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Efeitos Globais da Neuroproteção pela Administração Intranasal de Guanosina em um Modelo de Isquemia Cerebral Focal Permanente.

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“*There are more things in heaven* and earth, Horatio,
Than *are* dreamt of in your philosophy”
Shakespeare, W. *Hamlet*

Abreviações

A1 – Receptor de Adenosina 1

A2A – Receptor de Adenosina A2A

ACM – Artéria Cerebral Média

AKT/ PkB – Proteína Cinase B

ADP – 5´-Adenosina Difosfato

ATP – 5´-Adenosina Trifosfato

AVC – Acidente vascular cerebral

AVCh – Acidente vascular cerebral Hemorrágico

AVCi– Acidente vascular cerebral Isquêmico

BHE – Barreira Hematoencefálica

COX-2 – Ciclooxigenase 2

EEG – Eletroencefalograma

FLAIR – *Fluid-Attenuated Inversion Recovery*

Guo – Guanosina

GFAP – Proteína Ácida Fibrilar Glial

GSk3β – Cinase Glicogênio-Sintetase 3 Beta

H&E – Hematoxilina e Eosina

HIF-1α – Fator induzido por Hipóxia

In-Guo – Administração Intranasal de Guanosina

iNOS – Óxido Nítrico Sintase Induzida

I.N. – Administração Intranasal

I.P. – Administração Intraperitoneal

iNOS– Óxido-nítrico sintase induzível

PI3k – Cinase Fosfaditilinositol-3

PTEN – Fosfatase e Tensina Homóloga

MAPk – Cinase Ativada por Mitogênio

mTOR – *Mammalian Target of Rapamycin*

MMP-2 – Metaloproteinase-2

MMP-9 – Metaloproteinase-9

NMDA – N-metil-D-aspartato

OGD – Privaçãode Oxigênio e Glicose

PBS – Solução Tampão de Fosfato

PSD – Densidade de Potência Espectral

PI – Iodeto de Propídio

ROS – Radicais Livres de Oxigênio

rt-PA – Ativador de Plasminogênio Tecidual Recombinante

SOD – Superóxido dismutase

SNC – Sistema Nervoso Central

TBARS – Substâncias Reativas ao Ácido Tiobarbitúrico

TC – Tomografia Computadorizada

qEEG – Eletroencefalograma quantitativo

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Resumo

Guanosina (Guo) é um nucleosídeo com diversos efeitos neuroprotetores que apresentou resultados promissores, em modelos animais, para patologias como: crises epiléticas, sepse, encefalopatia hepática e doenças psiquiátricas. Resultados relevantes também foram achados nos modelos animais de isquemia cerebral focal transitória e permanente. Esses resultados, porém, ficaram limitados pela janela terapêutica, pois os efeitos, nos escores comportamentais observados, se manifestaram somente com a administração da Guo imediatamente após a indução de isquemia. Para aumentar a janela terapêutica, passou-se a considerar a administração pela via intranasal a partir da primeira dose administrada três horas após indução de isquemia no modelo de termocoagulação dos vasos piais; entre os resultados se destaca a melhora dos escores comportamentais a curto prazo. Este trabalho teve por objetivos avaliar parâmetros que pudessem reforçar os efeitos da via intranasal no modelo de termocoagulação utilizando-se de ratos Wistar machos adultos e estender a janela terapêutica por um prazo mais longo, levando em consideração outros parâmetros que também refletissem os efeitos neuroprotetores da Guo. A administração intranasal de Guo apresentou-se capaz de prevenir o déficit motor até mesmo com sua primeira dose administrada nove horas após a indução de isquemia, e seus efeitos mantidos por até 42 dias após a indução. Verificou-se, também, que o esquema terapêutico, com janela terapêutica de três horas, se mostrou eficaz na prevenção do déficit motor a curto e longo prazo ao apresentar efeitos

protetores relacionados ao EEG quantitativo, às vias de sinalização e à permeabilidade da barreira hematoencefálica.

Müller, GC. Global Effects of Neuroprotection by Intranasal Guanosine Administration in a Model of Focal Permanent

Abstract

Guanosine (Guo) is a nucleoside presenting several neuroprotective effects, with promising results on animal models of: seizures, sepsis, hepatic encephalopathy and psychiatric diseases. Further results also were shown on animal models of focal transient and permanent ischemia. However, these effects were restricted by its narrow therapeutic window, with its results on preventing motor deficit only when administered immediately after ischemia induction. In order to increase this therapeutical window, intranasal Guo administration (In-Guo) was evaluated on a ischemic stroke model, presenting partial damage prevention with administration up to three hours after ischemia induction by thermocoagulation of pial vessels using Wistar rats. In this actual work, the objective was to evaluate parameters reinforcing evidence on In-Guo neuroprotective effects after ischemic damage, using thermocoagulation of pial vessels, on a long term perspective to estabilish its therapeutical window, and evaluate other parameters related to Guo neuroprotection. Thereby, In-Guo prevented long-term motor deficit even when administered up to nine hours after ischemia induction. On other side, therapeutical scheme with first administration up to three hours presented itself effective in preventing motor deficit on both short and long term, also with neuroprotective effects related to quantitative electroencephalogram, signaling pathways and to Blood Brain Barrier permeability.

APRESENTAÇÃO

Esta tese de doutorado dividide-se em três partes.

Parte I - introdução e objetivos – traz o embasamento teórico, os objetivos e as hipóteses da tese.

Parte II - artigo científico e resultados complementares – aqui se encontram as metodologias utilizadas e os resultados obtidos apresentados em um artigo,assim como os resultados complementares.

Parte III - discussão, conclusão e perspectivas- disserta sobre as limitações, conclusões e perspectivas que podem ser consideradas a partir dos resultados desse trabalho.

As referências bibliográficas das citações feitas encontram-se inseridas no texto da introdução e discussão desse documento.

O anexo refere-se ao artigo, em caráter de autoria dividida, redigido e publicado durante a elaboração dessa tese.

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PARTE I INTRODUÇÃO E OBJETIVOS

INTRODUÇÃO

1. Acidentes Vasculares Cerebrais

1.1 Epidemiologia

Doenças vasculares, em termos mundiais,são as principais causas de morbimortalidade (Katan and Luft 2018, Collaborators 2019, Kaji 2019, Liu, Huang et al. 2019, Zhao and Huang 2019), com sua incidência aumentada na medida em que fatores de risco, como a idade avançada, obesidade, hipertensão e sedentarismo, tornam-se mais prevalentes nas populações (Feigin, Krishnamurthi et al. 2015).

Dentre essas doenças, destacam-se os Acidentes Vasculares Cerebrais/ Encefálicos (AVC ou AVE, doravante AVC) os quais isoladamente representam a segunda maior causa de mortalidade. Na América Latina, estima-se que uma a cada cinco pessoas acima dos 25 anos irá apresentar um AVC, conforme o Global Burden of Stroke (levantamento epidemiológico da Organização Mundial da Saúde) (Feigin, Krishnamurthi et al. 2015). Ao lado disso, existe um impacto econômico importante decorrente das sequelas pós-AVC (de Santana, Dos Santos Figueiredo et al. 2018, Gorelick 2019), uma vez que um percentual considerável dos pacientes acometidos necessita de cuidado especializado, internações recorrentes, inclusive em Unidades de Tratamento Intensivo, e aposentadoria por invalidez (Gorelick 2019).

Etiologicamente, dividem-se os AVCs em: Hemorrágicos (AVCh),quadro caracterizado por rompimento na parede das artérias envolvidas na circulação cerebral; Isquêmicos (AVCi), quadro caracterizado pela oclusão de um vaso

por coágulo. Ainda que os primeiros apresentem maior letalidade, os isquêmicos representam 80-90% de todos os casos de AVC (de Santana, Dos Santos Figueiredo et al. 2018) razão pela qual esse trabalho passará a abordar os AVCi.

1.2. Apresentação Clínica, Diagnóstico e Achados em Neuroimagem.

Nos AVCi, as apresentações clínicas correspondem à função do território encefálico acometido, sendo a área de irrigação da artéria cerebral média (ACM) predominantemente afetada (Donnan, Fisher et al. 2008). Nela, encontram-se as áreas motora e sensorial primária, bem como – no hemisfério dominante – a área de Wernicke. Por isso, são frequentes sintomas como perda abrupta de força e sensibilidade em hemicorpo, e alterações na fala (Kothari, Hall et al. 1997, Goldstein and Simel 2005). Essa tríade, quando presente, indica alto valor preditivo positivo para o diagnóstico de AVCi (Kothari, Hall et al. 1997, Goldstein and Simel 2005)**,** porém não é capaz de excluir a necessidade de neuroimagem.

Em vista disso, para o diagnóstico são necessários exame físico neurológico e diferenciação de etiologia (hemorrágica ou isquêmica) – essencial para definição de conduta – por meio de tomografia computadorizada (TC) (Zivin 1998, Dirnagl, Iadecola et al. 1999, Powers, Rabinstein et al. 2019).Como, no Brasil, existe apenas uma minoria de centros de saúde que dispõem de tomógrafo, as possibilidades de tramento ficam restritas (de Carvalho, Alves et al. 2011, Martins, Pontes-Neto et al. 2013, de Santana, Dos Santos Figueiredo et al. 2018).

A TC pode detectar precocemente sinais de oclusão arterial por meio de hiperdensidade do vaso acometido (Marks, Holmgren et al. 1999), tendo de moderada/alta capacidade para identificar sinais de infarto cerebral (**Figura 1**), como a presença de hipodensidade tecidual (Marks, Holmgren et al. 1999), Sua utilização, portanto, foca-se na exclusão da presença de hemorragias intracerebrais (2019, Powers, Rabinstein et al. 2019), já que o uso de trombolítico é contraindicado nesses casos (2019, Powers, Rabinstein et al. 2019).

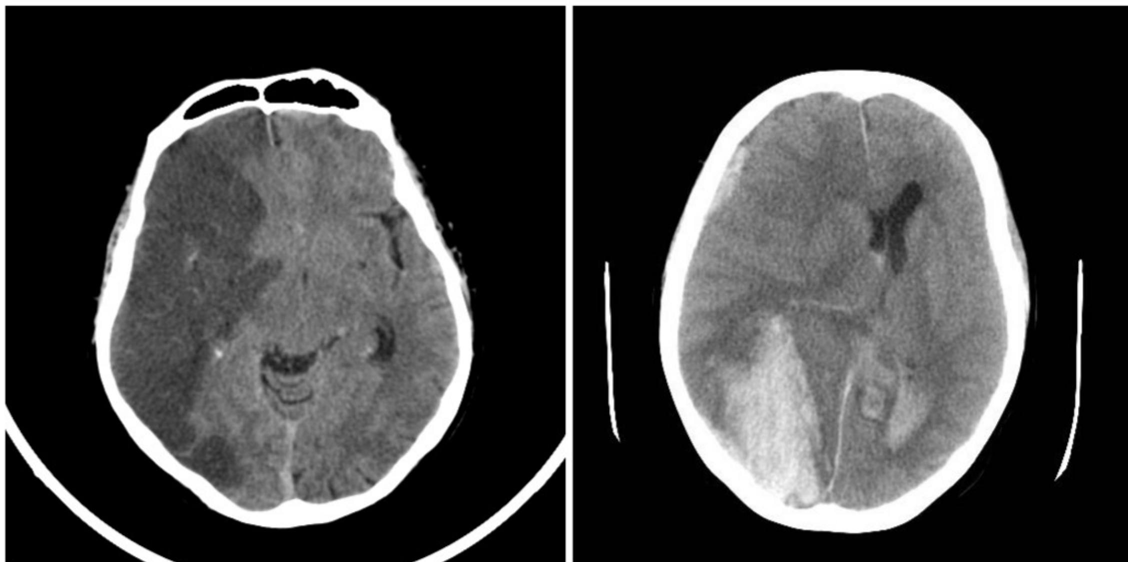

Figura 1.1 Imagens de Tomografias computadorizadas de crânio (TC), 48 horas após o evento isquêmico. À esquerda correspondente a um AVC isquêmico, extraída Radiopaedia.org, rID: 6153 (cortesia de Dr Jeremy Jones), à direita correspondente a um AVC hemorrágico, Radiopaedia.org, rID: 23669 (cortesia de Hugo Neves)

É possível, ainda, usar a imagem por ressonância magnética (RM) para detecção de sinais hiperagudos decorrentes da isquemia, sendo capaz de identificar com precisão consequências de edema citotóxico e morte celular aumentada, bem como hipoperfusão tecidual. Não obstante, a acessibilidade a

esse exame é ainda baixa e seus custos bastante elevados (Camilo and Goldstein 2003).

Recentemente, as análises de perfusão por meio de contraste em TC e RM foram propostas como complementares à avaliação dos pacientes, para definir prognóstico e determinar novas abordagens terapêuticas (Mokin, Levy et al. 2017, Wood 2018).

1.3 Mecanismos Fisiopatológicos do AVCi

A oclusão arterial no SNC leva, de maneira quase imediata, a uma série de eventos que podem ocorrer por horas ou dias após o início da oclusão (**Figura 2**), conhecida como “cascata isquêmica” (Durukan and Tatlisumak 2007, Brouns and De Deyn 2009).

Essa sequência de eventos envolve diversos processos bioquímicos que resultam na progressão de uma zona não salvável, conhecida como núcleo isquêmico, para zona salvável, parcialmente disfuncional, conhecida como “penumbra” (Durukan and Tatlisumak 2007, Brouns and De Deyn 2009).

1.3.1. Falha Bioenergética e Desequilíbrio Iônico

A hipoperfusão, no seu início, resulta em hipóxia e redução no aporte de glicose, o que determina importante falha bioenergética. Desenvolve-se daí, portanto, um desequilíbrio iônico por meio da disfunção de mecanismos homeostáticos celulares importantes, como a Na-K ATPase (Brouns and De Deyn 2009), o qual promoverá uma alteração do potencial de membrana e, consequentemente, a ativação aumentada dos receptores de glutamato.

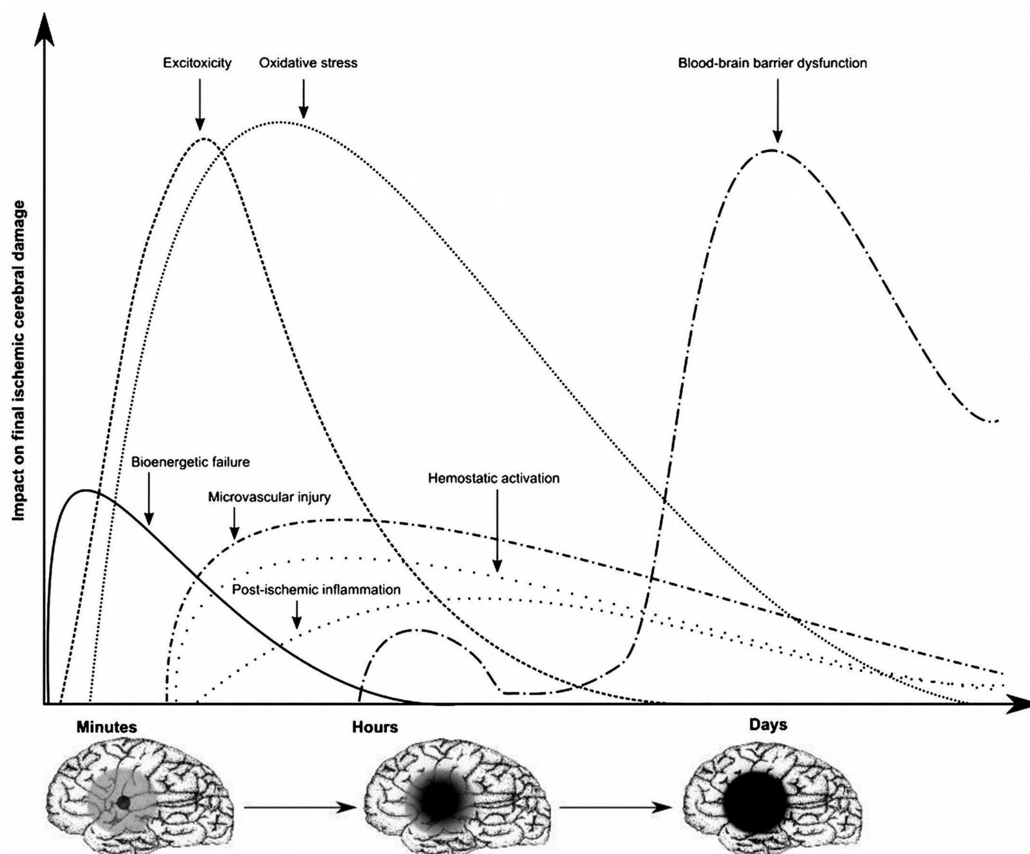

Figura 1.2: Cascata Isquêmica, conforme adaptado de Brouns and De Deyn 2009

1.3.2. Excitoxicidade Glutamatérgica

Diversos trabalhos apontam que o aumento abrupto da concentração intracerebral de glutamato e aspartato corresponde a uma resposta ao dano isquêmico, sendo a excitotoxicidade uma etapa importante para o aumento da morte celular (Dirnagl, Iadecola et al. 1999, Lo 2008, Brouns and De Deyn 2009, Lai, Zhang et al. 2014).

Como consequência da hiperestimulação glutamatérgica, diversas cascatas intracelulares são ativadas, principalmente as decorrentes da ação dos receptores NMDA. Pela ativação destes, observa-se um aumento no influxo intracelular de cátions, que se reflete na ativação da iNOS, responsável pela conversão de arginina em óxido nítrico, uma das principais fontes de ROS (Lo 2008, Lai, Zhang et al. 2014).

Outra consequência do influxo de cálcio aumentado é o desequilíbrio da sinalização envolvida na sobrevivência celular. Esse efeito decorre da inibição da fosforilação/ativação da PI3k e da consequente ativação de proteínas pró-apoptóticas, como PTEN, GSk3β, responsáveis pela sinalização de morte celular (Lo 2008, Lai, Zhang et al. 2014).

Em última análise, em curto prazo, tem-se, como resultado de todo esse processo, a morte celular aumentada se propagando pelo tecido cerebral potencialmente (Pisani, Bonsi et al. 2004, Lo 2008, Lai, Zhang et al. 2014).

1.3.3. Dano mitocondrial e Apoptose

A mitocôndria é um dos principais reguladores da morte celular, uma vez que se apresenta como um dos importantes depósitos do cálcio intracelular. Além disso, contém complexos enzimáticos essenciais para a manutenção da sobrevivência celular em sua membrana interna (Liu, Lu et al. 2018). Por isso, as mitocôndrias afetadas pelo dano isquêmico tornam-se componentes importantes na evolução desfavorável da zona de peri-infarto, na qual eventos, como excitotoxicidade glutamatérgica, disfunção bioenergética e iônica, afetam proeminentemente a funcionalidade mitocondrial (Liu, Lu et al. 2018).

