Effects of intranasal guanosine administration on brain function in a rat model of ischemic stroke

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Abstract

Ischemic stroke is a major cause of morbidity and mortality worldwide and only few affected patients are able to receive treatment, especially in developing countries. Detailed pathophysiology of brain ischemia has been extensively studied in order to discover new treatments with a broad therapeutic window and that are accessible to patients worldwide. The nucleoside guanosine (Guo) has been shown to have neuroprotective effects in animal models of brain diseases, including ischemic stroke. In a rat model of focal permanent ischemia, systemic administration of Guo was effective only when administered immediately after stroke induction. In contrast, intranasal administration of Guo (In-Guo) was effective even when the first administration was 3 h after stroke induction. In order to validate the neuroprotective effect in this larger time window and to investigate In-Guo neuroprotection under global brain dysfunction induced by ischemia, we used the model of thermocoagulation of pial vessels in Wistar rats. In our study, we have found that In-Guo administered 3 h after stroke was capable of preventing ischemia-induced dysfunction, such as bilateral suppression and synchronicity of brain oscillations and ipsilateral cell death signaling, and increased permeability of the blood-brain barrier. In addition, In-Guo had a long-lasting effect on preventing ischemia-induced motor impairment. Our data reinforce In-Guo administration as a potential new treatment for brain ischemia with a more suitable therapeutic window.

Keywords Stroke · Guanosine Intranasal administration · Neuroprotection · Quantitative electroencephalogram · Blood-brain barrier · Cell signaling

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Introduction

Cerebrovascular diseases are a major cause of death and morbidity worldwide, and ischemic brain stroke is the most prevalent among them [1–3]. So far, there are two therapies available for ischemic stroke: the recombinant tissue plasminogen activator (rtPA) and the more recently approved endovascular reperfusion [4–8]. The rtPA is the standard therapy and it

Table 1 Sample size of cohorts. Sample sizes described by its respective group and methodology

Group	Methodology					
	Behavior short term*	Behavior long term	EGG	Evans blue	Albumin in CSF	Flow cytometry
Sham [§]	47	11	6	11	12	7
Sham Guo	42	8	7	12	10	5
IS [§]	54	12	11	11	12	8
IG	45	11	6	12	9	7
Total	188	42	30	46	43	27

*Sum of all other cohorts

[§] Higher number of animals used for methodology standardization

works by dissolving the arterial clot through plasminogen activation [4]. Its use is restricted, however, due to the increased risk of intracerebral hemorrhaging and a narrow therapeutic window [4–6]. Alternatively, endovascular therapy with mechanical thrombectomy has had outstanding results reported in recent trials and within expanded therapeutic window [7, 8]. However, the criteria for using this therapeutic approach are based on imaging and require a highly skilled multi-professional team and infrastructure, which are lacking in most health centers in developing countries [7, 9, 10]. Therefore, the search for effective and broadly accessible treatments for ischemic stroke is a priority [3].

The physiopathology of ischemic stroke has been extensively studied in order to find new therapeutic approaches. Hence, it is known that an ischemic insult starts with an arterial thromboembolic occlusion, leading to bioenergetic failure, which ultimately triggers a signaling phenomenon known as ischemic cascade [11–13]. The ischemic cascade involves unbalanced ionic homeostasis, inflammatory cascade, enhanced cell death, glutamatergic excitotoxicity, astrocyte reactivity, and blood-brain barrier (BBB) damage [11], among other events. This series of events results in the progression of

ischemic damage from the necrotic region, or “core,” to the “penumbra zone,” which surrounds the core and presents collateral perfusion [12]. The penumbra is the potentially salvageable tissue [12] and is the usual target for traditional and innovative therapeutic strategies [12, 14].

In the clinical scenario, electroencephalogram (EEG) is a very sensitive method that may be used to monitor progression of cerebral injuries induced by ischemic insult [15, 16]. The topographical distribution of brain rhythms is extensively disrupted in ischemia [17–19]. A pathological pattern starts with decreased voltage of faster frequencies and, under intense hypoperfusion, progressively evolves into suppression of all frequencies in EEG [17, 20, 21]. As brain oscillations change with cerebral blood flow (CBF) variations, the EEG can promptly detect signs of cerebral cortex reperfusion during stroke therapy [18, 22–24].

