^{2+} concentrations (3.5mM). To further confirm the role of Ca^{2+} in GINI, we included a Ca^{2+} -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 20 μ M nimodipine, an L-type Ca^{2+} channel blocker, also attenuated GINI compared to control, further corroborating the data acquired with BAPTA. In contrast, depleting intracellular Ca^{2+} stores by incubating hippocampal slices for 1hr in 30 μ M cyclopiazonic acid (CPA), an inhibitor of intracellular Ca^{2+} 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 (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 μ M or 1mM glycine between wild type (WT) and GlyT1^{+/-} mice, bath application of 250 μ M glycine, which potentiated the NMDAR-EPSC amplitude in WT mice, significantly inhibited the NMDAR-EPSC amplitude in GlyT1^{+/-} mice (Fig. 1f). The decrease of the NMDAR-EPSC amplitude induced by 250 μ M glycine in GlyT1^{+/-} mice was abolished in the presence of dynasore (Fig. 1g). Therefore, the high levels of endogenous glycine in the 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 (10μM), 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 (Extended Data Fig. 1).

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. 1l-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 (Extended Data Fig. 2a-e), nor attributed to AMPA receptor activity (Extended Data Fig. 2f), and not limited to the hippocampal region (Extended Data Fig. 2g).

Glycine is released during oxygen-glucose deprivation.

Immunohistochemical data illustrated in extended data figure 3 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 (Extended Data Fig. 4a) and a significant increase

in open probability (P_{open}) compared to control (Extended Data Fig. 4b). 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 (Extended Fig. 4c–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 (Extended Data Fig. 4h), NMDAR-EPSC trains (20Hz) were recorded with GO and the decrease of the NMDAR-EPSC amplitude was abolished following OGD (Extended Data Fig. 4i–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 (Extended Data Fig. 5a). 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 Extended Data Fig. 5b–e and validation of ET-1 model in Extended Data Fig. 6a–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 (Extended Data Fig. 5f–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 (Extended Data Fig. 6e). 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 (Extended Data Fig. 7a–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; Extended Data Fig. 7c; Supplementary Video 1 and 2).

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 (Extended Data Fig. 7d). The spread of the virus occupied a volume that was comparable to the PT stroke (Fig. 3b; Extended Data Fig. 8a), 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 extended data figure 6d 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; Supplementary Video 3 and 4). We first used a deep learning segmentation model to automatically calculate stroke volume from our cleared tissue (Fig. 4d-e; Extended Data Fig. 8b). We observed a decrease in stroke volume in NFPS-treated mice compared to saline-treated mice (Fig. 4e). These results are in agreement 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

Despite evidence from animal studies that NMDAR antagonists minimize neuronal loss⁴³⁻⁴⁶, clinical trials targeting different binding sites of NMDARs failed due to poor tolerance and lack of efficacy⁴⁷⁻⁴⁹. This widespread inhibition of NMDAR function is not compatible with neuronal communication. 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. Several GlyT1-As have been tested and proven to be safe and well tolerated in human clinical trials⁵⁰ and therefore could lead to an innovative clinical approach for the treatment of stroke.

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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^{-/-}) mice⁵¹, 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^{52,53}. 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 μ M cyclopiazonic acid (CPA) (Tocris Bioscience), and 20 μ M nimodipine (Millipore Sigma). The inhibitors of clathrin-mediated endocytosis, 100 μ M dynasore (Millipore Sigma), 100 μ M dynamin blocking peptide (DBP; Tocris Bioscience), and 30 μ M Dynngo4a (Abcam) were included in the internal solution.

Sniffer-patch technique

To detect glycine release, we used the “sniffer patch” technique⁵⁴⁻⁵⁷. A Chinese Hamster Ovary (CHO) cell line were generated and stably transfected with the $\alpha 2$ subunit of the glycine receptor (GlyR)⁵⁸. 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 $P_{open} = 1$ 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.*⁵⁹. 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 described⁶⁰⁻⁶⁴.

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 (Extended Data Table 1)⁶⁵.

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)⁶⁶ or cortical endothelin-1 (ET-1)⁶⁷ 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 described⁶⁸. 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)^{69,70}. 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)⁷¹. 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 described⁷². 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 out⁷³. 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.*⁷⁴. 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 network⁷⁵⁻⁷⁷ which was deemed 98% accurate. The network was first pre-trained on a large data-set⁷⁸, then further trained using 906 experimental scans.