A indução de morte celular por meio das funções mitocondriais ocorre por mecanismos, como inibição da fosforilação oxidativa, abertura do poro de transição de permeabilidade mitocondrial e liberação do Citocromo C, ou outra proteína do espaço entre as membranas interna e externa (Lipton 1999).

Esses efeitos ocorrem principalmente porque, em situações como a excitoxicidade glutamatérgica induzida pela isquemia cerebral, nas quais há um

aumento expressivo das concentrações intra e extracelulares de cálcio, a mitocôndria pode funcionar como um “depósito” desse cátion (Szabadkai and Duchen 2008, Liu, Lu et al. 2018). Isso promove, em conjunto com os baixos níveis de ATP, uma variação do potencial de membrana mitocondrial (Hajnoczky, Csordas et al. 2006), a qual apresentará, como consequências, abertura do poro de transição de permeabilidade mitocondrial e desacoplamento nos mecanismos de produção de ATP (Leung and Halestrap 2008). Se este for mantido, poderá causar a morte celular por necrose; se controlado por níveis mínimos de ATP, com consequente fechamento do poro (Leung and Halestrap 2008), pode implicar a sinalização de vias intracelulares, promovendo a morte celular programada (Kim, He et al. 2003, Leung and Halestrap 2008).

Concomitantemente à sinalização de morte celular induzida pelo dano mitocondrial, outras vias de sinalização pró e anti apoptóticas são moduladas, uma vez que os ROS também ativam mecanismos de morte celular (Lo 2008, Lai, Zhang et al. 2014, Uzdensky 2019).

Cabe ressaltar que a zona isquêmica é essencialmente heterogênea, uma vez que a transição entre núcleo e zona de penumbra se faz de maneira gradual, na medida em que se afasta do foco isquêmico, visto que esse gradiente é definido pela perfusão tecidual, proporcional ao substrato energético (Fricker, Tolkovsky et al. 2018, Yang, Liu et al. 2019). Isso influencia diretamente o tipo de morte celular, predominantemente dividida em necrose e apoptose (Uzdensky 2019). As células capazes de entrar em apoptose estão disfuncionais, porém mantêm integridade de membrana plasmática e níveis

mínimos de ATP (Radak, Katsiki et al. 2017). Tendo em vista essas condições, os mecanismos de apoptose, regulados por diversas vias de sinalização, são alvos importantes a serem abordados para a investigação de potenciais fármacos neuroprotetores (Uzdensky 2019).

Especialmente afetadas por modelos de isquemia focal e envolvidas diretamente na morte celular são as caspases, enzimas responsáveis pela promoção e execução da apoptose (Bursch, Kleine et al. 1990, Schwartzman and Cidlowski 1993, Hengartner 2000). Essas enzimas, ativadas por diferentes mecanismos, fazem parte de uma cascata de reações que converge para as formas efetoras (Caspases 3, 6 e 9) as quais vão promover a morte por apoptose (Hengartner 2000).

Dessa forma, inibir os efeitos das caspases, especialmente das suas formas ativas, pode prevenir a morte celular e repercutir, de maneira favorável, em parâmetros funcionais nos modelos de AVCi (Schulz, Weller et al. 1999, Zehendner, Librizzi et al. 2011).

Outras vias de sinalização têm potencial modulatório sobre as caspases, principalmente em modelos de isquemia cerebral. Essas vias são aquelas envolvidas na sinalização da Akt/PI3k/mTOR, uma vez que essas proteínas têm papel importante na proliferação celular e na inibição de apoptose, inibindo proteínas como a GSK3β e a PTEN (Noshita, Lewen et al. 2001). A redução da atividade da via da mTOR,como exemplo em modelos animais de AVCi, está associada a indução de apoptose e piores desfechos. Além disso, a inibição dessa via tem capacidade de extinguir o efeito neuroprotetor de diversos fármacos (Wu, Reiter et al. 2004, Chong, Li et al. 2007, Gao, Zhang et al. 2010,

Chong, Hou et al. 2011). Isso sugere que modular essa via é fundamental para o controle da morte celular, a fim de promover a evolução favorável da zona de penumbra (Zhang and Chopp 2009, Chong, Yao et al. 2013, Taliyan and Ramagiri 2016).

Além disso, as cascatas das caspases e da Akt/PI3k não estão envolvidas somente na sinalização de apoptose, mas também em diversos outros parâmetros afetados pelo dano isquêmico, como a disfunção da BHE e o aumento das citocinas pró-inflamatórias (Zhao, Sapolsky et al. 2006, Jin, Song et al. 2011, Lopez-Ramirez, Fischer et al. 2012, Sifat, Vaidya et al. 2017, Qian, Dou et al. 2018).

1.3.4. Dano à BHE

O endotélio dos vasos cerebrais compõe a interface no sistema conhecido como "Barreira Hematoencefálica" (Liebner, Dijkhuizen et al. 2018).Essa barreira apresenta estruturas conhecidas como "tight junctions", compostas principalmente por famílias de proteínas como claudinas e ocludinas, as quais têm a função de reduzir a distância intercelular e de permitir uma passagem transendotelial extremamente seletiva (Liebner, Dijkhuizen et al. 2018).

Portanto, a BHE é um sistema com interações entre endotélio, astrócitos, neurônios, pericitos e micróglia, sendo fundamental para a manutenção do equilíbrio iônico e a atividade elétrica (Sifat, Vaidya et al. 2017, Liebner, Dijkhuizen et al. 2018).

Em vista disso, em um processo de isquemia cerebral, ocorrem mudanças importantes nesse sistema que vão desde disfunção precoce dos

astrócitos, reorganização das *tight junctions*, expressão aumentada de metaloproteinases, fatores, portanto, que culminam no aumento da permeabilidade da BHE (Yang and Rosenberg 2011).

A disfunção da BHE costuma ser bifásica em quadros de AVCi (Kuroiwa, Ting et al. 1985): inicial, sendo detectada de duas a seis horas do início do quadro isquêmico; tardio, de 48 a 72 horas depois do início do quadro isquêmico. Acredita-se que esse padrão ocorra por meio de distintos mecanismos que são influenciados por diferentes cascatas (Yang and Rosenberg 2011).

A primeira fase ocorre como consequência direta da isquemia com aumento do HIF-1α que promoverá a ativação da MMP-2, causando o aumento da permeabilidade da BHE (Yang and Rosenberg 2011).

A fase tardia decorre dos efeitos do processo inflamatório intensificado pela cascata isquêmica, já que este determina o aumento de fatores como citocinas e proteínas de adesão; como consequência, também há a indução da ativação da MMP-9, uma das principais responsáveis pelo dano “permanente” à BHE (Brouns and De Deyn 2009).

O dano à BHE se torna importante na medida em que ele está relacionado a processos como hipertensão intracraniana, resultante de edema vasogênico e transformação hemorrágica do AVCi. Ambos os quadros são de extrema gravidade, já que estão ligados à morte encefálica e ao coma (Feigin, Krishnamurthi et al. 2015, Truelsen, Krarup et al. 2015). Por conseguinte, terapêuticas que previnem o dano à BHE têm especial relevância para proteger os pacientes de desfechos desfavoráveis.

Autores sugerem, por exemplo, que a disfunção da BHE pode ser não somente um preditor de hemorragia intracraniana após administração de alteplase (Turner and Sharp 2016), mas também um mediador/moderador desse processo. Essa hipótese é reforçada pelo fato de que estudos de coorte apontaram eficácia moderada de fármacos moduladores do dano à BHE, já que aqueles apresentaram associações estatisticamente significativas na redução da chance de mortalidade intrahospitalar, no aumento da chance de alta com independência e na redução de eventos adversos em pacientes submetidos ao tratamento com alteplase (Jin, Cai et al. 2017, Sifat, Vaidya et al. 2017, Enomoto, Endo et al. 2019). Mesmo assim, esses achados necessitam de confirmação em ensaios clínicos randomizados (ECR).

Além de potencial alvo terapêutico, o aumento de permeabilidade da BHE pode ser usado para definição de conduta. Um dos principais exemplos disso é o ECR que propôs o critério de RM para determinar a possibilidade do tratamento com alteplase, em pacientes com janela terapêutica indeterminada (Thomalla, Simonsen et al. 2018, Zhao, Zhao et al. 2019).

A base fisiológica desse estudo se encontra no fato de que alterações como hiperintensidade na sequência FLAIR, em conjunto aumento de sinal em difusão (DWI, difusion weighted imaging) e redução do sinal no mapa de coeficientes aparentes de difusão (ADC), indicam um AVCi com presença de edema no tecido cerebral, o que representa restrição de difusão à água, comumente encontrada em situações de aumento da permeabilidade de BHE (Hjort, Wu et al. 2008, Weier, Fluri et al. 2009).O paciente, portanto, apresenta uma janela terapêutica inviável para o tratamento com alteplase.

1.3.5. Reatividade Astrocitária

Os astrócitos são responsáveis pela regulação de diversos processos no SNC, estando, por isso, envolvidos em diversas etapas do dano isquêmico (Nedergaard and Dirnagl 2005). Não obstante, os impactos da atividade astrocitária sobre o dano isquêmico ainda não foram completamente esclarecidos, bem como sua influência no prognóstico dos pacientes (Nedergaard and Dirnagl 2005).

Por conseguinte, abordar a função astrocitária em modelos de AVCi pode resultar no desenvolvimento de novas terapias e de novos marcadores prognósticos, uma vez que, minutos após o início do quadro isquêmico, os astrócitos sofrem alterações morfológicas e funcionais relacionadas a proliferação e hipertrofia, fenômeno conhecido como reatividade astrocitária (Liu and Chopp 2016). Ainda assim, os efeitos dessa resposta não são totalmente conhecidos. Alguns trabalhos testaram marcadores astrocitários como proteína ácida fibrilar glial (GFAP) e proteína B de liberação ao cálcio (s100ß) (Aurell, Rosengren et al. 1991, Herrmann, Vos et al. 2000)para avaliação de desfechos em longo prazo, apresentando correlações entre concentrações séricas da proteína ácida fibrilar glial e desfechos negativos, para um pequeno número de pacientes (Wunderlich, Wallesch et al. 2006).

Além disso, a avaliação de parâmetros astrocitários em modelos de isquemia cerebral se torna relevante, ao passo que fenômenos fundamentais para a evolução do quadro são modulados por células gliais. Entre esses fenômenos, encontram-se produção de fatores inflamatórios, mediação da tolerância isquêmica, recaptação de glutamato, homeostase iônica e,

consequentemente, progressão da morte celular na zona de penumbra (Giffard and Swanson 2005, Choudhury and Ding 2016).

Esse efeito, conhecido como “cicatriz glial”, é caracterizado pela proliferação aumentada e alterações morfológicas e funcionais dos astrócitos circundantes à zona isquêmica, que formam uma contenção física do dano (Silver and Miller 2004, Nedergaard and Dirnagl 2005). Tal efeito é reportado tanto em modelos animais de isquemia quanto em pacientes pós AVCi (Silver and Miller 2004, Nedergaard and Dirnagl 2005). A partir disso, estudos referem que o isolamento da lesão é uma maneira de proteger o tecido nervoso não afetado, ainda que, como consequência, restrinja a resposta regenerativa em estágios mais tardios e possa criar uma potencial zona epileptogênica (Wetherington, Serrano et al. 2008, Pekny, Wilhelmsson et al. 2014). Ainda assim, animais nocaute para a GFAP, submetidos a lesões isquêmicas, apresentam maiores infartos se comparados a animais *wild-type* (Li, Lundkvist et al. 2008, Barreto, White et al. 2011), o que sugere a importância da reatividade astrocitária e da cicatriz glial no controle da excitotoxicidade glutamatérgica e das espécies reativas de oxigênio (Pekny, Wilhelmsson et al. 2014).

Além disso, os efeitos da reatividade astrocitária não se restringem somente à zona peri-isquêmica, porquanto estudos em modelos animais de isquemia cerebral focal apontam para zonas de proliferação astroglial, tanto no hemisfério contralateral à lesão isquêmica, quanto em regiões distantes dela (Giffard and Swanson 2005, Nedergaard and Dirnagl 2005, Pekny, Wilhelmsson et al. 2014, Takatsuru, Nabekura et al. 2014, Liu and Chopp

2016). No entanto, os efeitos decorrentes desse processo não foram totalmente elucidados (Patience, Zouikr et al. 2015), ainda que possam ser observadas associações entre reatividade astrocitária contralateral e BHE, e parâmetros eletrofisiológicos (Garbuzova-Davis, Rodrigues et al. 2013, Takatsuru, Nakamura et al. 2013, Garbuzova-Davis, Haller et al. 2014, Takatsuru, Nabekura et al. 2014)

Assim sendo, prevenir os efeitos da isquemia sobre os parâmetros astrocitários, ainda que em regiões distantes da lesão isquêmica, pode ter repercussões em diversas outras etapas da cascata isquêmica (Alvarez, Katayama et al. 2013, Pekny, Wilhelmsson et al. 2014), o que representa um significativo impacto na recuperação da funcionalidade em modelos animais e em pacientes pós AVCi (Takatsuru, Nabekura et al. 2014).

1.3.6. Abordagens terapêuticas e prognósticas com base na fisiopatologia do AVCi

1.3.6.1. Tratamento

O tratamento para AVCi surgiu somente na década de 90, quando ensaios clínicos randomizados (ECR) validaram o alteplase ou rt-PA como uma alternativa para recanalização do vaso acometido (Albers, Bates et al. 2000, Donnan, Fisher et al. 2008).

O mecanismo desse fármaco se baseia na cascata de coagulação por ser um ativador do plasminogênio que o converte em sua forma ativa, a plasmina, responsável pela degradação da fibrina, principal componente do coágulo. O rt-PA também é responsável por ativar uma cascata de reações que

envolvem proteólise, adesão leucocitária e o consumo da lâmina basal, que pode apresentar diversas consequências. Disso decorre seu principal efeito adverso: o risco significativamente aumentado de hemorragia intracraniana, um quadro de péssimo prognóstico (Mazya, Egido et al. 2012).

Esse fármaco, no entanto, só pode ser oferecido a uma restrita porção dos pacientes afetados, visto seus critérios restritivos (comorbidades associadas, uso prévio de anticoagulantes e área cerebral acometida), além de sua janela terapêutica de 4,5h após o início do quadro isquêmico. Em vista disso, diversos estudos tentaram apontar quais pacientes se beneficiariam de tratamentos alternativos ou de uma expansão de janela. Recentemente, trabalhos apresentaram um software com boa acurácia para distinguir esse perfil de paciente. O propósito desse software é estimar uma relação entre zona necrótica e salvável – conhecida como “mismatch” - por meio da análise de perfusão em neuroimagem (Mokin, Levy et al. 2017).

Assim, utilizando-se o “mismatch”, foram realizados ECRs na tentativa de expandir a janela terapêutica do alteplase (Ma, Campbell et al. 2019, Powers, Rabinstein et al. 2019). Mesmo apresentando benefícios, esses estudos apontam necessidade de dados adicionais.

Como alternativa, a trombectomia mecânica se apresentou como um tratamento para além de 6h após a primeira manifestação do AVCi, com uma melhora importante de morbidade em pacientes tratados até 24h após a primeira manifestação dos sintomas (Albers, Marks et al. 2018, Damji and Lang 2018, Tsurukiri, Ota et al. 2019). No entanto, para obtenção de benefício, os pacientes também precisam ser selecionados pelo critério de perfusão (Mokin,

Levy et al. 2017, Vinny, Vishnu et al. 2018).Essa abordagem, entretanto, requer diversas condições para ser implantada, como equipamentos de ponta e equipe altamente especializada, ambos, em conjunto com o software de neuroimagem, indisponíveis na maioria dos centros de saúde do Brasil (Brinjikji, Rabinstein et al. 2014). Mesmo assim, essa terapêutica se mostrou viável e extremamente eficaz no Sistema Único de Saúde, em um ensaio recente (Martins, Mont'Alverne et al. 2020).

Como resultante desse panorama, a maioria dos pacientes diagnosticados com AVCi ainda não são efetivamente tratados no Brasil (de Carvalho, Alves et al. 2011), o que faz, geralmente,o quadro evoluir de maneira desfavorável e representa custos elevados para a sociedade (Christensen, Valiente et al. 2009, de Carvalho, Alves et al. 2011, Gorelick 2019)

Tendo em vista isso, na busca por novas terapias, a fisiopatologia dos AVCi foi detalhadamente estudada, e a “cascata isquêmica” determinada (Durukan and Tatlisumak 2007, Brouns and De Deyn 2009).

1.3.6.2. Prognóstico pelo EEG

Ainda que, no contexto de doenças cerebrovasculares, o EEG seja mais comumente utilizado para o diagnóstico de epilepsia pós-AVC (Myint, Staufenberg et al. 2006, Rabiller, He et al. 2015), mostra-se sensível para detectar mudanças na atividade elétrica decorrente de alterações sutis no fluxo sanguíneo cerebral de maneira rápida e eficiente (Laman, Wieneke et al. 2005, Foreman and Claassen 2012). Muitas vezes ele pode detectar anormalidades antes mesmo da neuroimagem (Laman, Wieneke et al. 2005).

O EEG quantitativo (qEEG), inclusive, pode detectar mudanças ainda mais sutis (Laman, Wieneke et al. 2005) no ritmo cerebral as quais apresentam correlações com estados patológicos. Por conseguinte, ele tem sido proposto para AVCi, tanto em modelos animais quanto em pacientes, como uma forma de predizer o prognóstico (Finnigan, Rose et al. 2004, Finnigan, Rose et al. 2006, Sheorajpanday, Nagels et al. 2009, Sheorajpanday, Nagels et al. 2011).

Isso se baseia na origem do sinal do EEG, proveniente da condução de volume decorrente do fluxo iônico gerado pelas sinapses no córtex cerebral, o que originará os diferentes ritmos cerebrais, que podem ser registrados por eletrodos de superfície. Cabe ressaltar que a classificação desses ritmos realiza-se por meio das faixas de frequências correspondentes às oscilações propostas por neurofisiologistas como Berger (Buzsaki and Draguhn 2004, Buzsaki 2006), sendo classicamente analisadas as seguintes: delta (1-4 Hz), teta (4-8 Hz), alfa (8-12 Hz), beta (13-30 Hz) e gama (30-120 Hz). Tendo em vista isso, a origem de algumas oscilações pode ser correlacionada a processos rítmicos, como exemplo as oscilações delta e teta com o sono de ondas lentas (originado predominantemente por neurônios localizados no tálamo), que precede a fase REM, alfa a inibição de estímulos visuais no córtex occipital (presente quando os olhos estão fechados), beta à vigília ativa e gama a estímulos cognitivos (predominantemente originado por interneurônios inibitórios) (Buzsaki and Wang 2012).

Diversas análises podem ser realizadas por meio do qEEG, entre elas quantificação da potência espectral (PSD) de cada uma das oscilações, simetria inter-hemisférica, razões entre os ritmos rápidos e lentos, coerência de uma frequencia entre diferentes locais e acoplamento entre diferentes

frequências na mesma região (Canolty, Edwards et al. 2006, Kahana 2006, van Vugt, Sederberg et al. 2007, Axmacher, Henseler et al. 2010, Sheorajpanday, Nagels et al. 2010).