Guanosine (Guo) prevents ischemic damage when administered immediately after permanent ischemia induction [25, 26]. Interestingly, intranasal administration of Guo (In-Guo) improves the neuroprotective potential of Guo in an ischemia model by expanding its therapeutic window [27]. In-Guo treatment was effective in preventing behavioral deficit and

Fig. 1 Forelimb symmetry score measured by cylinder test. **a** Symmetry score 48 h after focal ischemia induction, aggregating rats used in all further protocols of this work (42 to 54 animals per group **** $p < 0.001$ vs Sham and Sham Guo, ### $p < 0.005$ vs. IS by Kruskal-Wallis,

corrected by Dunn's multiple comparisons. **b** Timeline of symmetry score measured at each time point, **** $p < 0.001$ vs Sham and Sham Guo, ## $p < 0.01$ vs IS in the 42nd day after stroke induction, by two-way ANOVA (8 to 12 animals per group)

Fig. 2 Power spectral density (PSD) at electrode located on contralateral cortex unrelated to the ischemic lesion (E1). Representative PSD ($\mu\text{V}/\text{Hz}$) for each group: **a** 0 to 30 Hz and **b** 30 to 100 Hz. Plots of PSD of **c** delta, **d** theta, **e** beta, **f** slow gamma, and **g** middle gamma. **h** Electrode position:

black square, ischemic area; electrode 1 - a contralateral region unrelated to the ischemic lesion. No statistically significant differences were detected in all analyses of all groups by one-way ANOVA (6 to 11 animals per group)

Fig. 3 Power spectral density (PSD) at electrode located on ipsilateral peri-infarct cortex (E2). Representative PSD ($\mu\text{V}/\text{Hz}$) for each group: **a** 0 to 30 Hz and **b** 30 to 100 Hz. Plots of PSD of **c** delta, **d** theta, **e** beta, **f** slow gamma, and **g** middle gamma. **h** Electrode position: black square, ischemic area; electrode 2, ipsilateral peri-infarct cortex. All comparisons $*p < 0.05$ and $**p < 0.01$ vs Sham, $^{\&}p < 0.05$ vs Sham and Sham Guo, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs IS by one-way ANOVA, corrected by Tukey multiple comparisons or by Kruskal-Wallis, corrected by Dunn's multiple comparisons (6 to 11 animals per group)

brain damage when administrated up to 3 h after stroke [27] involving (i) modulation of astrocyte functions, by modulating the glutamatergic system (decreasing the glutamatergic excitotoxicity) [28–30]; (ii) modulating the adenosinergic system [31, 32]; (iii) repercussions on inflammatory cascade and on oxidative stress [26, 32]; and (iv) modulation of electrophysiological parameters [33–36]. The involvement of kinase pathways on these Guo effects has been also demonstrated [37, 38]. However, despite the evidence of all these effects by Guo, its neuroprotective mechanism is not completely clear.

Here, we introduce important evidence for the therapeutic approach of the neuroprotective potential of In-Guo in a stroke model. Firstly, we show that In-Guo presented a long-lasting protective effect, as behavioral improvement persisted up to 42 days after a stroke insult induced by thermocoagulation of the pial vessels. Moreover, we demonstrate that the neuroprotective effects of In-Guo were not restricted to the area around the core of the ischemic lesion. In fact, In-Guo treatment prevented ischemia-induced low-voltage EEG patterns in the peri-infarct area of both ipsilateral and contralateral hemispheres. The treatment also prevented the disruption of the BBB, and at the cellular level, it reduced pro-apoptotic signaling in the ipsilateral hemisphere.

Methodology

Materials/chemicals

Guanosine (Guo) and Evans blue were purchased from Sigma (St. Louis, MO, USA). The anesthetic ketamine hydrochloride and xylazine were obtained from Syntec Brazil (Cotia, SP, Brazil). All other chemicals were of analytical grade obtained from standard suppliers.

Animals

Male adult Wistar rats ($n = 188$; 90–120 days, weighing 350–450g, specified per protocol on Table 1), were obtained from the animal facility house of the Department of Biochemistry, ICBS, UFRGS, and from the University Animal Experimental and Reproduction Center (CREAL, UFRGS). Animals were kept under a 12-h light/dark cycle (light on at 7:00 am) at a temperature of $22 \pm 1^\circ\text{C}$. They were housed in plastic cages (4

or 5 per cage) with water and commercial food available ad libitum. No adverse effects were observed, and maximum care for reducing pain and distress of each animal was taken. The study design and procedures were reported according to the Animals in Research: Reporting in Vivo Experiments (ARRIVE) guidelines [39]. All protocols were in compliance with the approval of the Institutional Ethics Committee on Animal Use from our institution (CEUA-UFRGS-29028).

Induction of permanent focal ischemia

Ischemia was induced by thermocoagulation of the pial vessels at motor and sensorimotor cortices, as previously described [40, 41]. Briefly, rats were anesthetized by intraperitoneal administration of both ketamine hydrochloride (50 mg/kg) and methyl xylazine (10 mg/kg) and then placed on a stereotactic apparatus. Animals in the Sham groups were only exposed to craniotomy at the region of the frontoparietal cortex (+3 to –6 A.P and +2 L.L), while animals in the ischemia groups were exposed to thermocoagulation by close proximity to hot probe to the pial vessels.