Behavioural tests

Adhesive removal test:

The adhesive removal test was performed as described⁷⁹. 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^{80,81} 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^{82,83}, 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.

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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

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Figure 1

Figure 1: Increasing exogenous glycine and D-serine concentrations results in NMDAR internalization, which is dependent on Ca^{2+} 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 Ca^{2+} on NMDAR-EPSC amplitudes in the presence of 1mM glycine, as well as changing intracellular Ca^{2+} levels using BAPTA, nimodipine, or CPA. **e)** The effect of various concentrations of glycine on NMDAR-EPSC amplitudes in GlyT1^{-/-} mice, compared to WT. **f)** The dose-response curve of the

851 effects of glycine on NMDAR amplitudes in GlyT1^{+/-} mice. **g)** The effect of 250μM glycine on
852 NMDAR-EPSC amplitudes in GlyT1^{+/-} mice in the presence of dynasore. **h)** The effect of low
853 (10μM) and high (1mM) concentrations of glycine or D-serine concentrations on NMDAR-EPSC
854 amplitudes. **i)** The dose-response curve of NMDAR-EPSC amplitudes to D-serine compared to
855 glycine. **j)** The effect of 1mM D-serine in the presence of dynasore. **k)** The effect of 10μM D-serine
856 while elevating endogenous glycine levels with NFPS. **l)** A dose-response curve showing the effect
857 of exogenous D-serine levels on SR^{-/-} mice. **m)** The effect of a higher dose of D-serine (1mM vs.
858 2mM) on NMDAR-EPSC amplitudes in SR^{-/-} mice compared to their WT littermates. Data is mean
859 ± 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

872 post-stroke time to contact and time to remove in the adhesive removal task compared with saline
873 treatment, when evaluated 48hrs following PT stroke. **d)** Effect of various post-stroke
874 administration time-points of NFPS treatment on stroke volume with their corresponding box and
875 whisker plots following PT stroke. **e)** Representative cresyl violet sections (25µm thick) 48hrs
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878 plot depicting infarct volume. **f)** The effect of 24hrs pre-stroke NFPS administration on post-stroke
879 time to contact and time to remove in the adhesive removal task compared with saline treatment
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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-

stroke time to contact and time to remove in the adhesive removal task compared with saline treatment, in mice infected with AAV-GluN1-WT **(d)**, and in mice infected with AAV-GluN1-A714L **(e)** 48hrs following PT stroke. Data is mean $\pm$ SEM; statistical significance $p < 0.05$ *, $p < 0.01$ **, 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 *CollIV* 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 ± SEM; statistical significance $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and $p < 0.0001$ ****.

Extended Data Figure 1

Extended Data Figure 1: Priming NMDARs with a high dose of glycine or D-serine allows glycine-induced NMDAR internalization to occur at a low dose of glycine or D-serine.

a) Mean time-course data showing the continuous application of high dose (1mM) D-serine followed by low dose (10 μ M) D-serine in WT mice on NMDAR-EPSCs and its corresponding bar graph. **b)** Same as a) in the presence of dynasore. **c)** Mean time-course data showing the continuous application of high dose (1mM) glycine followed by low dose (250 μ M) glycine in WT mice on NMDAR-EPSCs and its corresponding bar graph. **d)** Same as c) in the presence of dynasore. Data is mean $\pm$ SEM; statistical significance $p < 0.05$ *.

Extended Data Figure 2

Extended Data Figure 2: Glycine-induced NMDAR internalization is not subunit-specific, nor dependent on AMPA receptor activation, and this effect is not restricted to the hippocampus.

a) Mean time-course data showing the effect of 15mins application of 1mM, 5mM and 10μM of exogenous D-serine on NMDAR-EPSCs in WT and GluN2A^{-/-} mice. Note: The mouse strain with a different background (C57Bl/6) required a higher dose of D-serine to initiate NMDAR internalization, hence the 5mM D-serine application. NMDAR decay kinetics in WT and GluN2A^{-/-} mice was also measured to verify the lack of GluN2A subunit-containing NMDARs and this was made evident with the slower decay tau. **b–e)** NMDAR decay kinetics in WT, SR^{-/-}, and GlyT1^{+/-} mice following application of 1mM or 10μM glycine or D-serine. No differences in decay tau were seen in any of the conditions. **f)** Mean time-course data showing the effect of 15mins application of 1mM of exogenous glycine on AMPA receptor-EPSCs and corresponding bar graph; internalization was not observed. **g)** Mean time-course data showing the effect of 15mins application of 1mM and 10μM of exogenous glycine on NMDAR-EPSCs recorded from the cortex with its corresponding bar graph and normalized raw data traces. Data is mean ± SEM; statistical significance $p < 0.05$ *.