Para realizar essas análises, é necessário decompor o sinal complexo do EEG por meio da Transformada de Fourier, uma série temporal com domínio em frequência. O ponto fundamental dessa análise decorre que o sinal natural complexo é resultante de funções periódicas (senos, cossenos) conhecidas, com diferentes fases e amplitudes. Essas funções são comparadas em relação à sua similaridade com o sinal registrado a fim de quantificar o componente da faixa de freqüência especificada. A partir disso, descreve-se o sinal do EEG – localizado no domínio de tempo - por uma função no domínio de freqüência, i.e., pode-se representar esse sinal em um gráfico com abcissa correspondente às frequências e a ordenada correspondente à amplitude ou densidade de potência espectral (PSD).

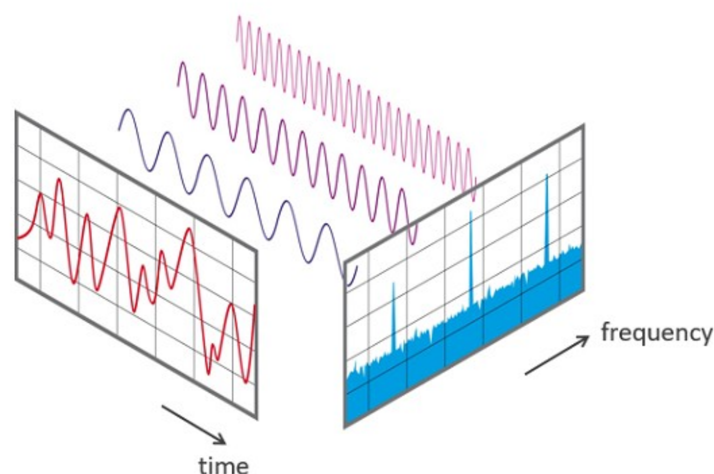

Fig 1.3. Figura representativa da decomposição do EEG pela Transformada de Fourier. Adaptado de:

https://www.nti-audio.com/pt/suporte/saber-como/transformacao-rapida-de-fourier-fft

Ainda que haja diferenças na literatura em relação às bases biológicas da variação da PSD de cada oscilação (Buzsaki 2006, Canolty and Knight 2010, Schomer and Lopes da Silva 2017), podem ser estabelecidas algumas correlações, entre elas, alterações como o predomínio de ondas lentas no EEG, a redução na potência relativa das oscilações “rápidas” (como beta e gama) e/ou o aumento na PSD das oscilações “lentas” (como Delta e Teta). Tais alterações são encontradas à medida que o fluxo sanguíneo cerebral reduz (Nuwer, Jordan et al. 1987, Laman, Wieneke et al. 2005, Sheorajpanday, Nagels et al. 2011).

Posteriormente, se a hipoperfusão for mantida, a amplitude das oscilações registradas no EEG também reduz, observando-se, então, a atenuação do poder de todas as oscilações (Nuwer, Jordan et al. 1987, Laman, Wieneke et al. 2005). Outras alterações relevantes também podem ser achadas à medida que se distancia o registro do núcleo isquêmico, como redução na PSD de Beta, um dia após o início do evento isquêmico **(**Foreman and Claassen 2012), cuja magnitude é proporcional ao tamanho e à proximidade da área infartada. É possível também se observar que oscilações Gama (lenta, média e rápida), de mais alta frequência e relacionadas classicamente a processos cognitivos, têm sua PSD rapidamente alterada devido aos eventos decorrentes da isquemia cerebral (Rabiller, He et al. 2015).

Além dessas, outras etapas da cascata isquêmica, como a hiperestimulação glutamatérgica, podem ser associadas às anormalidades na PSD de Teta, na zona de penumbra (Gallinat, Kunz et al. 2006, Torres, da Silva Filho et al. 2010, Vazana, Veksler et al. 2016).

Modelos experimentais/animais de AVCi focais apresentam, de mesmo modo, resultados relevantes quando se comparam os dois hemisférios, já que alterações funcionais ocorrem não somente no hemisfério ipsilateral à isquemia, mas também no hemisfério contralateral (Takatsuru, Nakamura et al. 2013, Takatsuru, Nabekura et al. 2014). Em vista disso, o hemisfério contralateral também apresenta alterações eletroencefalográficas, ainda que reportadas com divergências na literatura, mostrando diferentes padrões na zona peri-infarto ipsi e contralateral, os quais se justificam principalmente pela heterogeneidade de abordagens e de faixas de tempo estudadas.

No entanto, mesmo em pacientes, acredita-se que algumas alterações na quantificação das oscilações corticais no hemisfério contralateral estão relacionadas a quadros de maior gravidade (Gloor, Ball et al. 1977, Finnigan, Walsh et al. 2007). Nesse contexto, publicações avaliando medidas, como: simetria inter-hemisférica,a razão entre alfa e delta, a razão delta-teta e alfa-beta, em pacientes pós AVCi, apresentam valores preditivos moderados para predizer o desfecho em diferentes tipos de AVCi. Esse quadro sugere o uso do qEEG como potencial marcador (Finnigan, Rose et al. 2004, Finnigan, Rose et al. 2006, Finnigan, Walsh et al. 2007), que está sendo gradualmente mais utilizado principalmente na avaliação de pacientes em unidades de tratamento intensivo (Jordan 2004, Hajat, Ahmad et al. 2017).

2. Guanosina e Seu Potencial Neuroprotetor

Diante de todo esse contexto, apresenta-se a Guo, uma purina com base na Guanina, apresenta efeitos extracelulares correspondentes a funções neurotróficas e neuroprotetoras (Rathbone, Pilutti et al. 2008, Tasca, Lanznaster et al. 2018).

Diante disso, testou-se sua eficácia, nos modelos animais, para diversas patologias, como doenças psiquiátricas (Bettio, Cunha et al. 2012, Almeida, Comasseto et al. 2017), crises epilépticas (Schmidt, Tort et al. 2008) e encefalopatia hepática (Paniz, Calcagnotto et al. 2014, Cittolin-Santos, de Assis et al. 2017), sepse (Bellaver, Souza et al. 2015), doença de Parkinson (Li, Yao et al. 2014), trauma crânio encefálico (Dobrachinski, Gerbatin et al. 2019), doença de Alzheimer (da Silva, Nonose et al. 2020) e dor neuropática (Schmidt, Paniz et al. 2010). Nesses testes, os resultados se mostraram relevantes em diversos desfechos, como nos parâmetros comportamentais, na redução de crises epilépticas e até mesmo na mortalidade (Lara, Schmidt et al. 2001, Schmidt, Tort et al. 2008, Hansel, Ramos et al. 2014, Cittolin-Santos, de Assis et al. 2017, Massari, Lopez-Cano et al. 2017).

2.1. Repercussões da Guanosina na Atividade Elétrica Cerebral

A avaliação eletrofisiológica dos efeitos da Guo foi realizada predominantemente em modelos de crises epilépticas induzidas por agonistas glutamatérgicos (Schmidt, Tort et al. 2009, Torres, da Silva Filho et al. 2010). Nesses modelos, a administração de Guo mostrou-se capaz de prevenir a redução da PSD, induzida pelas crises epilépticas decorrentes do tratamento com ácido quinolínico (Torres, da Silva Filho et al. 2010).

Até então, na literatura, haviam sido registradas poucas alterações eletroencefalográficas nos animais controle tratados com Guo (Tort, Mantese et al. 2004, Torres, da Silva Filho et al. 2010).

Foram encontrados, também, efeitos neuroprotetores da Guo em modelos animais de encefalopatia hepática, nos quais comumente se observam alterações eletroencefalográficas, principalmente a predominância

de oscilações de baixa frequência e de baixa amplitude registradas no EEG, fortemente correlacionadas, nesses modelos, a estados comatosos e a morte (Paniz, Calcagnotto et al. 2014, Cittolin-Santos, de Assis et al. 2017). Nestes, o tratamento com Guo preveniu alterações eletroencefalográficas, como redução da frequência generalizada das oscilações corticais e presença de ondas trifásicas (Cittolin-Santos, de Assis et al. 2017), o que pode justificar a menor mortalidade e menor duração do com a dos animais tratados com Guo (Cittolin-Santos, de Assis et al. 2017). No entanto, os parâmetros do EEG não foram diretamente correlacionados aos parâmetros comportamentais.

Diante disso, a quantificação do EEG aparece como um parâmetro válido para avaliação de modelos animais referentes às patologias do SNC, e, ainda, apresenta-se como válido para avaliar o efeito neuroprotetor da Guo nessas patologias.

2.2. Guanosina e Isquemia Cerebral

Além dos efeitos supracitados, esse nucleosídeo dá evidências de resultados relevantes também em modelos animais de hipoperfusão (Ganzella, de Oliveira et al. 2012), isquemia focal transitória e permanente (Rathbone, Saleh et al. 2011, Connell, Di Iorio et al. 2013, Hansel, Ramos et al. 2014, Hansel, Tonon et al. 2015). Assim sendo, dentre os efeitos observados, destacaram-se a prevenção dos déficits motores comportamentais referentes ao dano isquêmico, bem como a redução do volume de infarto. Nos modelos de OGD, classicamente utilizados para reproduzir *in vitro* alterações decorrentes da isquemia (Tornabene, Helms et al. 2019), observa-se a proteção da Guo em fatias hipocampais por meio de parâmetros de mortalidade celular, como marcadores de disfunção mitocondrial e de

rompimento da membrana celular (Thomazi, Godinho et al. 2004, Thomazi, Boff et al. 2008, Dal-Cim, Martins et al. 2016, Thomaz, Dal-Cim et al. 2016).

No modelo de isquemia focal transitória induzida pela oclusão da ACM, a administração combinada de Guo, em regime de pré e pós indução, mostrou-se capaz de reduzir o déficit neurológico induzido pela isquemia, embora não apresentasse a capacidade de afetar o dano ao retículo endoplasmático (Connell, Di Iorio et al. 2013).

Observou-se que, no modelo de isquemia focal permanente induzido por termocoagulação, a administração sistêmica/intraperitoneal de Guo também se mostrou capaz de prevenir o dano induzido pela isquemia, como: aumento de células neuronais e astrocitárias marcadas positivamente para PI (comumente usado para medir morte celular), aumento na proporção de células positivas para micróglia ativada(Hansel, Ramos et al. 2014, Hansel, Tonon et al. 2015).

Todos esses efeitos, porém, se mostram eficazes a partir de uma importante restrição: somente quando a Guo é administrada imediatamente após a indução de isquemia (Rathbone, Saleh et al. 2011, Connell, Di Iorio et al. 2013, Hansel, Ramos et al. 2014, Hansel, Tonon et al. 2015).

2.3. Guanosina e seus Aspectos Mecanísticos em Modelos de Isquemia

Diversos trabalhos postulam efeitos do tratamento com Guo em etapas do dano isquêmico, como produção de ROS, cascata inflamatória, apoptose e, principalmente, na excitoxicidade glutamatérgica, quando analisados em modelos *in vivo* (Hansel, Ramos et al. 2014, Hansel, Tonon et al. 2015).

Ainda assim, majoritariamente esses trabalhos descrevem os efeitos da Guo de maneira associativa, postulando alterações provocadas pela isquemia e prevenidas pela administração de Guo (Hansel, Ramos et al. 2014). Em vista disso, Hansel e colaboradores mostraram os efeitos da Guo na expressão de transportadores glutamatérgicos, especialmente na prevenção da redução de GLT-1, principal transportador de glutamato astrocitário, o que reforça, em parte, os efeitos da Guo na excitotoxicidade (Hansel, Ramos et al. 2014). Além disso, também sugeriram que efeitos neuroprotetores fossem moderados pela redução na formação de ROS, já que a Guo também preveniu os efeitos da isquemia em parâmetros, como a expressão e atividade da enzima SOD, catalase e ácido ascórbico/vitamina C. Esse estudo também mostrou correlações significativas entre o escore de simetria e os níveis de ROS, TBARS e ácido ascórbico/vitamina C (Hansel, Ramos et al. 2014)

Da mesma forma, o efeito da Guo no estresse oxidativo também foi testado no modelo de OGD (Thomaz, Dal-Cim et al. 2016). Nesse estudo, a OGD aumentou os níveis de marcadores relacionados ao estresse oxidativo e nitrosativo, bem como elevou a quantidade de células com potencial de membrana disfuncional e em vias de apoptose. Uma vez que a Guo se mostrou capaz de prevenir esses efeitos nesse modelo e apresentou também efeitos antioxidantes em isquemia *in vivo*, havia a possibilidade de que, tal qual seu metabólito ácido úrico (Becker, Reinholz et al. 1991), agisse como "*scavenger*". Nesse mesmo trabalho, no entanto, a Guo não apresentou atividade antioxidante *per se*, sendo esta uma evidência contrária à hipótese de que o mecanismo de neuroproteção da Guo se desse por sua interação direta com as ROS (Thomaz, Dal-Cim et al. 2016).

Assim, em busca de um denominador comum para justificar os diversos efeitos da Guo no dano isquêmico, investigaram-se as vias de sinalização ligadas à sobrevivência celular. Foi, então, demonstrado que inibidores das vias PI3k e Mapa podem bloquear os efeitos neuroprotetores da Guo (Dal-Cim, Martins et al. 2011, Bettio, Cunha et al. 2012, Lanznaster, Dal-Cim et al. 2016). O efeito de modulação da Guo nessas vias, no entanto, aparentou ser mediado/moderado por receptores de adenosina e canais de potássio (Dal-Cim, Martins et al. 2011, Lanznaster, Massari et al. 2019).

Esses resultados ganham relevância, pois a sinalização dessas vias pode explicar grande parte dos efeitos da Guo, como redução do apoptose, disfunção mitocondrial e até mesmo aumento na recaptação do glutamato (Frizzo, Lara et al. 2002, Molz, Dal-Cim et al. 2011, Chojnowski, Opielka et al. 2021).

2.4. Guanosina Administrada Via Intranasal como Alternativa Terapêutica

Estudos anteriores apontavam que a via intranasal se mostrava como válida para diversas substâncias endógenas que têm efeito sobre o SNC (Chavanpatil and Vavia 2004, Ma, Ma et al. 2008, Wang, Cheng et al. 2008, Fletcher, Kohli et al. 2009). Isso se deve pelo fato de essas substâncias atingirem maior concentração comparativamente às vias sistêmicas em seu alvo terapêutico. As justificativas para isso são: menor metabolização periférica (Lochhead and Thorne 2012, Djupesland, Messina et al. 2014) e utilização de transportadores nas vias olfatórias localizadas na placa cribriforme onde há descontinuidade de BHE (Rhim, Lee et al. 2013, Djupesland, Messina et al. 2014).

Foi realizado, então, um estudo (Ramos, Muller et al. 2016) para verificar o perfil farmacocinético da administração intranasal de Guo (In-Guo). Sua radioatividade foi detectada cinco minutos após a administração, com uma importante modificação do conteúdo de purinas no líquido cerebrospinal e no soro, não observada no grupo que recebeu a administração intraperitoneal.

Nesse mesmo trabalho, o estudo da determinação de volume e dose efetivas indicou, pela análise de conteúdo radioativo, o volume ótimo de 50 µL por narina e a concentração ótima de 30 mg/ml (Ramos, Muller et al. 2016). Cabe ressaltar que uma estimativa aproximada de concentração medida em miligramas de Guo por peso de animal é uma dose efetiva de 8,5 mg/kg, muito inferior à concentração utilizada por Hansel e colaboradores (60 mg/kg) (Hansel, Ramos et al. 2014), que obteve melhora em escore de simetria somente em regimes de pré tratamento e quando a Guo foi administrada imediatamente após a indução do dano isquêmico. Esse estudo (Ramos, Muller et al. 2016) também mostrou resultados promissores dessa molécula já com a primeira dose administrada 3h após a indução de isquemia em um modelo de isquemia focal permanente, o que preveniu déficit motor e dano mitocondrial induzido pela isquemia a curto prazo (Ramos, Muller et al. 2016).

Com isso, impôs-se a necessidade de validar a eficácia do tratamento In-Guo em um modelo de isquemia cerebral.

3. Hipóteses

O presente trabalho baseia-se na hipótese de que o modelo de isquemia por termocoagulação unilateral implica alterações no hemisfério contralateral, como alterações eletrofisiológicas, astrocitárias, vias de sinalização e disfunção

da BHE, as quais podem ser prevenidas pela Guo,quando administrada por via intranasal.

4. Objetivos

4.1. Objetivos Gerais

Investigar a eficácia do tratamento intranasal de GUO para avaliar, em curto e longo prazo, o seu efeito na recuperação dos animais submetidos ao modelo de isquemia cerebral por termocoagulação.

Determinar os efeitos da Guo em parâmetros das oscilações corticais, comportamentais, histológicos e citológicos diante do insulto isquêmico.

Avaliar o dano do modelo de termocoagulação e o potencial de prevenção ao dano isquêmico pelo tratamento com In-Guo na disfunção da BHE.

4.2. Objetivos Específicos

1. Confirmar o efeito neuroprotetor da Guo no modelo de isquemia cerebral por termocoagulação.

2. Determinar a janela terapêutica dessa molécula quando administrada via intranasal, para aumentar seu valor translacional.

3. Avaliar alvos terapêuticos envolvidos na neuroproteção desse potencial fármaco, em modelos animais de AVCi/Isquemia cerebral.

PARTE II - METODOLOGIAS E RESULTADOS

O presente trabalho está dividido em dois capítulos.

O capítulo I traz o artigo "Effects of Intranasal Guanosine Administration on Brain Function in a Rat Model of Ischemic Stroke", publicado na revista científica Purinergic Signalling. Relaciona-se ao objetivo específico dois e três.

O capítulo II relaciona-se a dados suplementares aos do capítulo I, os quais, embora não enviados para submissão, foram coletados com as mesmas metodologias e que justificam os parâmetros escolhidos para constarem no artigo. Relaciona-se ao objetivo específico um e dois, e ao objetivo geral de determinar a janela terapêutica da In-Guo.

Capítulo I

Effects of Intranasal Guanosine Administration on Brain Function in a Rat Model of Ischemic Stroke

Purinergic Signalling
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ORIGINAL ARTICLE

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

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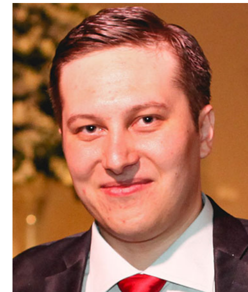
Abstract
Ischemic stroke is a major cause of morbidity and mortality worldwide and only few affected patients are able to receive treatment, especially in developing countries. Detailed pathophysiology of brain ischemia has been extensively studied in order to discover new treatments with a broad therapeutic 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

Muller, G.C is a MD and Phd student in Biochemistry. His main work is focused on neuroprotective effects of nucleoside Guanosine in stroke models.