Treatment with intranasal guanosine after ischemia induction

Before the procedure, the animals were randomly allocated into four groups: Sham; Sham Guo; ischemic saline (IS); and Ischemic guanosine (IG). During all performed analyses, blind experiments were conducted.

Based on a previous study [27], treatment consisted of three intranasal administrations of either Guo or saline solution at 3, 5, and 8 h after surgery. The animals were anesthetized with ketamine (25 mg/kg) and xylazine (5 mg/kg) before each administration [27]. Fifty microliters per nostril of saline solution 0.9% or Guo at 30 mg/ml diluted in saline was administered at the bottom of the nasal septae using a long edge pipette tip [27].

Forepaw symmetry evaluation (cylinder test)

As previously determined [40], the cylinder test is the most sensitive test for detecting motor impairment observed after ischemia induction in this experimental model. Cylinder test is performed by placing the animal in a glass cylinder (20 cm diameter and 30 cm high), and an experimenter counts the first 20 forepaw contacts with the cylinder walls. Each contact using either exclusively right or left forepaw, as well as both simultaneously, was recorded and further used to calculate a symmetry score by the Vasconcelos et al. method [40]. This task was performed with each animal before surgery (to exclude pre-surgery asymmetric rats), at the 48th hour and consecutively each 7 days until the 42nd day after stroke induction.

Fig. 4 Power spectral density (PSD) at electrode located on contra-lateral peri-infarct cortex (E3). Representative PSD ($\mu\text{V}/\text{Hz}$) for each group: **a** 0 to 30 Hz and **b** 30 to 100 Hz. Plots of quantitative PSD of **c** delta, **d** theta, **e** beta, **f** slow gamma, and **g** middle gamma. **h** Electrode position: black square, ischemic area; electrode 3, contra-lateral peri-infarct cortex. All comparisons $*p < 0.05$ and $**p < 0.01$ vs Sham, $^{\#}p < 0.05$ vs Sham and Sham Guo, $^{\$}p < 0.05$ vs IG, $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ vs IS by one-way ANOVA, corrected by Tukey multiple comparisons by Kruskal-Wallis, corrected by Dunn's multiple comparisons (6 to 11 animals per group)

Fig. 5 Correlation plots of qEEG. Abbreviations used for each oscillation PSD: DE1 delta electrode 1, TE1 theta E1, BE1 beta E1, SG E1 slow gamma E1, MG middle gamma E1, DE2 delta electrode 2, TE2 theta E2, BE2 beta E2, SGE2 slow gamma E2, MGE2 middle gamma E2, DE3 delta electrode 3, TE3 theta E3, BE3 beta E3, SGE3 slow gamma E3, MGE3 middle gamma E3. **a–d** Correlation plots per group of PSD of all analyzed oscillations at 3 areas for all groups. Horizontal and vertical labels represent each oscillation followed by respective electrode (E1, E2, and E3). Correlation coefficients are expressed by colors according to its color scale, represented from blue (negative/inverse correlation) to

In vivo electrophysiology

Since brain ischemia has a significant impact on brain electrical activity and quantitative analysis of the EEG (qEEG) is used to monitor and to predict long-term stroke progress [16, 22], we performed the qEEG on all groups.

red (positive/direct correlation). **a** Sham correlation plot, **b** Sham Guo correlation plot, **c** IS correlation plot, and **d** IG correlation plot. Significant correlations (p -adjusted < 0.05) are displayed with asterisk (*) in the corresponding square. **e–h** Network analysis per group of brain oscillation PSD at 3 different areas for all groups. Each oscillation PSD and the electrode location (E1, E2, and E3) are described in the respective nodes. Each tie represents a correlation coefficient higher than 0.8 between nodes, and the tie width is proportional to correlation coefficient among both nodes (6 to 11 animals per group). **e** Sham network, **f** Sham Guo network, **g** IS network, and **h** IG network

Fig. 6 Network analysis of q EEG. Abbreviations used for each oscillation PSD: DE1 delta electrode 1, TE1 theta E1, BE1 beta E1, SG E1 slow gamma E1, MG middle gamma E1, DE2 delta electrode 2, TE2 theta E2, BE2 beta E2, SGE2 slow gamma E2, MGE2 middle gamma E2, DE3 delta electrode 3, TE3 theta E3, BE3 beta E3, SGE3 slow gamma E3, MGE3 middle gamma E3. **e–h** Network analysis per group of brain

oscillation PSD at 3 different areas for all groups. Each oscillation PSD and the electrode location (E1, E2, and E3) are described in the respective nodes. Each tie represents a correlation coefficient higher than 0.8 between nodes, and the tie width is proportional to correlation coefficient among both nodes (6 to 11 animals per group). **e** Sham network, **f** Sham Guo network, **g** IS network, and **h** IG network