Extended Data Figure 3

Extended Data Figure 3: Glycine is co-localized with markers for glutamatergic terminals in the CA1 region of the hippocampus.

a–d) Confocal images of a hippocampal slice with triple immunohistological co-staining showing the localization of vesicular glutamate transporters 1 and 2 (**a**; VGLUTs1-2; green), vesicular inhibitory amino acid transporters (**b**; VGAT; red) and the neuronal cell body marker NeuN (**c**; blue), and the three-channel merged composite image (**d**). **e–h)** High magnification images of the asterisked region in panel **a**, showing segregation of VGLUTs1-2 to extrasomatic regions (**e**), and VGATs to somatic regions (**f**), separated by neuronal cell bodies (**g**; NeuN). Merged image shown in **h**. **i)** High magnification images of the CA1 region of a different slice co-stained with antibodies against VGLUTs 1-2 (green), glycine (red) and VGAT (blue). **j)** Zoomed image of the perisomatic region illustrated by the asterisk shown in **g**, illustrating the region of interest used for triple blob co-localization. **k)** Graph showing the extent of glycine blob colocalization to VGLUTs1-2, VGAT, and VGLUTs1-2 and VGAT together, accounting for ~3% of co-localization.

Extended Data Figure 4

Extended Data Figure 4: Glycine-induced NMDAR internalization is triggered during oxygen-glucose deprivation conditions and is blocked by dynamin and glycine oxidase.

a) The effect of oxygen-glucose deprivation (OGD) on P_{open} of homomeric $\alpha 2$ glycine receptors. **b)** Changes in P_{open} in the presence of OGD alone or 300 μ M cadmium, a non-specific Ca^{2+} channel blocker. The robust response to OGD was abolished suggesting a Ca^{2+} -dependent glycine release process. As a positive control, 35mM K^+ was applied to the slice to induce glycine release *via* neuronal depolarization and a comparable increase in P_{open} was observed. **c)** Raw traces showing the effect of OGD on NMDAR-EPSCs evoked at low-frequency and the normalized NMDAR-EPSC amplitudes during OGD in the presence of dynamin blocking peptide (DBP), which inhibits NMDAR internalization. The OGD paradigm induced a decrease in the amplitude of NMDAR-EPSCs during the single pulse stimulation paradigm. Application of DBP did not attenuate the effect of OGD. Therefore, the decreased NMDAR-EPSC amplitudes resulting from single pulse stimulation of the presynaptic terminal was not due to GINI **d–e)** The effect of OGD on NMDAR-EPSC amplitudes evoked by a 20Hz, 10 stimulus train, compared with control. When the amplitude of the last EPSC in the train (EPSC₁₀) was normalized to the first amplitude of the train (EPSC₁), the EPSC₁₀/EPSC₁ ratio, there was a significant smaller ratio during OGD than in control conditions, likely due to NMDAR internalization. **f–g)** The effect of OGD on EPSC₁₀/EPSC₁ ratio for trains elicited in OGD conditions in the presence of various internalization blockers, DBP, dynasore (dyna), and Dyn4a (Dyn4a), showing similar results to that observed in control conditions. **h)** The effect of purified glycine oxidase (GO; an enzyme that catalyzes the breakdown of glycine) with 1 μ M glycine on P_{open} of homomeric $\alpha 2$ glycine receptors expressed in outside-out patches obtained from CHO cells during OGD. **i–j)** The presence of GO abolished the combined

983 inhibitory effect of OGD and a 20Hz train on NMDAR-EPSC amplitudes. Data is mean $\pm$ SEM;
984 statistical significance $p < 0.05$ *.

Extended Data Figure 5

Extended Data Figure 5: Supplementary data of the effect of NFPS administration on cell death and behavioural tasks following photothrombosis and endothelin-1 strokes.

a) Cell death assay (Fluoro Jade C; green) on photothrombotic (PT) stroke following NFPS administration. **b)** Training curves for the adhesive task following PT stroke with NFPS. **c)** Cylinder task data following NFPS administration and PT stroke. **d)** Training curves for the adhesive task following endothelin-1 (ET-1) stroke with NFPS. **e)** Cylinder task data following NFPS administration and ET-1 stroke. **f–g)** The effect of pre-stroke NFPS administration on post-stroke deficits in the horizontal ladder test following ET-1 stroke; percentage of misses both pre- and post- ET-1 stroke for mice treated with saline or NFPS is depicted. Data is mean $\pm$ SEM; statistical significance $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and $p < 0.0001$ ****.