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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

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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 multiprofessional 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

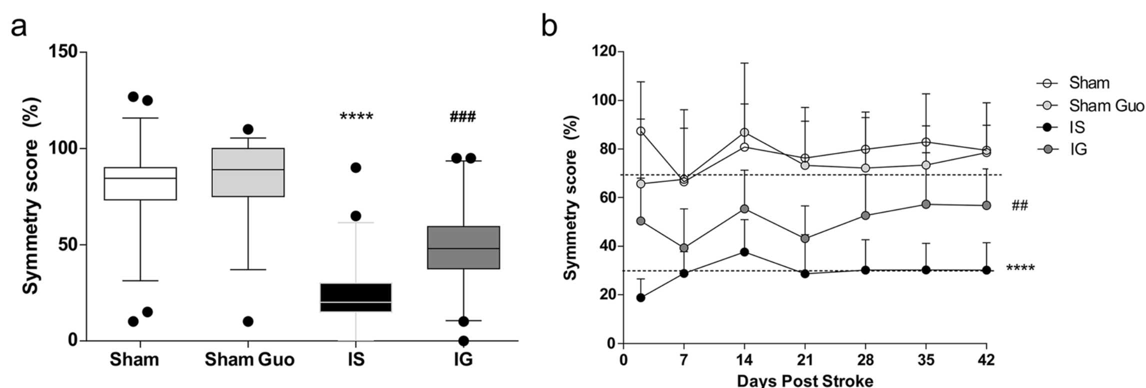


Fig. 1 Forelimb symmetry score measured by cylinder test. **a** Symmetry score 48 h after focal ischemia induction, aggregating rats used in all further protocols of this work (42 to 54 animals per group $^{****}p < 0.001$ vs Sham and Sham Guo, $^{\#\#\#}p < 0.005$ vs. IS by Kruskal-Wallis, corrected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

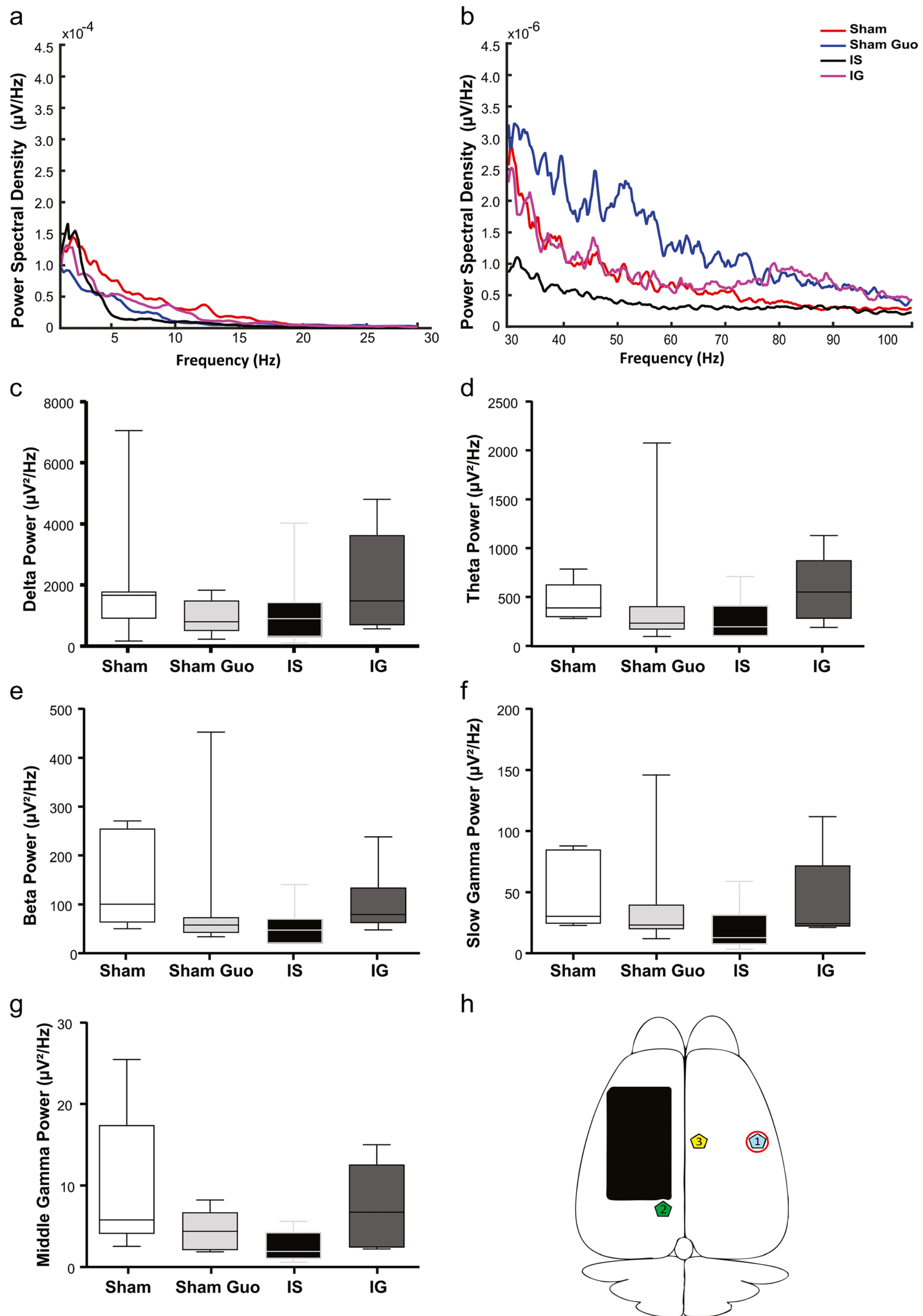
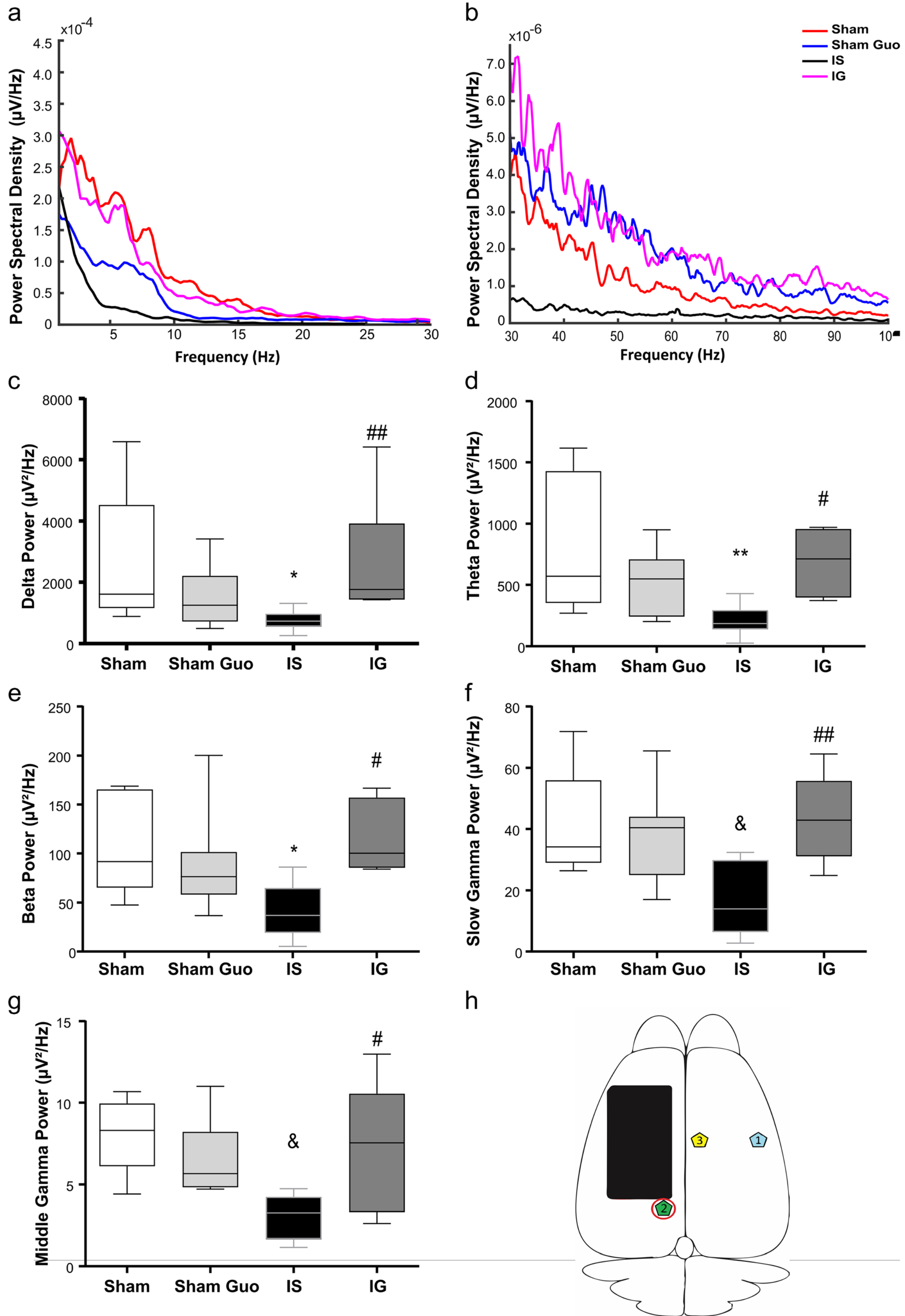


Fig. 2 Power spectral density (PSD) at electrode located on contralateral cortex unrelated to the ischemic lesion (E1). Representative PSD (µV/Hz) for each group: **a** 0 to 30 Hz and **b** 30 to 100 Hz. Plots of PSD of **c** delta, **d** theta, **e** beta, **f** slow gamma, and **g** middle gamma. **h** 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)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x10-4
4.5
4.0
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Power Spectral Density (μV/Hz)
5
10
15
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25
30
Frequency (Hz)
b
x10-6
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
Power Spectral Density (μV/Hz)
30
40
50
60
70
80
90
100
Frequency (Hz)
Sham
Sham Guo
IS
IG
c
8000
6000
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2000
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Delta Power (μV²/Hz)
##
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Theta Power (μV²/Hz)
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Beta Power (μV²/Hz)
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Slow Gamma Power (μV²/Hz)
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Sham Guo
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Middle Gamma Power (μV²/Hz)
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2

◀ **Fig. 3** Power spectral density (PSD) at electrode located on ipsilateral peri-infarct cortex (E2). Representative PSD (μV/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 ± 1 °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.

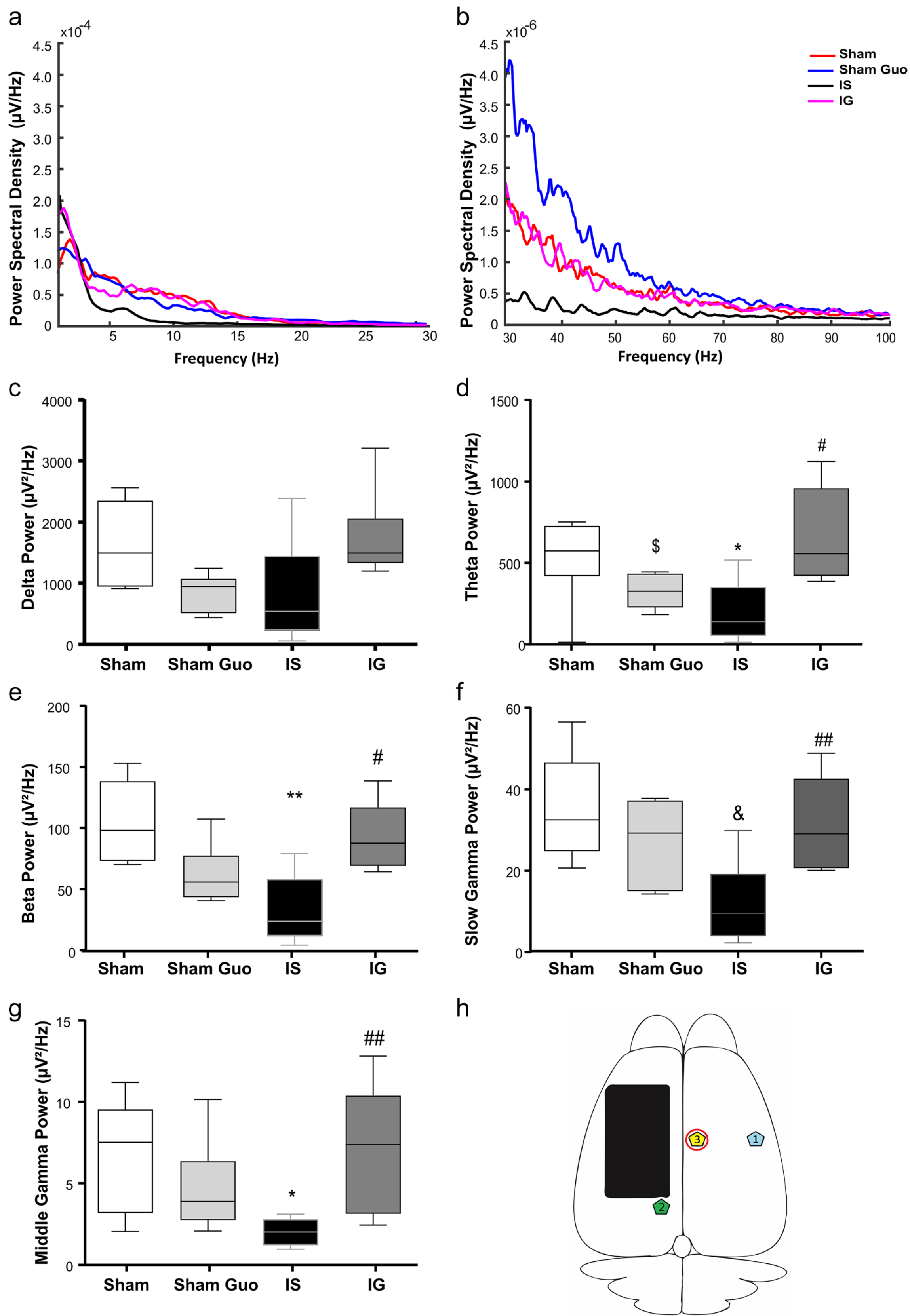
Treatment with intranasal guanosine after ischemia induction

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.



a
x10^-4
Power Spectral Density (µV/Hz)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
5
10
15
20
25
30
Frequency (Hz)
b
x10^-6
Sham
Sham Guo
IS
IG
30
40
50
60
70
80
90
100
c
Delta Power (µV²/Hz)
4000
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2000
1000
Sham
Sham Guo
IS
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Theta Power (µV²/Hz)
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◀ **Fig. 4** Power spectral density (PSD) at electrode located on contralateral peri-infarct cortex (E3). Representative PSD (μV/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, contralateral 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)

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.

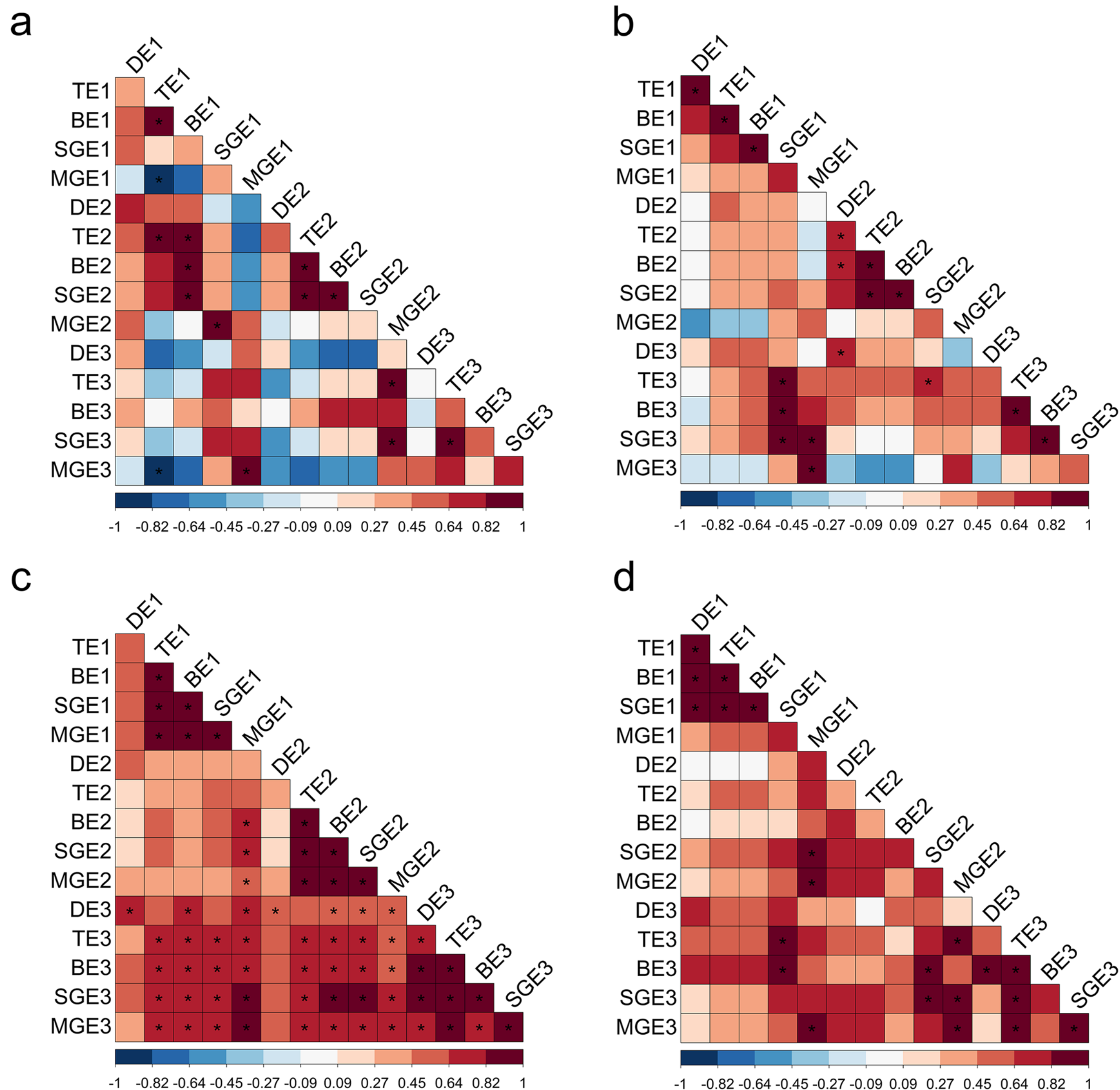

Fig. 5 Correlation plots of qEEG. Abbreviations used for each oscillation PSD: DE1 delta electrode 1, TE1 theta E1, BE1 beta E1, SG E1 slow gamma E1, MG middle gamma E1, DE2 delta electrode 2, TE2 theta E2, BE2 beta E2, SGE2 slow gamma E2, MGE2 middle gamma E2, DE3 delta electrode 3, TE3 theta E3, BE3 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 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