Electrode implantation

Immediately after the induction of permanent focal ischemia, all rats allocated for this protocol were maintained on the apparatus for implanting four stainless steel subdural electrodes. These electrodes were implanted at following areas (respective coordinates): contralateral unrelated to the ischemic lesion (E1, A.P—1.88, L.L—3.90); ipsilateral peri-infarct cortex (E2, A.P—7.0, L.L + 2.0); contralateral peri-infarct cortex (E3, A.P—1.88, L.L—1.10) [42]; a reference electrode was positioned above the bregma line between sagittal

sutures. Electrodes were fixed with a small screw (also used for grounding) in the occipital bone and with a dental acrylic helmet on the skull.

Quantitative electroencephalogram and data processing

After forepaw symmetry evaluation, animals were immediately transferred to the in vivo electrophysiology room. Acquisition of video-EEG was made through an amplification and recording system (MAP-32, Plexon Inc).

Video-EEG was recorded for approximately 18 min and EEG signals were filtered through 0.1 to 500 Hz band and

Fig. 7 Evaluation of BBB permeability. **a** Representative slices of Evans blue staining for each group. **b** Quantification of blue-covered area. **c** Correlation (each dot as an animal, 11–12 animals per group) of symmetry score with blue-stained area ($R^2 = 0.26$, $p < 0.005$). **d** Albumin CSF levels and **e** correlation (each dot as an animal, 9 to 12 animals per group) of symmetry score with albumin CSF levels (spearman $Rho = -0.22$, $p < 0.05$). To all comparisons: $*p < 0.01$ and $***p < 0.005$ vs Sham and Sham Guo, $##p < 0.01$ and $###p < 0.005$ vs IS. In **c** and **e**, the line represents overall Pearson R between both parameters.

Fig. 8 Flow Cytometry of Signaling Pathways in the peri-infarct area of the Ipsilateral Cortex. **a** Ratio of pPI3k/PI3k-positive cells. **b** pPI3k/PI3kMFI ratio. **c** Dot plot correlating symmetry score and pPI3k/PI3k-positive cells ($R^2 = 0.46$, $p = 0.0001$). **d** Cleaved Caspase 3/Caspase 3 ratio of positive cells. **e** Cleaved Caspase 3/Caspase 3 MFI ratio. **f** Dot plot correlating symmetry score Cleaved Caspase 3/Caspase 3 MFI ratio

($R^2 = 0.44$, $p = 0.002$). In **c** and **f**, the line represents overall Pearson R . The following symbols represent $*p < 0.05$, $***p < 0.005$, and $SSS p < 0.005$ statistically different from Sham and IG and $#p < 0.05$ and $###p < 0.005$ statistically different from IS; all comparisons made by one-way ANOVA and corrected by Tukey multiple comparisons (5 to 8 animals per group)

digitalized at 1 kHz. Video and EEG were evaluated simultaneously. To perform the qEEG, an interval of 60 s was selected during awake immobility state. We used custom-written and built-in routines in MATLAB (version 2016a, MathWorks Inc.) to perform the analyses. Power spectral density (PSD) was computed using the *pwelch.m* function (Signal Processing Toolbox). The frequency bands were separated into delta (1–4 Hz), theta (5–8 Hz), beta (13–30 Hz), slow gamma (20–50 Hz), and middle gamma (60–100 Hz). The animals used in the EEG experiments were not used for biochemical parameter analyses and were euthanized by exsanguination through cardiac puncture after the experiment.

Evaluation of blood-brain barrier permeability

Aiming to identify if our stroke model, and In-Guo administration, could have effects on the BBB, we evaluated its permeability/leakage with the following methodologies: Evans blue (EB) staining and measurement of cerebrospinal fluid (CSF) albumin levels.

Evans blue

Immediately after the cylinder test, EB (2% dissolved in saline solution, 3 ml/kg) was administered in the tail vein [43]. After 90 min of EB administration, animals were anesthetized and then euthanized by exsanguination through cardiac puncture [27]. The brains were removed and weighed. Each sample was frozen and sliced with a blade (thickness of 2 mm) guided by a ruled sheet of rat brain matrix (Insight LTDA, Ribeirão Preto, SP, Brazil) [43, 44]. Afterwards, slices were photographed and the EB stained area was calculated using ImageJ software (NIH, Bethesda, MD, USA).