Extended Data Figure 6

Extended Data Figure 6: Control data for *in vivo* experiments.

a–c) Endothelin-1 injection induced stroke and behavioural deficits in the adhesive removal task, compared to sham stroke (saline injection). **d)** Effect of adeno-associated virus (AAV)-GluN1-WT and AAV-GluN1-A714L intracortical injection on post-stroke behavioural performance in the

1003 adhesive removal task. **e)** Temperature time-course experiment following photothrombotic stroke,
1004 showing no contribution of hypothermia on the therapeutic effect of NFPS. Data is mean $\pm$ SEM;
1005 statistical significance $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and $p < 0.0001$ ****.

Extended Data Figure 7

Extended Data Figure 7: Electrophysiological representation and live-cell imaging visualization of glycine-induced NMDAR internalization blockade following application of 1mM glycine.

a) Normalized NMDAR-EPSC amplitudes and normalized raw data traces recorded from GluN1-WT and GluN1-A714L transfected HEK293 cells with and without the NMDAR competitive antagonist, APV. **b)** Normalized NMDAR-EPSC amplitudes mean time-course data showing the effect of 1mM glycine application in GluN1-WT and GluN1-A714L transfected HEK293 cells. **c)** Visual representation of the internalization of NMDARs into the cell in GluN1-WT and GluN1-A714L transfected HEK293 cells. Following application of 1mM glycine, internalization of NMDARs was observed in the GluN1-WT transfected HEK293 cells but not in the GluN1-A714L transfected cells. Transfected NMDARs are labeled in green, while extracellular NMDARs are

1019 additionally labeled in red, with cell impermeable nanobody. **d)** Normalized NMDAR-EPSC
1020 amplitudes mean time-course data and bar graph showing the effect of 1mM glycine application
1021 on cortical slices in mice infected with either the AAV-GluN1-WT or AAV-GluN1-A714L
1022 constructs. Data is mean $\pm$ SEM; statistical significance $p < 0.05$ *.

Extended Data Figure 8

Extended Data Figure 8: 3D vascular imaging and viral infection visualization.

a) Various angles from 3D datasets acquired with the light sheet microscope demonstrating vasculature (red) and extent of viral infection (green) following injection of the adeno-associated virus GluN1-WT in the left hemisphere of a mouse having been induced by a photothrombotic stroke. **b)** Various angles from 3D datasets acquired with the light sheet microscope demonstrating vasculature in the left hemisphere of a mouse having been induced a photothrombotic stroke.

Extended Data Table 1

Primary Antibody	Source	Dilution
Glycine (rabbit)	Abcam	1:100
Synaptophysin (mouse)	Millipore Sigma	1:1000
Vesicular glutamate transporter 1 and 2 (VGLUT1 and VGLUT2; guinea pig)	Millipore Sigma	1:1000
Vesicular GABA transporter (VGAT; mouse)	Synaptic Systems	1:250
CD31 (rabbit)	Abcam	1:50
Collagen IV (rabbit)	Abcam	1:400
Neuronal nuclei (mouse)	Millipore Sigma	1:1000
Secondary Antibody	Source	Dilution
CY3 (donkey anti-rabbit)	Jackson ImmunoResearch	1:800
Alexa-647 (donkey anti-mouse)	Molecular Probes	1:200
FITC (goat anti-guinea pig)	Jackson ImmunoResearch	1:100

Extended Data Table 1. List of antibodies.

Extended Data Table 2

Primer	Sequence
NheIGluNI-F	5'-AGCTAGCATGAGCACCATGCACCTGCT-3'
EcoRVGluN1-R	5'-AGATATCTCAGCTCTCCCTATGACGGG-3'
A714L-F	5'P-ACAATTACGAGAGCCTGGCTGAGGCCATCCA-3'
A714L-R	5'P-GCTCTCGTAATTGTGTTTTCCATGTGCCG-3'

Extended Data Table 2. Primers used for GluN1 variants subcloning into pcDNA3.1. F indicates a forward primer, R indicates a reverse primer.

Figures

Figure 1

Increasing exogenous glycine and D-serine concentrations results in NMDAR internalization, which is dependent on Ca^{2+} 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

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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

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