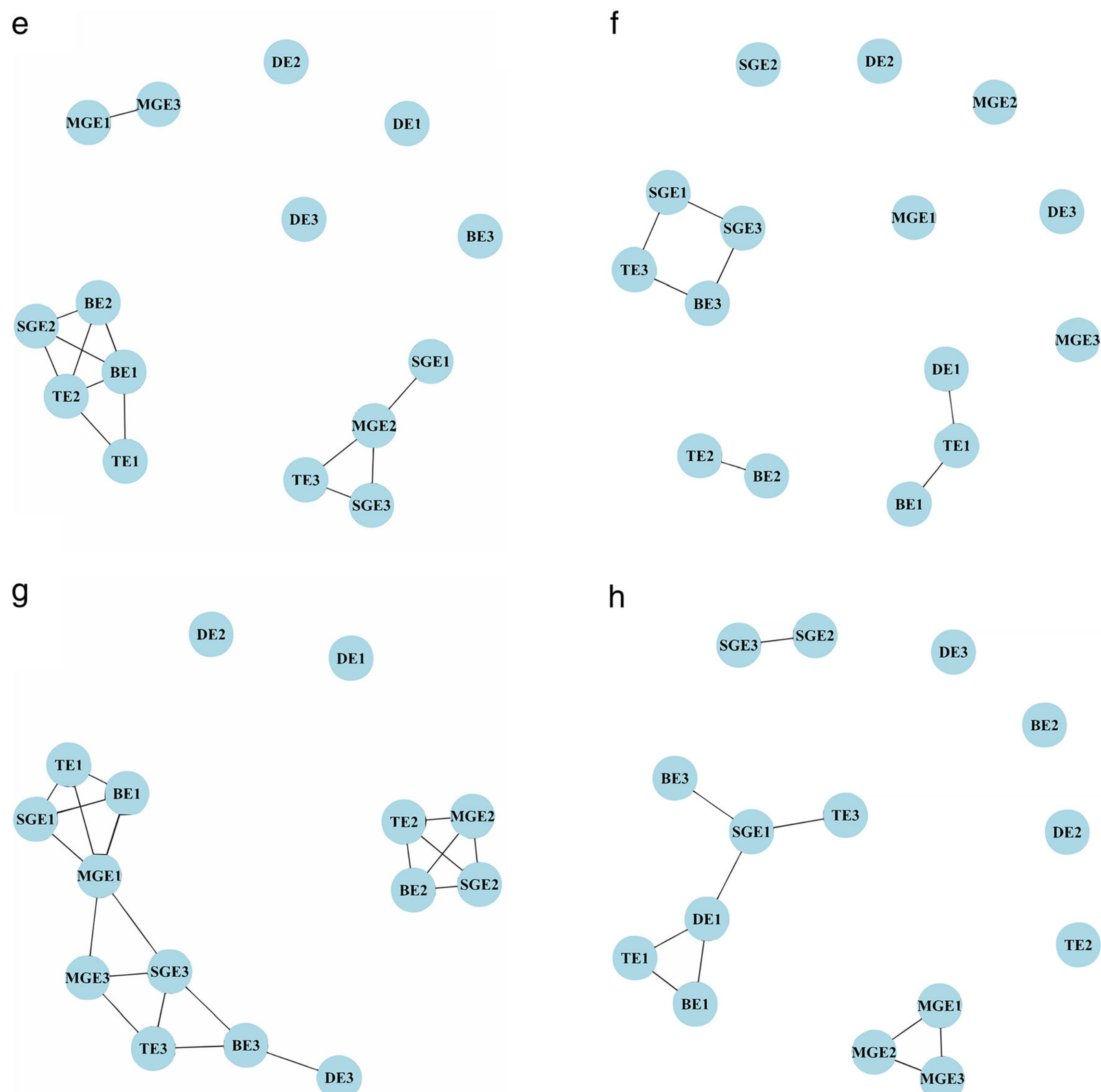

Fig. 6 Network analysis of q EEG. Abbreviations used for each oscillation PSD: DE1 delta electrode 1, TE1 theta E1, BE1 beta E1, SG E1 slow gamma E1, MG middle gamma E1, DE2 delta electrode 2, TE2 theta E2, BE2 beta E2, SGE2 slow gamma E2, MGE2 middle gamma E2, DE3 delta electrode 3, TE3 theta E3, BE3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

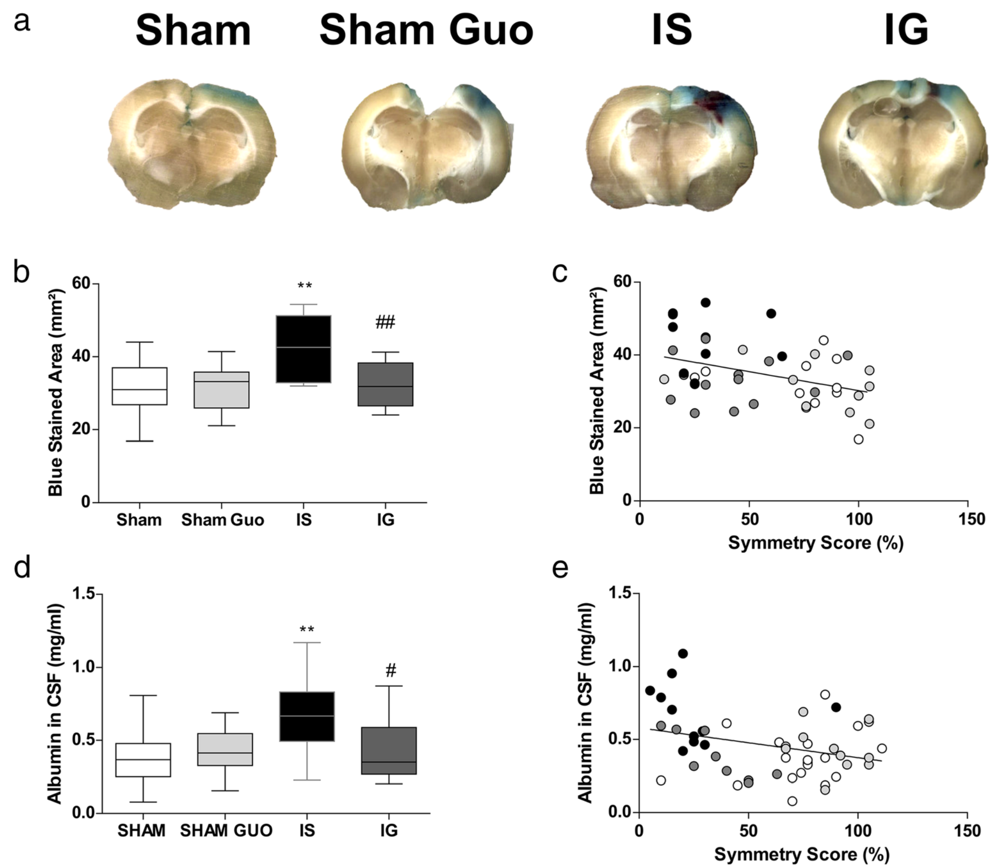

Fig. 7 Evaluation of BBB permeability. **a** Representative slices of Evans blue staining for each group. **b** Quantification of blue-covered area. **c** Correlation (each dot as an animal, 11–12 animals per group) of symmetry score with blue-stained area ($R^2 = 0.26$, $p < 0.005$). **d** Albumin CSF 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.

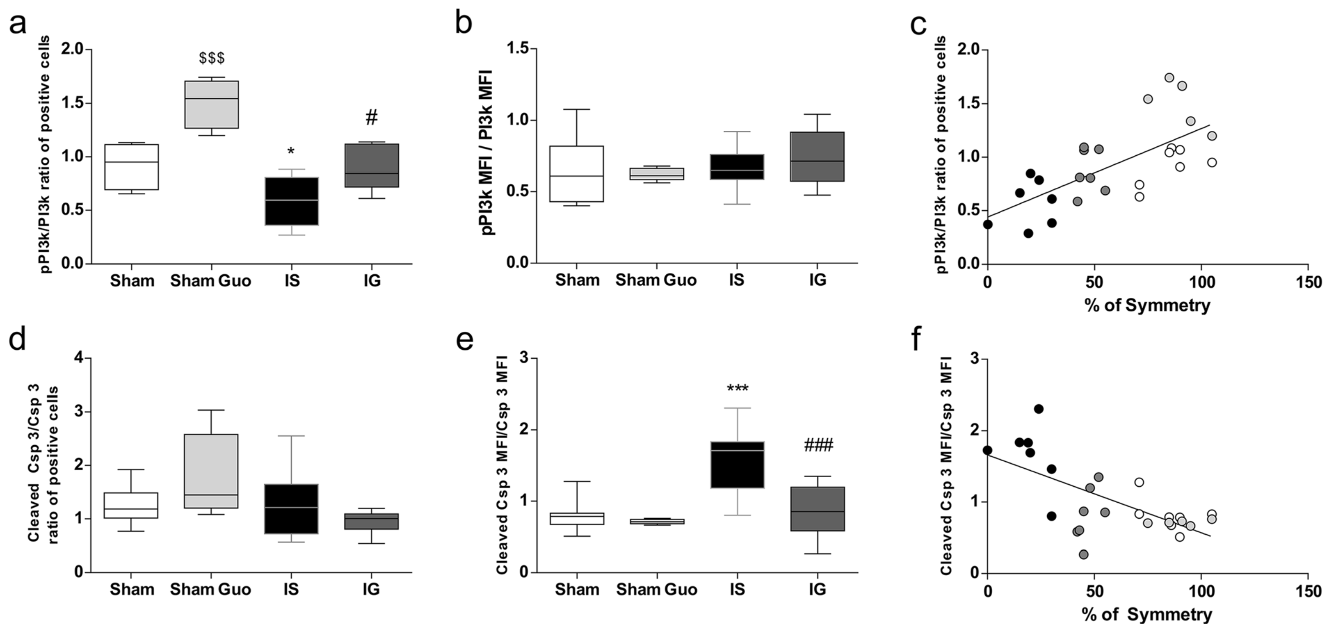

Fig. 8 Flow Cytometry of Signaling Pathways in the peri-infarct area of the Ipsilateral Cortex. **a** Ratio of pPI3k/PI3k-positive cells. **b** pPI3k/PI3kMFI ratio. **c** Dot plot correlating symmetry score and pPI3k/PI3k-positive cells ($R^2 = 0.46$, $p = 0.0001$). **d** Cleaved Caspase 3/Caspase 3 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 $^{\$\$\$}p < 0.005$ 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 × 1/2 in. length) [31] and immediately put on ice and centrifuged at 10,000×*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 μl of CSF sample was mixed with 500 μl of reagent from the bromocresol green commercial kit (Doles, 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×*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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Complementos aos Resultados

Em relação aos animais alocados no protocolo de EEG, um período de imobilidade em vigília de sessenta segundos foi escolhido com o objetivo de reduzir o efeito de artefatos no sinal eletroencefalográfico. Esses animais, em geral, apresentam um comportamento letárgico 48 horas após o protocolo de indução de isquemia. Durante os trechos observados não foram detectados traçados compatíveis com períodos ictais/correspondentes a crises epiléticas, bem como comportamentos estereotipados característicos desses estados.

Além disso, para obtenção da Fig. 5, foram utilizadas as PSD de cada faixa de freqüência analisadas, sendo correlacionadas em pares. O coeficiente de correlação de cada um destes pares originou um dos quadrados, sendo graficamente representados pelo gradiente de cores, sendo as cores "frias" correspondentes a coeficientes negativos, e as "quentes" aos coeficientes positivos.

A fim de esclarecer esse processo, abaixo foi representado um gráfico de pontos com dois parâmetros (PSD de Gama Médio [MG PSD] em E1 e E3) correlacionados.

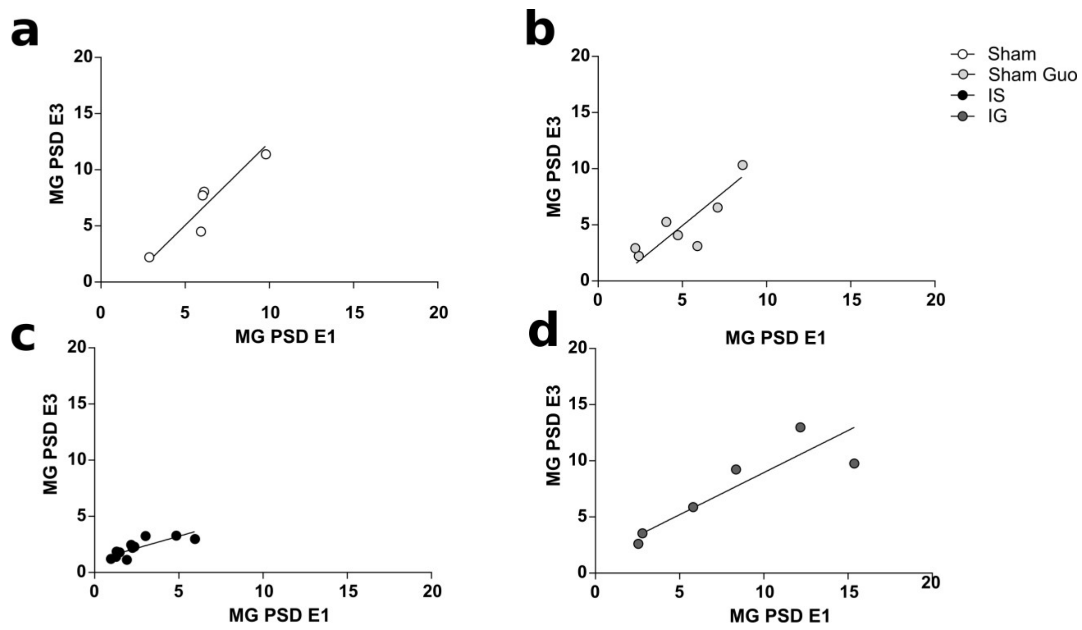

Fig 2.1. Gráfico de pontos representando as correlações entre PSD de dois eletrodos (E1 e E3) e as linhas de tendência respectivas dos quatro grupos analisados: **a.** Sham (Spearman rho = 0.96), **b.**Sham Guo (Spearman rho = 0.82), **c.** IS (Spearman rho = 0.84) e **d.** IG (Spearman rho = 0.94).

Capítulo II

Resultados complementares ao capítulo I, não submetidos à publicação em revista científica. Este capítulo está ligado ao objetivo geral de determinar a janela terapêutica ótima para In-Guo. Também se relaciona com objetivo específico 2: determinar os efeitos da Guo em parâmetros histológicos e citológicos, perante o dano isquêmico.

1. Metodologias Complementares.

1.1. Animais e Protocolos Experimentais de Isquemia

Nesse estudo, foram utilizados ratos Wistar machos, pesando de 350 a 450 gramas, obtidos do biotério do Departamento de Bioquímica, Instituto de Ciências Básicas, UFRGS, e do Centro de Reprodução de Animais de Laboratório, mantidos em ciclos claro/escuro de 12h, temperatura de 22 ± 1° C, com alimentação e água fornecidas *ad libitum*.

Para indução de isquemia, utilizou-se a metodologia previamente descrita no **Capítulo I** dessa tese.

1.2. Escolha De Esquemas de Administração Intranasal de Guo.

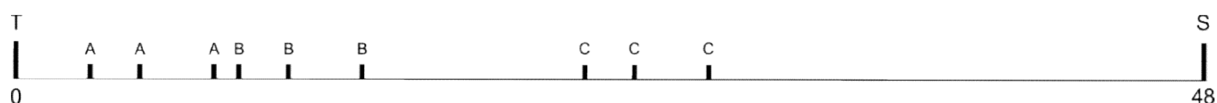

Fig. 2.1 Esquemas de administração intranasal de Guo/Solução salina. **T**, momento de termocoagulação dos vasos piais (tempo 0). **S**, primeira avaliação de simetria de patas anteriores (tempo 48h). **A** - 3h,5h,8h após T0, **B** - 9h,11h,14h após T0, **C** - 24h,26h,29h após T0.

Inicialmente, a fim de fundamentar a escolha do esquema de administração de Guo, realizou-se a avaliação comportamental a longo prazo, com diferentes janelas terapêuticas **(Fig 2.1)**. Além disso, os animais também

foram alocados para os grupos Sham (somente craniotomia) e IS (craniotomia e termocoagulação, com tratamento com solução salina).

1.3. Teste de Simetria dos Membros Anteriores

A análise comportamental foi realizada por meio do teste do cilindro, descrito anteriormente no capítulo I (de Vasconcelos Dos Santos, da Costa Reis et al. 2010, Hansel, Ramos et al. 2014, Hansel, Tonon et al. 2015). Esse teste consiste em determinar o número de vezes que o animal coloca as patas dianteiras na parede do cilindro, de 20 x 30 cm, onde está contido. Ele é dado por concluído quando o animal atinge um total de 20 colocações, contando-se a quantidade das patas, esquerdas, direitas e ambas simultaneamente, que se apoiaram na parede do cilindro. O escore de simetria é calculado conforme metodologia descrita no artigo do capítulo I.

Os testes comportamentais, sempre conduzidos pelo mesmo experimentador sem conhecimento dos grupos experimentais, foram realizados dois dias antes da cirurgia e nos seguintes períodos posteriores a ela: 2, 7, 14, 21, 28, 35 e 42 dias.

1.4. Processamento e Coleta de Amostras

As amostras foram coletadas 48 horas após a indução de isquemia, sendo realizada perfusão cardíaca e exsanguinação, com a utilização de solução fisiológica salina (0,9%), até que a ausência de conteúdo hemático fosse visualmente confirmada. Após, caso a amostra fosse designada para o protocolo de histologia, administrou-se, da mesma maneira, aproximadamente 50 ml de paraformaldeído 4% (Sigma, St. Louis, MO,USA) em PBS 0,1M pH 7,3.

Para os animais alocados no protocolo de citometria de fluxo, amostras do tecido cerebral adjacente à lesão isquêmica e da região equivalente no hemisfério contralateral foram coletadas, conforme descrito por Hansel e colaboradores (Hansel, Tonon et al. 2015), e posteriormente dissociadas em 1 ml de PBS 0,1 M, pH 7,3 contendo 1 mg/ml de colagenase IV.

1.5. Histologia

Após a coleta, as amostras foram fixadas em solução contendo paraformaldeído a 4% (Sigma, St. Louis, MO, USA), diluído em PBS 0,1M pH 7,3 por 48 horas; posteriormente realizaram-se secções coronais nas coordenadas centrais relativas à lesão isquêmica (A.P -2 to -4). Essas secções foram parafinizadas, sendo novamente seccionadas em 4µm por meio de micrótomo, e, em seguidas, fixadas em lâminas.

As lâminas, após desparafinização em estufa a 55ºC durante 45 minutos,foram mergulhadas em xilol 100% por 10 minutos. Depois disso, passaram por um processo de hidratação gradual do tecido, sendo imersas, por 10 minutos em cada passo, nas seguintes soluções: álcool 90%, 80%, 70%, 50% e água destilada.