Quantification of albumin concentration in cerebrospinal fluid

Immediately after the cylinder test, animals were anesthetized by inhaled isoflurane and placed in a stereotaxic apparatus. CSF samples were collected by direct puncture of the cisterna magna with an insulin syringe (27 gauge $\times$ 1/2 in. length) [31] and immediately put on ice and centrifuged at 10,000 $\times$ g at 4 °C for 5 min; the supernatant was collected and then stored at – 80 °C [31]. Samples contaminated with blood were excluded from the study. In accordance with Durgawale et al. [45], 50 μ l of CSF sample was mixed with 500 μ l of reagent from the bromocresol green commercial kit (Doses, GO, Brazil) [46]. To enhance sensitivity, the mixture was incubated for 10 min at 37 °C. Absorbance was measured at 546 nm using a spectrophotometer [45, 46]. A standard curve with pre-established albumin concentrations was used to determine concentration in mg/ml.

Flow cytometry

We performed flow cytometry to investigate putative alterations caused by ischemia in PI3K (pro-survival) and Caspase (pro-apoptotic) signaling pathways in the ipsilateral peri-infarct area and the potential neuroprotective effects of Guo.

The rats were euthanized 48 h after ischemia, immediately after forepaw symmetry evaluation, by exsanguination. Animals were anesthetized and perfused by cardiac puncture with saline solution. Brain tissue samples were obtained from the peri-infarct area of the ipsilateral cortex, based on Hansel et al.'s protocol [26, 27]. Samples were incubated in 1 ml of phosphate-buffered saline (PBS 0.1 M, pH 7.4) containing 0.01 mg/ml collagenase IV for 10 min and then mechanically dissociated. After dissociation and in every washing process with PBS, homogenates were centrifuged at 1000 $\times$ g for 10 min at 4 °C, and the pelleted cells were washed twice with PBS with the same centrifugation protocol [26].

Cells were permeabilized with Triton X-100 0.1% for 10 min and then blocked with bovine serum albumin 5% solution for 15 min. After blocking, samples from the ipsilateral peri-infarct area were incubated for 1 h in the same solution containing the following antibodies at a proportion of 1 to 200: anti-Caspase 3, anti-Cleaved Caspase 3, anti-PI3k, and anti-phosphorylated PI3k (all antibodies are from Cell Signaling Inc.).

After incubation, all samples were washed with PBS twice and incubated again with blocking solution containing AlexaFluor 488 anti-Rabbit and AlexaFluor 635 anti-Mouse in a proportion of 1 to 500 (Jackson ImmunoResearch Laboratories, Inc., PA, USA). Finally, samples were washed twice, and the pellet was resuspended in PBS.

The number of positive cells for each antibody labeling was determined by a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA), considering size (SSC (side scatter), 6 to 30 micrometers) and granularity (FSC (forward scatter)). Emission of fluorochromes was registered to specific channels by their respective wavelengths using software CellQuest Pro (Becton Dickinson, Franklin Lakes, NJ, and USA). All data were collected using FL1 (excitation laser 488, filter 530/30) and FL4 (excitation laser 635, filter 661/16) channels and because of that, no compensation was applied. Using software FlowJo 7.6.3 (Tree Star, Ashland, OR, USA), data from 10,000 events were analyzed by density dot plots. We created gates considered negative for each protein using cells labeled only with the secondary antibody, using logarithmic scale dot plots. Events labeled in FL1 or FL4 with higher fluorescence than the cells labeled only with the secondary antibody were considered positive. Data were expressed by percentage of positive cells and by the mean fluorescence intensity (MFI) that was correlated with the protein content in these positive cells.

To evaluate proteins related to apoptotic and survival signaling in the peri-infarct area of the ipsilateral hemisphere, the active forms (Cleaved Caspase 3 and phosphorylated PI3k) were normalized by the total content of Caspase 3 and PI3K, respectively.

Statistics

To estimate animal sample size for experiments, the primary outcome of forepaw symmetry was used considering 4 groups, with 7 animals per group, with 80% power, and an alpha of 0.05, without estimating losses. We used Minitab 22.0 software for this assessment, using variances described by earlier published papers as parameters [27]. Animals from the ischemic saline group with a forepaw symmetry score above 30% were excluded from flow cytometry, EEG, and BBB permeability analyses.

Using GraphPad Prism 6.0 software (GraphPad Software, La Jolla California USA), D'Agostino-Pearson Normality test was conducted to determine the normality of groups. Analyses of parametric variables were made by one-way analysis of variance (ANOVA) test, corrected for multiple comparisons with Tukey post hoc. Analyses of non-parametric parameters were made by Kruskal-Wallis tests and corrected by Dunn's test. To make correlations between parameters, Pearson and Spearman correlations to parametric and non-parametric variables were employed, respectively, and multiple comparisons were corrected by the Holm-Bonferroni method in the correlation plots. All differences were considered statistically significant when the p value was less than 0.05.