Em seguida, cada lâmina foi imersa em hematoxilina de Harris por 5 minutos com o objetivo de corar componentes ácidos, predominantes no núcleo das células. As amostras foram lavadas em água destilada por 10 minutos e imersas em eosina por 10 minutos, corante que reage a componentes básicos, predominantes no citoplasma celular.

Posteriormente, realizaram-se duas lavagens em água destilada por 10 minutos para retirar o excesso de corante; as lâminas foram submetidas à

desidratação gradual com álcool (até 90%) e, então, mergulhadas em xilol. Finalmente, a montagem foi feita com meio DPX (Distrene Plasticiser Xylene) e a cobertura, com lamínulas de vidro.

1.6. Mensuração de Volume de Infarto

As imagens foram capturadas pelo microscópio Olympus FLUOVIEW FV1000, com aumentos de 4x, 10x e 40x. Para se estimar o volume de infarto, um observador com experiência em histologia, cegado para a alocação dos grupos, avaliou as de 4x e delimitou as áreas correspondentes ao núcleo da lesão e à parte circundante, com predominância de apoptose, referente ao peri-infarto. Dessa forma, as áreas demarcadas foram posteriormente quantificadas com o software Image J (NIH, Bethesda, MD, USA) e normalizadas em mm^2 por meio do diâmetro da objetiva em cada campo de aumento 4x.

1.7. Contagem de Células por Citometria de Fluxo

Posteriormente à dissociação em PBS contendo colagenase IV, as amostras foram filtradas, centrifugadas a 10000 g por 10 minutos a 4ºC, lavadas duas vezes com PBS, permeabilizadas com Triton X-100 e bloqueadas com soro de albumina bovina a 1% por 15 minutos.

Essas amostras foram incubadas por uma hora em solução de bloqueio contendo anticorpo anti-GFAP e anti-β-Tubulina III, ambos na proporção de 1:100 (Dako, CA, USA). As células, lavadas novamente duas vezes com PBS, foram, então, incubadas por uma hora com solução de bloqueio contendo AlexaFluor 488 anti-Rabbit e anti-Mouse na proporção de 1:200 (Jackson ImmunoResearch Laboratories, Inc, PA, USA).

A quantidade de eventos positivos para GFAP e β Tubulina III foi determinada pelo citômetro de fluxo (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA). A emissão dos fluorocromos foi registrada pelos canais específicos, de acordo com o seu comprimento de onda, usando-se o software CellQuest Pro (Becton Dickinson, Franklin Lakes, NJ, USA).

Dados de 10000 eventos foram adquiridos – descontados os artefatos, definidos por tamanho e limiar superiores ao especificado – utilizando-se de amplificação logarítmica. As análises foram realizadas por meio do software FlowJo7.6.3 (Treestar, Ashland, OR), a partir de gráfico de pontos (“*dotplots”)* de densidades, com eixo x de FL1 e eixo y de FL3. Analisaram-se dois parâmetros: a porcentagem de eventos positivos para cada marcação (expressada por meio de porcentagem do controle) e a média de intensidade de fluorescência (MFI).

1.8. Estatística

Para análise dos dados, utilizaram-se os softwares: Prism 6.0, R (v3.6.2, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Áustria) e Rstudio (RStudio: Integrated Development Environment for R).

As variáveis foram avaliadas quanto à simetria de suas distribuições; para aquelas que fossem simétricas, foi utilizada a análise de variância (ANOVA) de uma via, com correção para comparações múltiplas pelo método de Tukey. Para analisar variáveis com dependência temporal, utilizou-se a ANOVA de duas vias com medidas repetidas, sendo também utilizado o método de Tukey.

Aquelas variáveis com amostra pequena (i.e, volume de infarto n=3 por grupo, com três fatias por animal) tiveram suas diferenças analisadas pela diferença entre as medianas e o cruzamento de seu intervalo de confiança pela unidade, obtido por 10000 simulações, com uso de reamostragem (“*bootstrap*”).

2. Resultados

2.1. Simetria das Patas Anteriores

Em vista do exposto, observou-se que os esquemas terapêuticos, cuja primeira dose foi administrada três e nove horas após a termocoagulação dos vasos piais, foram efetivos na prevenção do déficit motor no escore de simetria em 42 dias. No entanto, 48 horas após a indução de isquemia, somente o grupo tratado com Guo 3 horas após o insulto apresentou diferença estatisticamente significativa em relação ao tratado com placebo **(Fig 2.2)**.

Dessa forma, determinou-se que, para avaliações de curto prazo, cujo objetivo seria encontrar parâmetros associados à neuroproteção da Guo, como os apresentados no capítulo I, seria mais adequado o uso da janela de três horas, ainda que o esquema terapêutico iniciado em nove horas apresente maior potencial translacional. Além disso, também foi observado que a administração de Guo 24 horas após o dano induzido por termocoagulação não apresentou capacidade de prevenir o déficit motor em nenhum dos tempos avaliados **(Fig. 2.2)**.

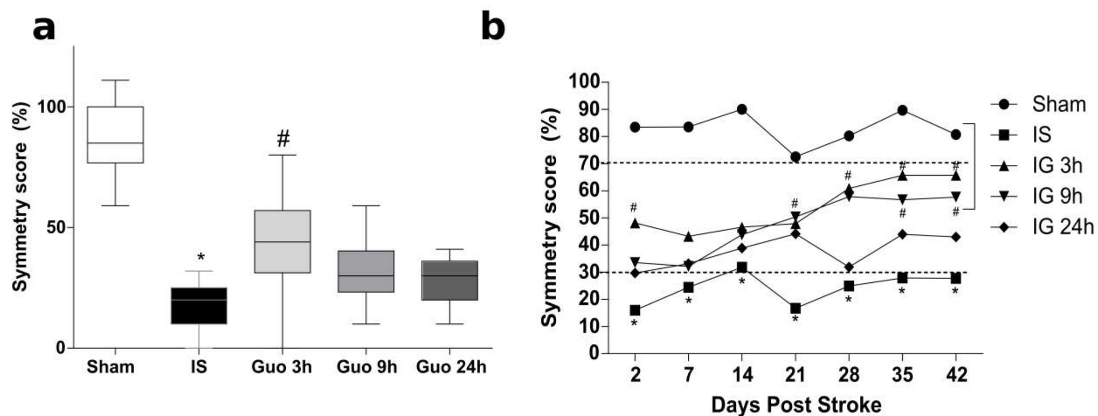

Fig. 2.2. Simetria das patas anteriores pelo teste do Cilindro. **a.** Escore de simetria 48 horas após a indução de isquemia. **b.** Linha temporal da Simetria das patas dianteiras até 42 dias após a indução de isquemia. Expressadas médias nos pontos, sendo omitidos os desvios padrão a fim de melhorar visualização.*p<0.05 comparado com o grupo Sham,# p<0,05 comparado com o grupo IS, ambos em cada ponto de tempo, análise de duas vias de variância para medidas repetidas (Tempo e Tratamento), comparações múltiplas corrigidas pelo método de Tukey.

2.2. Avaliação Histológica por H&E e Área de Infarto

Qualitativamente, as fatias dos animais correspondentes ao grupo Sham não apresentaram neurônios e células da glia com sinais de picnose/núcleos aumentados, restritos à área I. Todavia, os animais alocados no grupo IS (isquemia por termocoagulação tratado com solução salina) apresentaram predominância de células anucleadas/núcleo picnótico inteiramente basófilas em todas as áreas avaliadas (Fig 2.3), predominância de tipos celulares característicos de astrócitos, presença dos tipos celulares gemistocísticos (células astrocitárias caracterizadas por pouca marcação por eosina em seu citoplasma e considerável marcação por hematoxilina em seus limites).

Comparativamente, aqueles animais submetidos ao protocolo de isquemia e tratados com Guo evidenciaram esses tipos celulares restritos

às áreas I e II, também sendo possível observar uma camada de células fibrosas na região III e delimitar células consideradas apoptóticas e saudáveis.

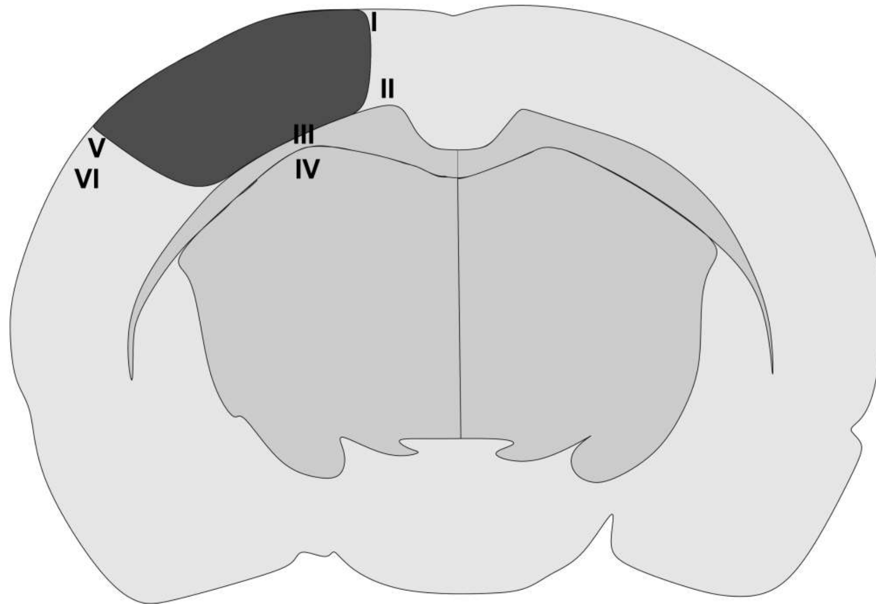

Fig. 2.3. Figura representativa das fatias analisadas por microscopia óptica, a área escura é correspondente à área afetada pela lesão provocada pela termocoagulação dos vasos pia. As letras representamos campos analisados.

Uma vez que se tornou possível delimitar essas áreas, realizou-se a quantificação da área de infarto, sendo utilizadas 10000 simulações pelo método de "*bootstrap*" para apresentar intervalos de confiança robustos, devido ao pequeno tamanho amostral. Observou-se, inclusive, um aumento significativo da área de infarto do grupo IS em relação ao Sham **(Fig. 2.4)**, da mesma maneira que foi percebida uma redução significativa daqueles animais alocados no grupo IG em relação aos do grupo IS **(Fig. 2.4)**.

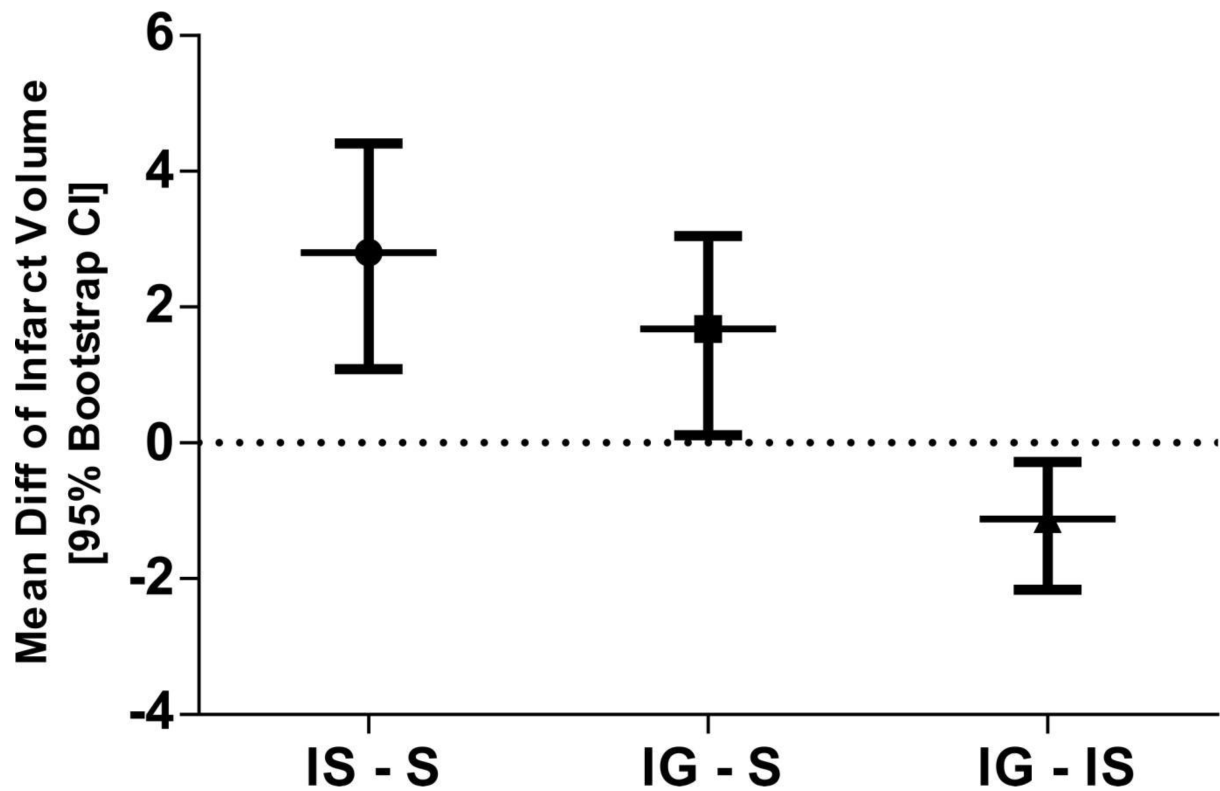

Fig. 2.4. Diferença entre as médias das áreas de infarto. No eixo y estão apresentadas as médias pontuais, com seus limites inferiores e superiores estimados por 10000 simulações “bootstrap”, sendo consideradas estatisticamente significantes as diferenças que não cruzassem ou contivessem no seu intervalo de confiança 95% ao valor nulo.

2.3. Citometria de Fluxo/Parâmetros celulares

Inicialmente foi realizada a avaliação dos astrócitos e neurônios pelos marcadores GFAP e β Tubulina III respectivamente, ambas relativas ao citoesqueleto desses tipos celulares, 48 horas após a indução de isquemia por termocoagulação.

Não se observou diferença estatisticamente significativa no número de eventos positivos para β Tubulina entre os grupos em ambos os hemisférios **(Fig. 2.5)**. Porém, há diferença entre os controles Sham e os grupos isquemia no conteúdo correspondente à média de intensidade de fluorescência (MFI)

das células positivas para o hemisfério ipsilateral à lesão, medida proporcional ao conteúdo desse marcador nas células analisadas **(Fig. 2.5)**. Possivelmente, isso pode ser explicado pelo fato de que há uma redução nos processos dendríticos no dano agudo.

Em relação aos eventos positivos para GFAP na zona peri-infarto contralateral **(Fig. 2.5)**, o grupo isquemia, tratado com solução salina, apresentou um aumento estatisticamente significativo em relação aos grupos Sham. O tratamento com In-Guo mostrou-se capaz de prevenir esse efeito. Assim sendo, foi reforçado o aspecto global do modelo de termocoagulação e do tratamento com Guo, o que motivou a realização de análises em outros parâmetros para confirmação desse efeito, sendo alguns já expostos no capítulo I.

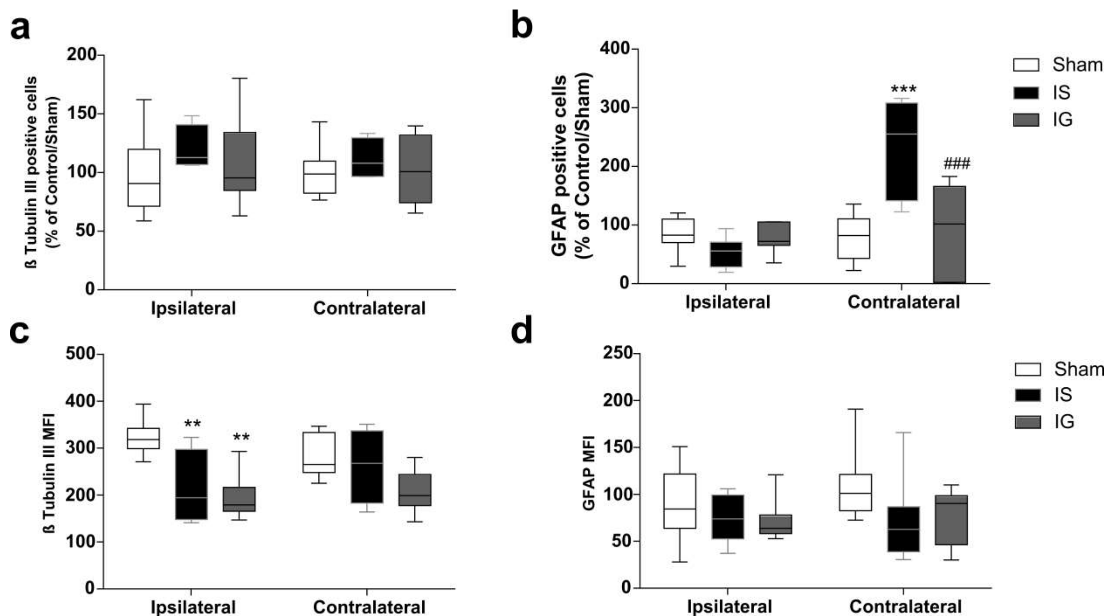

Fig. 2.5. Análise de citometria de fluxo de marcadores de astrócitos (GFAP) e neurônios (β Tubulina III) (Sham n = 9, IS n =7, IG=7). **a.** percentagem do controle de células marcadas positivamente para β Tubulina III. **b.** percentagem do controle de células marcadas positivamente para GFAP. **c.** Média de fluorescência de intensidade das células marcadas positivamente para β Tubulina III. **d.** Média de fluorescência de intensidade das células marcadas positivamente para GFAP.

PARTE III - DISCUSSÃO E CONCLUSÕES

1. Análise Comportamental a Longo Prazo

Já há algum tempo, tinham sido descritos os efeitos do tratamento com Guo a curto e médio prazo (Hansel, Ramos et al. 2014), com prevenção parcial da simetria das patas anteriores, uma vez que os grupos tratados com diferentes concentrações de Guo apresentaram escores inferiores aos grupos controle (Sham).

Em contrapartida, no presente trabalho, o uso da via intranasal preveniu o déficit comportamental induzido pela isquemia cerebral, já que os animais tratados com Guo apresentaram recuperação gradual, atingindo escores semelhantes aos daqueles alocados nos grupos Sham, no 42º dia após a indução de isquemia. Nesse protocolo, particularmente, foram destacados o cegamento dos experimentadores e a randomização dos animais alocados.

Além disso, os dados não publicados mostraram uma recuperação parcial a longo prazo dos animais isquêmicos tratados com Guo, mesmo quando esta foi administrada pela via intranasal, nove horas após a termocoagulação.