To perform correlation plots and network analyses, we used R and R studio version 3.6.2 (R, R Foundation for Statistical Computing, Vienna, Austria) with packages "corrplot," "igraph," and "Hmisc". In network analysis, we used a Spearman correlation coefficient above 0.8 to form edges between nodes.

Results

Symmetry score measured by cylinder test

In accordance with our previous data [27], no motor deficit was detected in Sham groups. Measured at 48 h after ischemia (Fig. 1a), the IS group presented a significant reduction of the symmetry score ($p < 0.0001$, when compared to both Sham groups), which was improved by In-Guo administration (IG group, $p < 0.005$ vs IS, Sham and Sham Guo groups).

The follow-up evaluations, made until the 42nd day after ischemia induction, showed a long-lasting reduction in the symmetry score in the IS group ($p < 0.0001$, when compared to both Sham groups); in the IG group, we observed a gradual improvement in the score ($p < 0.01$, IG vs IS on overall curve),

reaching total recovery from motor deficit on the 42nd day ($p < 0.05$ vs IS, $p > 0.05$ vs the Sham and SG group) (Fig. 1b).

EEG power spectral analysis

We used qEEG as a tool to evaluate brain electrical activity and In-Guo treatment 48 h after ischemia induction. Accordingly, we analyzed a contralateral region unrelated to ischemia (E1, Fig. 2), the ipsilateral peri-infarct area (E2, Fig. 3) and the peri-infarct area (E3, Fig. 4).

The power spectral density (PSD) analysis of EEG, recorded from a contralateral brain region unrelated to the ischemic lesion (E1), showed no significant differences among all groups in all analyzed frequencies (Suppl. Table 1 and Fig. 2).

In contrast, in the peri-infarct area zone of the ipsilateral cortex (E2), IS animals presented a reduction in all PSD oscillations (delta, theta, beta, slow gamma and middle gamma), when compared to the Sham group ($p < 0.05$). In-Guo treatment in ischemic animals (IG group) prevented the reduction of all PSD oscillations compared to the IS group ($p < 0.05$, IG vs IS) (Suppl. Table 1 and Fig. 3).

Furthermore, in the peri-infarct area of the contralateral hemisphere (E3), IS animals presented a reduction in PSD oscillations of theta, beta, slow gamma, and middle gamma, when compared to the Sham group ($p < 0.05$). In-Guo treatment in ischemic animals (IG group) prevented the reduction of all affected PSD oscillations compared to the IS group ($p < 0.05$) (Suppl. Table 1 and Fig. 4). Noteworthy, in theta PSD, we observed an increase of IG in comparison with the Sham Guo group ($p < 0.05$) (Suppl. Table 1 and Fig. 4).

We also performed correlation analyses (Suppl. Fig. 1) to evaluate association between the forepaw symmetry score and the PSD oscillations in all brain regions analyzed (E1, E2, and E3). We observed that the symmetry score increased with the increase in PSD of theta, beta, slow gamma, and middle gamma oscillations in all analyzed areas. The significant positive correlations ($p < 0.05$) suggest that the improvement in behavioral performance (forepaw symmetry) was associated with higher PSD oscillations in both hemispheres (Suppl. Fig. 1d-o).

Correlations of PSD oscillations at each electrode and between electrodes were performed to evaluate synchrony between the analyzed regions. Areas with hyperexcitability in the brain, such as those caused by brain injury, present hypersynchrony [47–49]. Indeed, the IS group (Fig. 5c) presented a greater number of significant positive correlations of PSD oscillations compared to the Sham (Fig. 5a) and Sham Guo groups (Fig. 5b), suggesting that ischemia induced synchronicity and thus global dysfunction. The IG group (ischemic group treated with In-Guo) (Fig. 5d) had less significant positive correlations of PSD oscillations. These positive correlations between PSD oscillations indicate an increase in synchronicity. Therefore, In-Guo partially prevented the state of synchronicity induced by ischemia.

To better evaluate the brain synchronicity, we performed network analysis with the qEEG data. In a network analysis, a node represents a PSD parameter and edges represent a correlation coefficient above 0.8. Thus, a network with a larger number of connections (higher density) and with between nodes of both hemispheres represents higher synchronicity and thus global dysfunction. The network analysis showed that the Sham group (Fig. 6e) and Sham Guo group (Fig. 6f) presented a network pattern with lower density compared to the IS group (Fig. 6g). Therefore, the correlation coefficients among the PSD oscillations in all analyzed electrodes were similar to each other and higher than the Sham and Sham Guo groups. This suggests that both hemispheres of the IS (Fig. 6g) group were altered with increased synchronous activity. On the other hand, the IG group (Fig. 6h) presented a network pattern similar to Sham Guo (Fig. 6f), with an absence of ties between PSD oscillations in E1 (unrelated to the ischemic lesion) and E2 (ipsilateral peri-infarct area), suggesting a decreased stroke induced-synchrony in the In-Guo treated group. This reinforces the effect Guo on the area unrelated to ischemia.