2. Efeitos da Guo nas Oscilações Corticais em Modelos de Isquemia e Injúria Cerebral.

Pela primeira vez, o efeito da isquemia por termocoagulação foi caracterizado em parâmetros das oscilações corticais registradas no EEG, tendo apresentado resultados distintos de outros modelos de isquemia focal permanente. Em modelos com a oclusão da ACM, observaram-se resultados diversos, sendo, no entanto, predominantes os estudos que demonstraram

atenuação das ondas de frequência mais alta (Gama Alta, Média e Baixa, Beta) e aumento relativo das ondas de frequência mais baixa (Alfa e Delta) (Sheorajpanday, Nagels et al. 2009, Sheorajpanday, Nagels et al. 2011, Sheorajpanday, Nagels et al. 2011).

Verificou-se, então, a supressão de todas as ondas nos animais isquêmicos tratados com solução salina, um padrão similar ao observado na redução abrupta de fluxo sanguíneo cerebral nos modelos animais com isquemia global (Schmidt-Kastner, Paschen et al. 1989) e no clampeamento da carótida, em humanos (Tan, Garcia-Toca et al. 2009). Esse padrão é reforçado pelo aumento da correlação global entre as potências espectrais das ondas em diferentes regiões, a qual mostrou um acometimento geral da rede, causado pelo modelo de isquemia por termocoagulação. Outros estudos observaram um padrão semelhante em casos de hipoperfusão severa, nos quais há um aumento transitório da sincronia global do EEG (Hofmeijer, Tjepkema-Cloostermans et al. 2014).

Da mesma forma, a administração de Guo, nesse trabalho, também apresentou efeitos diferentes dos previamente observado. Já foram descritos, em abundância, os efeitos da Guo sobre o sistema glutamatérgico, os quais podem estar correlacionados às alterações eletrofisiológicas demonstradas por Torres e colaboradores, com um efeito da Guo na modulação da PSD das ondas Teta em relação a animais tratados somente com ácido quinolínico (Torres, da Silva Filho et al. 2010).

O tratamento com Guo preveniu a supressão das PSD causada pelo dano isquêmico com poucas restrições a faixas de frequência, assim como teve

efeito protetor no hemisfério contralateral em relação à lesão isquêmica. Além disso, também apresentou uma correlação entre a melhora do escore de simetria e as potências espectrais das frequências avaliadas, o que reforça o efeito neuroprotetor dessa molécula. A Guo teve, ainda, efeito na prevenção do aumento de GFAP pela isquemia, na zona peri-isquêmica contralateral, corroborando seu efeito “global”.

O contraste entre os efeitos da Guo, descritos em estudos eletrofisiológicos anteriores, e os verificados nesse trabalho pode estar relacionado a alguns fatores: primeiro, a intensidade do dano causado pelo modelo, uma vez que os efeitos da termocoagulação não se restringem somente ao sistema glutamatérgico (Hansel, Ramos et al. 2014, Hansel, Tonon et al. 2015), diferentemente do tratamento com ácido quinolínico. O segundo fator pode ter relação com a administração de Guo pela via intraperitoneal, afetada pelo metabolismo periférico e de primeira passagem, apresentando, portanto, menor biodisponibilidade em relação à via intranasal (Ramos, Muller et al. 2016). As alterações de supressão das oscilações corticais registradas no EEG podem estar relacionadas ao edema consequente à ruptura da BHE, tendo em vista que autores mostraram alterações na análise quantitativa das EEG, quando administradas soluções hipertônicas com a finalidade de aumentar a permeabilidade da BHE (Huang, Dong et al. 2002).

3. Alterações da BHE em Modelos de Isquemia Cerebral e Efeitos da Guo em sua Permeabilidade

Os modelos de isquemia cerebral propõem diversas abordagens para a avaliação da disfunção da BHE em modelos animais, sendo predominantemente usados o azul de Evans e a Sódio Fluoresceína (Saunders, Dziegielewska et al. 2015). Há diversas críticas em relação à acurácia dessas avaliações, ainda que revisões as proponham como medidas qualitativas (Saunders, Dziegielewska et al. 2015).

Nesse trabalho, optou-se por duas avaliações da permeabilidade da BHE: uma histológica/qualitativa; outra relacionada a aspectos clínicos. Dessa maneira, observou-se, no presente modelo, a presença de disfunção da BHE por dois métodos e a prevenção dessa disfunção pelo tratamento com Guo. Esse efeito está correlacionado aos aspectos abordados por Hansel e colaboradores (Hansel, Ramos et al. 2014, Hansel, Tonon et al. 2015) nos quais níveis de interleucinas pró e anti-inflamatórias mostraram-se diferentes entre os dois grupos. Ainda assim, é difícil dissociar essas duas alterações, no sentido de que a inflamação sistêmica causa o aumento da permeabilidade da BHE (Muresanu, Sharma et al. 2019). Aqueles autores avaliaram parâmetros relacionados ao estresse oxidativo (Hansel, Ramos et al. 2014, Hansel, Tonon et al. 2015, que também resulta em dano endotelial (Muresanu, Sharma et al. 2019). Além disso, a hiperestimulação dos receptores NMDA está parcialmente ligada às vias de produção de ROS, reagindo sinergicamente (Vazana, Veksler et al. 2016) a outros estímulos,o que culmina em um aumento significativo da permeabilidade capilar.

Também relacionadas ao efeito da Guo sobre o dano isquêmico e a BHE estão as vias de sinalização ligadas à rota mTOR e à apoptose, sendo que a PI3k e as caspases 3 e 9 foram particularmente estudadas (Jin, Song et al. 2011, Zehendner, Librizzi et al. 2011, Lopez-Ramirez, Fischer et al. 2012, Qian, Dou et al. 2018).

Por conseguinte, os efeitos da Guo encontram-se ligados a diversas vias que convergem para prevenir o dano à BHE, dado que foi confirmado pelos achados desse trabalho. Ainda assim, não há evidência de que esses efeitos sobre a BHE não sejam mediados, fazendo-se necessários, por isso, testes para averiguar o efeito direto da Guo sobre a BHE e desta sobre o escore de simetria.

4. Parâmetros Celulares e de Sinalização Relacionados à Neuroproteção da Guo

Alguns autores sugerem que mecanismos de ação da Guo sejam mediados pelas vias da proteína alvo molecular da rapamicina (mTOR), com estudos conduzidos em modelos de OGD. Neles, a administração de inibidores dessa rota, como LY294002, rapamicina e wortmanina, é capaz de inibir o efeito antiapoptótico da Guo em fatias hipocampais (Molz, Dal-Cim et al. 2011, Tasca, Lanznaster et al. 2018).

Tendo em vista isso, foram abordados vários parâmetros relacionados a vias de sinalização pró e anti-apoptóticas, sendo escolhidas para publicação aquelas afetadas pela termocoagulação de vasos piais e pelo tratamento com Guo: PI3k e Caspase 3. Diante disso, foi possível observar efeitos semelhantes aos apresentados na literatura, uma vez que o tratamento com Guo mostrou-se

capaz de prevenir o aumento relativo nas formas ativas da Caspase 3 e a redução na forma ativa da PI3k, causada pela indução de isquemia. Além disso, também se percebeu a presença de correlações entre esses parâmetros e o escore comportamental, o que reforça o papel dessas vias de sinalização na recuperação dos animais tratados.

Como consequência dos efeitos nas vias de sinalização resultantes do tratamento com Guo, também se observou uma prevenção do dano por parâmetros histológicos, uma vez que, comparado com o grupo IS, aqueles animais tratados com In-Guo apresentaram menor quantidade de núcleos disfuncionais, maior integridade dos tipos celulares na adjacência da área infartada que tem, em última instância, como resultado, menor área de infarto. Ainda que usualmente sejam apresentados os volumes de infarto medidos pela marcação com cloreto de trifenil-tetrazólio (metabolizado pela succinato desidrogenase, com produto um metabólito de cor vermelha, em tecidos “saudáveis”) (Bederson, Pitts et al. 1986) alguns autores sugerem que esse método tem baixa sensibilidade, quando utilizado em amostras extraídas 24 horas após a indução de isquemia (Zille, Farr et al. 2012). Em vista disso, esses autores sugerem que a avaliação da região infartada seja feita com colorações como H&E ou cresil violeta (Zille, Farr et al. 2012), particularmente para avaliações seriadas a longo prazo. Algumas características que advogam a favor do uso dessas colorações, principalmente da H&E, encontram-se na facilidade de como são realizadas e na possibilidade de estabelecerem correlações com exames anatomopatológicos. Como exemplo, a constatação da perda de afinidade por hematoxilina dos neurônios após 48 horas de

progressão de quadros isquêmicos, fenômeno conhecido como "*ghost-neurons*" (ou "*ghost-cells"*) (Jortner 2006).

Reforçando a avaliação predominantemente qualitativa das lâminas, os parâmetros da citometria de fluxo também foram afetados pela isquemia por termocoagulação. No entanto, não foram observadas diferenças no número de células no hemisfério ipsilateral à lesão, o que pode ser justificado por idiossincrasias da técnica, com baixa acurácia para células com integridade citoplasmática comprometida. Ainda assim, detectou-se redução no conteúdo de proteínas de citoesqueleto neuronais na região peri-infarto ipsilateral dos animais submetidos à isquemia, o que pode estar relacionado a achados de autores que mostraram um aumento do "turnover" de dendritos, com redução da densidade deles em uma semana após o evento isquêmico (Biernaskie and Corbett 2001, Brown, Li et al. 2007). Ainda assim, a fim de confirmar ambos os resultados, são necessárias abordagens com imunofluorescência com marcação para GFAP e ß Tubulina III, atualmente em fase de execução.

No hemisfério contralateral, a diferença foi relativa aos eventos classificados como astrócitos, que aumentaram no grupo IS, um fenômeno já reportado na literatura cujas consequências ainda não foram completamente esclarecidas (Pekny, Wilhelmsson et al. 2014). Esse fenômeno pode estar, porém, relacionado à formação da cicatriz glial e representar elevado grau de dano tecidual, ainda que haja divergências importantes entre os autores sobre esse aspecto (Giffard and Swanson 2005, Nedergaard and Dirnagl 2005, Pekny, Wilhelmsson et al. 2014, Takatsuru, Nabekura et al. 2014, Liu and Chopp 2016). O tratamento com In-Guo, por outro lado, preveniu esse aumento, o que, em conjunto com a formação de uma camada de células

fibróticas entre o tecido saudável e o disfuncional, apontada pela coloração de H&E, reforça o efeito protetor da Guo e sugere que esse tratamento é capaz de prevenir as alterações globais causadas pelo dano isquêmico. Reforçando esses achados, foram observados dados relativos ao qEEG que mostraram um padrão de redução de sincronicidade global nos animais tratados com In-Guo.

5. Conclusões e Perspectivas

Nessa tese foi demonstrado e reforçado o potencial neuroprotetor da Guanosina, em um modelo de isquemia cerebral. Claramente, notou-se que a In-Guo apresentou aumento da janela terapêutica, tendo sido capaz de reverter o déficit comportamental, mesmo quando administrada até nove horas após a indução do dano isquêmico. Ainda assim, para o estudo de alterações bioquímicas e morfológicas, a janela de três horas seja mais vantajosa, já se observaram resultados nos parâmetros comportamentais no primeiro momento de avaliação (48 horas). Diferentemente ocorreu quando se usou o esquema terapêutico com a primeira dose nove horas, pois somente podem ser observadas diferenças comportamentais significativas em relação ao grupo isquêmico tratado com placebo quatorze dias após a indução de isquemia.

Em relação ao qEEG, observa-se que o modelo de isquemia por termocoagulação afetou as oscilações corticais em ambos os hemisférios com uma importante redução no PSD das diferentes faixas de freqüência avaliadas. Por outro lado, o tratamento com In-Guo três horas após o dano foi capaz de prevenir essas alterações, o que é reforçado pelos resultados mostrados pelo mapa de correlações e a análise de redes, onde se percebe uma redução importante do estado de sincronia nos animais tratados com Guo em relação àqueles tratados com solução salina.

Foi reforçado, também, o aspecto global relacionado ao dano do modelo de termocoagulação dos vasos piais – considerado um modelo de isquemia focal –, uma vez que se apresentaram alterações no hemisfério contralateral à lesão. Além disso, observou-se que a Guo também foi capaz de prevenir esse dano, com uma janela terapêutica de três horas. Hipotetizou-se, portanto, que a prevenção global desse dano poderia estar relacionada ao impacto direto do tratamento com Guo na disfunção da BHE.

Em vista disso, o tratamento com Guo via intranasal (In-Guo), em janela terapêutica expandida, mostrou-se relevante a longo prazo, aspecto fundamental para a validação do uso desse agente neuroprotetor. Cabe ressaltar, também, a facilidade de administração da via Intranasal em relação a outras vias, o que aumenta o potencial translacional dessa abordagem. No entanto, tornam-se necessárias abordagens confirmatórias no modelo de ECRs, conforme proposto por outros autores (Llovera, Hofmann et al. 2015).

A interação entre a Guo e a BHE também precisa ser explorada, tendo em vista que mecanismos específicos não foram abordados nesse trabalho, sendo importantes, por isso, avaliação da inibição de receptores A1 e A2, bem como vias de sinalização relacionadas a mTOR e a resultante desses eventos no aumento de permeabilidade da BHE (Chojnowski, Opielka et al. 2021). Igualmente fazem-se necessárias elucidações sobre o efeito da Guo em um dano restrito ao endotélio e, por conseguinte, à BHE.

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ANEXOS

1. Artigo publicado na revista Purinergic Signalling:

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ORIGINAL ARTICLE

Intranasal guanosine administration presents a wide therapeutic time window to reduce brain damage induced by permanent ischemia in rats

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Abstract In addition to its intracellular roles, the nucleoside guanosine (GUO) also has extracellular effects that identify it as a putative neuromodulator signaling molecule in the central nervous system. Indeed, GUO can modulate glutamatergic neurotransmission, and it can promote neuroprotective effects in animal models involving glutamate neurotoxicity, which is the case in brain ischemia. In the present study, we aimed to investigate a new in vivo GUO administration route (intranasal, IN) to determine putative improvement of GUO neuroprotective effects against an experimental model of permanent focal cerebral ischemia. Initially, we demonstrated that IN [^{3}H] GUO administration reached the brain in a dose-dependent and saturable pattern in as few as 5 min, presenting a higher cerebrospinal GUO level compared with systemic administration. IN GUO treatment started immediately or even 3 h after ischemia onset prevented behavior impairment. The behavior recovery was not correlated to decreased brain infarct volume, but it was correlated to reduced mitochondrial dysfunction in the penumbra area. Therefore, we showed that the IN route is an efficient way to promptly deliver GUO to the CNS and that IN GUO

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treatment prevented behavioral and brain impairment caused by ischemia in a therapeutically wide time window.

Keywords Guanosine · Intranasal · Purines · Ischemia · Neuroprotection

Introduction

Guanine-based purines (GBPs), specifically the nucleoside guanosine (GUO) and the nucleotides guanosine-5′-mono-, di-, and triphosphate (GMP, GDP, and GTP, respectively), are endogenous molecules and members of the purinergic system, which are known to participate in several intracellular processes. In the central nervous system (CNS), in addition to their intracellular roles, GBPs present extracellular effects that suggest they are putative neuromodulator signaling molecules [1]. Indeed, GBPs can modulate neurotransmitter systems, particularly glutamatergic neurotransmission. Specifically, the nucleoside GUO has been shown to stimulate astrocytic glutamate (GLU) uptake [2, 3] and to inhibit GLU vesicular storage [4], thus modulating glutamatergic synaptic transmission. As GLU is the main excitatory neurotransmitter in the CNS, and excessive GLU signaling (excitotoxicity) is involved with several brain diseases [5, 6], the neuroprotective potential of GUO against excitotoxicity has been investigated. Accordingly, the exogenous administration of GUO has shown neuroprotective effects on CNS injury in several animal models linked to excitotoxicity events, including models of dementia [7], seizures [8, 9], neuropathic pain [10], stroke [11–15], and hepatic encephalopathy [16].

Although modulatory and neuroprotective potentials of GUO were strongly demonstrated, GUO mechanism of action is not fully understood. A putative binding site for GUO was already described in rat brain membranes [17], but in opposite, several evidences point to an adenosine (ADO) receptors contribution for GUO effects. Indeed, some, but not all of the observed neuroprotective effects of GUO, were blocked by ADO receptor antagonists, suggesting multiple mechanisms of action for GUO [18–22]. Certainly, GUO effects seem to be directly mediated by action on the brain, as intracerebroventricular or intracortical GUO infusion in experimental models of excitotoxicity have demonstrated neuroprotection [8, 13]. Nevertheless, most of the neuroprotective effects exerted by GUO were observed after its systemic (intraperitoneal [IP] or oral) administration [7, 9–16]. In this regard, systemic administration routes present important limitations for protocols with endogenous drugs that aim to reach the brain but can be metabolized in the systemic pathway, which is the case for GUO. GUO can be enzymatically degraded to guanine both extra- and intracellularly and subsequently reduced to xanthine (XAN) and uric acid (UA). Moreover, the interconversion among guanine- and adenine-based purines, such as ADO and its derivatives inosine (INO) and hypoxanthine (HYPO), plays an important role in maintaining purine homeostasis [23, 24]. Therefore, a long systemic pathway could decrease the amount of GUO that reaches the brain by metabolization and/or uptake, consequently decreasing the effectiveness of the administered dose [25, 26].

An intranasal (IN) route of administration appears to be a relevant alternative for delivering compounds to the CNS. Several works have demonstrated transport of macromolecules across the nasal epithelia, sometimes faster and higher than the conventional intravascular pathway [27–31]. Currently, it is thought that substances that are able to cross the nasal epithelial barrier can reach the brain through components associated with the olfactory and trigeminal nerves after IN administration. Subsequently, they are rapidly distributed throughout the brain by convective perivascular space flow. Hence, the IN route of administration may be a particularly attractive drug delivery method to manage cerebrovascular diseases, such as stroke [27, 28]. Accordingly, therapeutic proteins and other molecules were delivered to ischemic brains, suggesting that IN administration is clearly a promising route for stroke therapy [29–31]. Thus, IN GUO administration could offer a potential means of improved delivery to the brain.

Recently, we have shown neuroprotective effects of IP treatment with GUO early after permanent focal cerebral ischemia [11, 12], which caused a significant and long-lasting recovery of the impaired behavioral function and decreased the extension of brain infarct volume. These effects were related to modulation of the glutamatergic system, cellular redox environment, and inflammatory cytokine balance in the penumbra area (periphery of lesion), the main presumptive site of endogenous restorative processes and, thus, of the therapeutic target site. Like the conventional drugs to manage stroke, IP GUO was shown to be effective when administered immediately after the induction of experimental brain ischemia [11, 12].

Taken together, in the present work, we aimed to investigate the neuroprotective potential of IN GUO following brain ischemia as an alternative strategy to avoid GUO metabolization and compromised blood circulation as a consequence of ischemia. Thus, we induced a permanent focal cerebral ischemia in rats searching for a therapeutic time window for IN GUO treatment. Preliminarily, we evaluated pharmacokinetic parameters of IN GUO administration and compared them with an IP route using radiolabeled [^{3}H] GUO administration and purine levels determination in brain structures, blood plasma, and cerebrospinal fluid (CSF).