Evaluation of BBB permeability

The CSF albumin content and Evans blue staining were measured to evaluate BBB dysfunction.

The blue-stained area in both Sham and Sham Guo groups (Fig. 7a and b) was significantly smaller than in the IS group ($p < 0.005$, IS vs Sham and Sham Guo). In-Guo treatment abolished this difference ($p < 0.005$, IG vs IS). Accordingly, there was a significant inverse correlation between the forepaw symmetry and the Evans blue stained area (Fig. 7c, $R^2 = 0.26$, $p < 0.005$).

The ischemic insult caused a significant increase in the CSF albumin content when compared to both Sham groups ($p < 0.005$, IS vs Sham and Sham Guo) (Fig. 7d). In-Guo administration prevented this increase ($p < 0.01$, IS vs IG), presenting values similar to Sham groups (Fig. 7d). In addition, a significant, but weak, correlation between albumin content and symmetry score was observed (Fig. 7e, Spearman $Rho = -0.22$, $p < 0.05$).

Flow cytometry

In ipsilateral peri-infarct area samples, the ratio of active isoforms was calculated to evaluate pro-apoptotic (Cleaved Caspase 3/total Caspase 3) and pro-survival (pPI3k/PI3k) signaling. The ischemic insult decreased the pPI3k/PI3k ratio of positive cells ($p < 0.05$, Fig. 8a) and increased the ratio of Cleaved Caspase 3 MFI/total Caspase 3 MFI ($p < 0.005$, Fig. 8e) compared to the Sham group. Both effects were abolished by In-Guo treatment, indicating a protective effect of Guo in our

ischemia model. Interestingly, Guo also increased the ratio in the Sham Guo group ($p < 0.001$) (Fig. 8a). However, there was no statistical difference in the content ratio of active PI3k between groups (Fig. 8b).

The IS group exhibited a statistically higher content ratio of Cleaved and total Caspase 3 when compared to Sham groups ($p < 0.001$), an effect prevented by In-Guo ($p < 0.001$) (Fig. 8e). Nevertheless, no statistical difference was detected in the percentage of positive cells for Cleaved Caspase 3/Caspase 3 for all groups (Fig. 8d).

The reduction in the active forms of positive cells of PI3k (Fig. 8a) and the enhanced expression of Cleaved Caspase 3 (Fig. 8e) suggest that our ischemic model induced cell death and In-Guo treatment prevented this apoptotic state. To further explore this hypothesis, correlations between these parameters and the symmetry score were made. Accordingly, forepaw symmetry and the ratio of positive cells of phosphorylated and total PI3k were positively correlated (Fig. 7c, $R^2 = 0.46$, $p < 0.005$). Also, the content ratio of active and total Caspase 3 was negatively correlated with the symmetry score (Fig. 7f, $R^2 = 0.44$, $p < 0.0005$).

Discussion

Here, we show that early In-Guo treatment promoted a neuroprotective effect that persisted up to 42 days after injury. Our present results validate previous data for a therapeutic window of 3 h for In-Guo treatment [27]. Additionally, we demonstrated that acute In-Guo treatment did not cause potentially harmful effects to Sham animals. In-Guo partially prevented short-term motor deficits induced by ischemia and accelerated the long-lasting recovery, in such way that the animals presented normal forepaw symmetry from the 35th day after ischemic injury.

Our electrophysiological data showed that the injury altered brain oscillations recorded by EEG. This methodology can be used in certain contexts to evaluate the outcomes and management of patients with stroke [19, 50, 51]. Indeed, routine EEG detects changes in brain oscillations associated to altered CBF and cellular metabolism observed in ischemic brain [17, 21]. Interestingly, quantitative EEG (qEEG) analysis has been able to detect similar and subtle changes even more effectively (as fast as 28 to 104 s after ischemic injury) [24]. In this way, a decrease in brain oscillation amplitude was demonstrated in the ipsilateral hemisphere at the lesion [21] that gradually recovered after 48 to 72 h following middle cerebral artery occlusion in rats [21]. In patients, the qEEG analysis is shown to be related with the severity of stroke and to the response to treatment [23]. Changes in delta and theta and faster oscillations were previously described during ischemia [15, 17, 20, 22, 23, 52].

In our study, the qEEG showed a decrease in the power spectral density (PSD) for delta, theta, beta, and gamma oscillations in the peri-infarct area of the ipsilateral cortex (E2) and most of the oscillations in the peri-infarct area of contralateral cortex (E3), measured at 48 h after ischemia induction. Similar results were found in the presence of severe hypoperfusion, i.e., an EEG suppression pattern of all frequencies [18]. Therefore, this synchronic decrease in power density of brain oscillations (global EEG suppression) is probably associated with the decrease in CBF and with brain edema, which is closely linked to early BBB disruption [53, 54].

Heatmap and network analyses were made to evaluate correlations between qEEG parameters and, thus, their associations with increased synchronicity in EEGs, i.e., characteristic of hyperexcitability [47, 55]. In both analyses, the correlation coefficients among the PSD in all three electrodes for the IS group were similar to each other and higher than the Sham group. This implies that both hemispheres were altered, thus presenting increased synchronous activity in the IS group and pointing to a globally dysfunctional brain. Interestingly, similar patterns of increased synchronous activity were found in animal models and patients with strokes and seizures at various moments [56]. In this study, the effect of In-Guo administration on brain oscillations during ischemia was associated with a decrease in synchrony, suggesting an improvement in global brain functionality apparently caused by a modulation of ipsilateral and contralateral peri-infarct area and in those unrelated to the ischemic lesion (as E1). Since it has been shown that abnormal cortical oscillations affect behavioral parameters, the maintenance of brain rhythms similar to the normal/Sham pattern in both hemispheres seems to contribute to the better and long-lasting behavioral outcome of ischemic animals treated with Guo [33–35].

Recent publications have shown that some abnormal EEG patterns, especially when presenting reduced EEG activity, may be associated with BBB impairment [54, 57–59]. Preventing BBB damage in stroke promotes better outcomes in patients [60, 61] and also reduces harmful rtPA effects on the BBB [11], especially the increased risk of hemorrhagic transformation [62–64]. Accordingly, randomized clinical trials using drugs to prevent BBB dysfunction in stroke have shown improvement in outcomes, even in patients treated with rtPA or subjected to mechanical thrombectomy [60, 61]. Our present results support this proposition. We detected that In-Guo administration prevented the impairment of BBB integrity, both in tissue and in CSF, 48 h after ischemic surgery. These data suggest a global effect of Guo on preventing the spread of harmful signaling factors from the peri-infarct area to the whole brain. Since BBB disruption is involved in many neuropathologies [11, 65–67] and Guo presented a general neuroprotective effect without a clear mechanism [32], we hypothesize that Guo acts by contributing, at least partially, to maintain BBB integrity.

It has been shown that PI3k/Caspase 3 pathway activities modulate BBB stabilization [68, 69], especially in models of stroke, hypoxia-ischemia, and intracerebral hemorrhage [70–73], though the mechanisms are not fully understood. Previous data show active Caspase 3 regulating other functions than cell death [72, 74] and influencing disorganization of BBB components [69, 74, 75]. Our data in the ipsilateral peri-infarct area indicated that our ischemia model simultaneously decreased the phosphorylated pPI3k/PI3k-positive cells and increased the Cleaved Caspase 3/Caspase 3 ratio, suggesting an enhancement of apoptotic processes, which may result in mitochondrial damage and ultimately apoptosis [76]. In-Guo treatment enhanced PI3k phosphorylation even in non-pathological scenarios, such as in the Sham-Guo group, which was a finding similar to previous *in vitro* results [75]. Therefore, In-Guo treatment prevented the effects of ischemia on PI3k phosphorylation and on activation of Caspase 3, and both effects were correlated with the improvement of the symmetry score.

Limitations on our study included that one should keep in mind that ketamine may have neuroprotective effects [77]. Nevertheless, previous work from our group and others demonstrated the suitability of ketamine in anesthesia in this animal model of stroke [40, 41] whereas in this study and other studies from our group [25–27], all animals were exposed to same anesthetic setting.

In summary, we demonstrated that In-Guo administration presented a long-term neuroprotective effect that could be related to the short-term prevention of the impairments to brain functionality. Interestingly, the EEG, BBB, and the survival/apoptotic cellular pathway data demonstrated that Guo did not act only in the ipsilateral peri-infarct area but also on brain regions far from the lesion core. Taken altogether, we hope to move a step further in order to consolidate the neuroprotective effectiveness of Guo against stroke.

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Author's contribution All authors contributed to material preparation and data collection. Study conception and design and data analysis were performed by Gabriel Cardozo Müller, Samanta Oliveira Loureiro, Mayara Vendramin Pasquetti, Marcelo Ganzella, Letícia Pettenuzzo, Maria Elisa Calcagnotto, and Diogo Souza. The first draft of the manuscript was written by Gabriel Cardozo Müller and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript and approved its publication.

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Data availability Data will be made available if requested to corresponding authors or to the first author.

Declarations

Ethical approval of animal use All experiments were performed following the rules and with approval of the Institutional Ethics Committee on Animal Use, CEUA-UFRGS, Project No. 29028. These rules were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Conflict of interest The authors declare no conflicts of interest.

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