Materials and methods

Chemicals

GUO, acetonitrile (ACN), trifluoroacetic acid (TFA), and methyl triphenyl tetrazolium chloride (TTC) were purchased

from Sigma (St. Louis, MO, USA). Methacrylate resin was from Leica®, and methylene blue was obtained from Merck®. [^{3}H] GUO (specific activity 15 Ci/mmol) was obtained from American Radiolabeled Chemicals (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 and obtained from standard suppliers.

Animals

One hundred fifty adult male Wistar rats (60–90 days old) were obtained from the animal facility house of the Department of Biochemistry, ICBS, UFRGS. The animals were kept under a 12-h light/dark cycle (light on at 7:00 am) at a temperature of 22 ± 1° C. They were housed in plastic cages (five per cage) with water and commercial food available ad libitum. The animals were anesthetized by IP administration of both ketamine hydrochloride (50 mg/kg) and methyl xylazine (10 mg/kg). The IP injection volume was 1 ml/kg. Animals were sacrificed by decapitation using a guillotine. All experiments were performed following the rules of the Ethics Committee on Animal Use - CEUA-UFRGS. The protocols were approved by the CEUA-UFRGS, Project No.: 22319.

Preparation and administration of IN [^{3}H] GUO solution

A mother solution of unlabeled GUO (120 mg/ml) was prepared on the day of each experiment, and it was used to prepare all the different concentrations of GUO used in the study. Unlabeled GUO at each concentration specified was mixed with a fixed amount of radiolabeled [^{3}H] GUO (0.5 mCi/ml, specific activity 15 Ci/mmol). The mixture of unlabeled GUO with tracer amounts of [^{3}H] GUO was named as [^{3}H] GUO solution.

Six rats per each condition analyzed were anesthetized, and the [^{3}H] GUO solution was administered to both nostrils of each animal at the bottom of the nasal septum with a pipette tip with a long edge. The administered volume of [^{3}H] GUO solution was applied slowly, and the interval between each nostril administration was approximately 2 min. After the time interval specified for each group protocol, blood samples were transcardially collected and the animals were decapitated and the brain structures (i.e., olfactory bulb, parietal cortex, hippocampus, and cerebellum) were dissected and processed.

Rats that received no interference, such as anesthesia, surgery, and/or treatment, were considered control animals that were designated as "naïve" group in each analyses. At the end of the experiments, all naïve animals were grouped into a single population to consider inter-experimental variability (eight rats in total). Thus, the control group was identical for all comparisons in this work.

Evaluation of pharmacokinetic parameters of IN [^{3}H] GUO solution administration

We investigated dose curve and time distribution of IN [^{3}H] GUO into the rat brain and blood plasma. The dose curve of IN [^{3}H] GUO solution was evaluated by applying 0.05 ml per nostril of different concentrations of [^{3}H] GUO solution (2.5, 7.5, 15, 30, and 60 mg/ml) and assessing the radioactivity content and purine levels in brain structures and blood plasma 15 min after administration. Based on the unlabeled GUO concentration and the radioactivity detected for each solution administered, we estimated the dose curve for [^{3}H] GUO exposure of each brain structures and blood plasma. To achieve the IN [^{3}H] GUO time curve, we applied 30 mg/ml IN [^{3}H] GUO solution, 0.05 ml per nostril, and evaluated the radioactivity content and purine levels after 5, 15, 60, 120, and 180 min.

Comparison between IN and IP [^{3}H] GUO administration

The [^{3}H] GUO solution (30 mg/ml) was injected IN (IN GUO, 0.05 ml per nostril) or IP (IP GUO 0.1 ml). After 5 min, the radioactivity and purine levels of the brain structures, CSF, and plasma samples were determined.

Brain structure processing

The olfactory bulb, parietal cortex, hippocampus, and cerebellum were dissected from the brain, weighed, and the right hemisphere structures were used for quantification of radioactivity (by liquid scintillation counter Hidex 300 SL), and the left hemisphere structures were used for determination of purines by high performance liquid chromatography (HPLC) measurements. The following procedure was performed for both hemispheres according to [25] with minor modifications; the structures were homogenized in 0.6 ml of 7 % TFA, except the bulb, which was homogenized in 0.3 ml of 7 % TFA for deproteinization. Next, the homogenates were centrifuged at 10,000×*g* at 4 °C for 10 min, and the supernatants were collected. Aliquots of 0.2 and 0.4 ml from the olfactory bulb and other structures, respectively, were used to measure the radioactivity content. To analyze purines by the HPLC method, an aliquot of 0.1 ml was neutralized with 0.075 ml of 1.5 M potassium bicarbonate ($KHCO_3$). After, the samples were filtered through cellulose 0.22 μm pore size membrane and finally stored at −70 °C.

CSF and plasma sampling

CSF samples (approximately 0.1 ml) were removed by direct puncture of the cisterna magna with an insulin needle in a stereotactic apparatus. Each sample was centrifuged at 10, 000×*g* at 4 °C for 10 min to obtain a cell-free supernatant.

This supernatant was filtered through a cellulose 0.22 μm pore size membrane, and the samples were individually stored at −70 °C.

Blood was withdrawn (approximately 2 ml) by cardiac puncture and added to collection tubes containing sodium citrate as an anticoagulant. The samples were centrifuged at 5000×*g* for 10 min and the supernatant (plasma) was collected. An aliquot of 0.4 ml was added to 0.6 ml of 7 % TFA to deproteinization. After, the samples were centrifuged at 10, 000×*g* at 4 °C for 10 min and the supernatant was collected. A supernatant aliquot (0.4 ml) was collected for radioactivity analysis. Another aliquot (0.1 ml) was submitted to the same process of neutralization and storage as brain structures and it was used for purines analysis.

HPLC

Determination of the purines concentration in CSF, blood plasma, and brain structures was performed using the methodology of HPLC described previously [7]. The injected sample volume was 0.02 ml. The following purines were analyzed: ADO, INO, HYP, GUO, XAN, and UA.

Evaluation of the neuroprotective potential of IN GUO treatment in the cerebral ischemia model

To investigate the neuroprotective potential of IN GUO treatment, we used a permanent focal cerebral ischemia model in rats and evaluated parameters of behavioral functionality (cylinder test), histological damage (infarct volume by TTC stained), and mitochondrial dysfunction at the penumbra area (MitoTracker^{TM} analyses).

Before any surgical manipulation, we performed cylinder test (described below) in all animals to exclude potential asymmetric rats. Following this preliminary test, the animals were randomly separated into two main groups: ischemia saline (IS; craniotomy with induced ischemia followed by saline solution administration) and GUO ischemia (IG; craniotomy with induction of ischemia followed by GUO treatment). Of note, we previously showed that animals submitted only to surgical procedures (craniotomy) without thermocoagulation of pial vessels (SHAM) showed no behavioral impairment or brain histological damage [11, 12]. The IG group was subdivided according to the administration schedule: IG 0 h (immediately, 1, 3, and 6 h after surgery), IG 1 h (1, 3, and 6 h after surgery), or IG 3 h (3, 5, and 8 h after surgery). Saline or 30 mg/ml GUO solutions were IN administered (0.05 ml per nostril). The saline group received four administrations of saline solution (NaCl 0.9 %) immediately, 1, 3, and 6 h after surgery. For saline and/or GUO administration 3 to 8 h after the surgery, animals were anesthetized again with half of the initial anesthetic dose. The concentration of GUO solution for neuroprotective experiments was chosen based on the pharmacokinetics profile of IN administration. The administration schedule was initially chosen based on previous studies by our research group [11, 12]. After induction of ischemia, further analyses were performed by investigators blind to the treatments.

Induction of permanent focal ischemia

Ischemic injury was induced by thermocoagulation of pial blood vessels in the motor and sensorimotor cortices by approaching a hot probe to the meninges [11, 12]. This procedure resulted in the degeneration of the six cortical layers just below the affected blood vessels [32]. Briefly, the animals were anesthetized and placed in a stereotaxic apparatus. The skull was surgically exposed and a craniotomy was performed, exposing the left fronto-parietal cortex (+3–6 mm and AP + LL 2–0 mm bregma). After the procedure, the skin was sutured and the body temperature was maintained at 37 °C using a heating pad until recovery from anesthesia.

Measurement of sensorimotor activity

This procedure was based on the spontaneous exploratory behavior of rodents. It consisted of placing the animal into a glass cylinder (20 cm in diameter and 30 cm high) and counting the first 20 forelimb contacts with the cylinder walls. Occurrences of contact with exclusive use of the ipsilateral limb (to the injury) or contralateral limb or the use of both limbs were recorded separately. The animals were submitted to a pre-test prior to surgery and a post-test 48 h after surgery. The score of symmetry for each animal was calculated each day by the formula previously described [11, 12, 32].

Measurement of the volume of the brain infarcted area

After the cylinder procedure, the animals were euthanized and the brains were quickly removed from the skulls and sectioned in a coronal plane at 2 mm thickness using a rat brain matrix (Insight LTDA, Ribeirão Preto, SP, Brazil). The slices were immersed for 30 min in 2 % TTC at 37 °C followed by overnight fixation in 4 % paraformaldehyde. The infarct volume was calculated by the formula: volume = measurement of infarct area × slice thickness (2 mm). The brain slices were analyzed by the software Image J. The results are expressed in mm^3.

Dysfunctional mitochondria measurement

Tissue samples (30 mg) from the side of the lesion of parietal cortex, located between the lesion and the cerebral longitudinal fissure (a piece measuring approximately 8 mm × 6.2 mm) was dissected after rat transcardial perfusion with saline in accordance with recent studies by our group [11, 12]. This

region was chosen because it has characteristics similar to the penumbra area [11, 12, 32]; therefore, it was designated as the penumbra area. The samples were dissociated in 0.1 M phosphate-buffered solution (PBS), pH 7.4, containing 0.1 mg/ml of collagenase IV. After the dissociation, the samples were decanted for 15 min and an aliquot of supernatant was collected and incubated with MitoTracker™ Green FM and Red FM dyes (100 nM each). Mitotracker™ green provides an estimation of mitochondrial quantity and mass, and red provides a measure of the mitochondrial potential. Taken together, these data (higher mitochondrial mass and lower potential) indicate mitochondria dysfunction [33]. The emission of fluorescence was measured by a band pass green (FL-1; 530 nm/30) and red (FL-3; 670 nm long pass) using CellQuest Pro software (Becton Dickinson, Franklin Lakes, NJ, USA) and data from 10,000 events were acquired. All flow cytometric analyses were performed using Flow Jo software 7.6.3 (Treestar, Ashland, OR).

Statistics

An unpaired Student's t test was used to compare radioactivity measurements between IN and IP [^{3}H] GUO groups. Purine levels evaluated by HPLC in brain structures, blood samples, and CSF were analyzed by a one-way ANOVA followed by Bonferroni's post-test. Kruskal Wallis test followed by Dunn's post-test was used to analyze the neuroprotective effects of different administration schedules of IN GUO treatment on sensorimotor activity and on brain infarct volume, comparing each different treatment protocol with IS. Dysfunctional mitochondrial cell data were analyzed by one-way ANOVA followed by a Bonferroni's post-test. Post-test statistical powers were calculated after each analysis of neuroprotective effects and are shown in the Supplementary Material. Correlations were analyzed by a Pearson's correlation analysis. Differences were considered significant at $p \leq 0.05$ probability.

Results

Pharmacokinetic analysis following IN [^{3}H] GUO solution administration

Radioactivity detection

Radioactivity was detected in all investigated brain structures (i.e., olfactory bulb, parietal cortex, hippocampus, and cerebellum) with the olfactory bulb presenting higher radioactivity levels (Fig. 1). The dose curve indicated that IN [^{3}H] GUO exposure was increased in brain structures by increasing the GUO amount administered (Fig. 1a), pointing to a dose-dependent profile. Saturation was achieved with the dose of 30 mg/ml IN [^{3}H] GUO solution. Regarding the time curve, no significant difference was observed in radioactivity content from 5 to 180 min after IN [^{3}H] GUO solution administration (Fig. 1b).

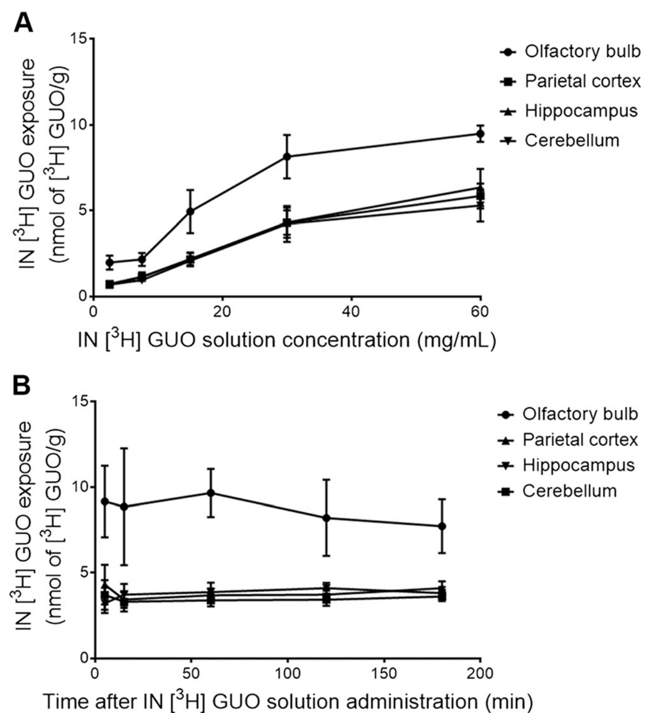

Fig. 1 Brain structures. **a** Dose curve of IN [^{3}H] GUO administration. Radioactivity detected 15 min after administration of 0.05 ml per nostril. **b** Time curve. Radioactivity detected after administration of 30 mg/ml of IN [^{3}H] GUO solution, 0.05 ml per nostril. Each point represents the mean ± SE. $N = 6$ animals per group

Similar to brain structures, the dose curve indicated a dose-dependent profile of [^{3}H] GUO after IN [^{3}H] GUO solution administration in blood plasma (Fig. 2a). In contrast to brain structures, no saturation level was reached even in the higher doses administered (Fig. 2a), and higher radioactivity levels were observed over time (Fig. 2b).

HPLC measurements

No significant difference in GUO levels analyzed by HPLC was observed in any brain structure between naïve rats and rats that received IN [^{3}H] GUO solution of each different tested conditions (concentration and time curves; Supplementary Tables 1 and 2). Only XAN and UA profiles showed statistically significant differences at specific conditions; a significant increase in XAN level in the olfactory bulb, 15 min after 7.5 mg/ml of IN [H^3] GUO solution (Supplementary Table 1), and an increase in UA level in the parietal cortex, 60 min following 30 mg/ml of IN [H^3] GUO solution administration (Supplementary Table 2).

We observed that only ADO plasma levels were significantly increased 15 min after animals received 15 to

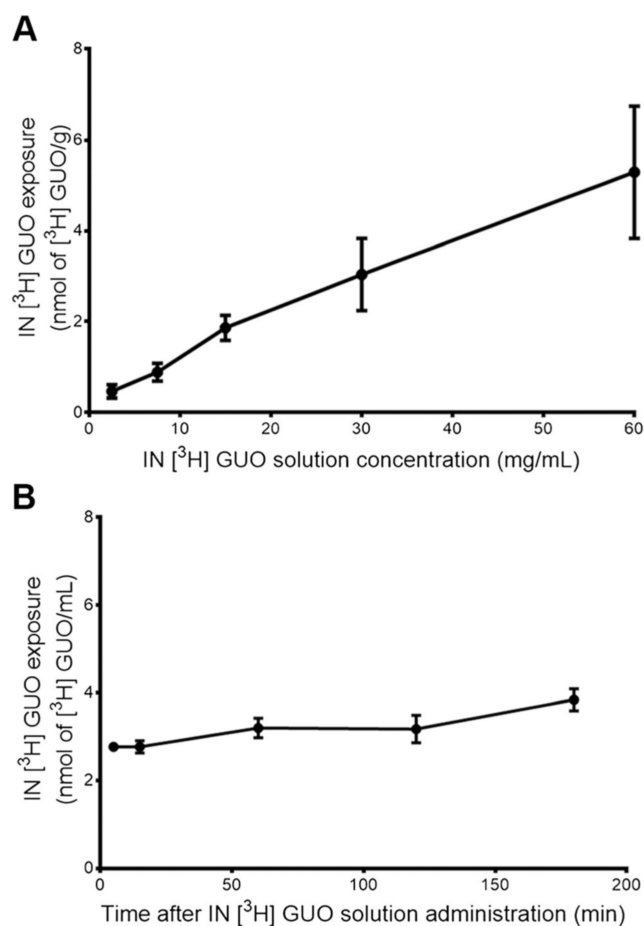

Fig. 2 Blood plasma. **a** Dose curve of IN [^{3}H] GUO administration. Radioactivity detected 15 min after administration of 0.05 ml per nostril. **b** Time curve. Radioactivity detected after administration of 30 mg/ml of IN [^{3}H] GUO solution, 0.05 ml per nostril. Each point represents the mean ± SE. $N = 6$ animals per group. *$P < 0.05$ compared to the naïve group

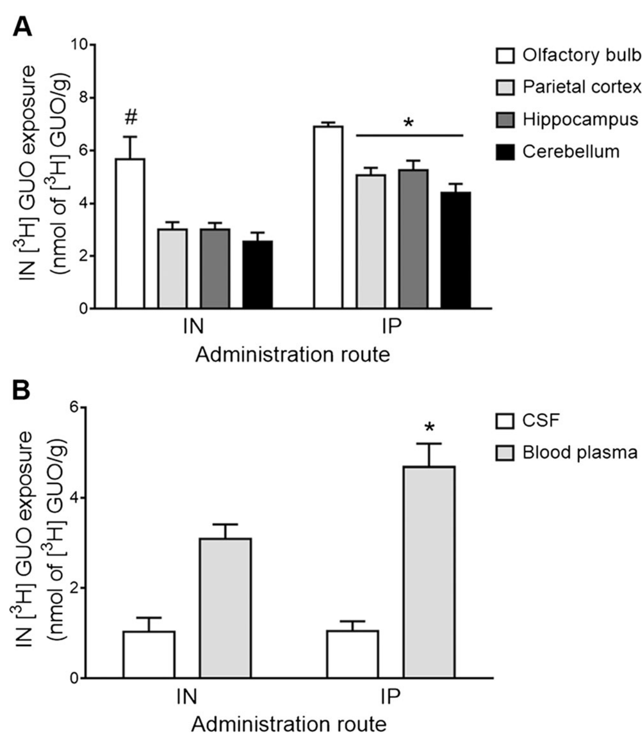

Fig. 3 Comparison of injection routes for [^{3}H] GUO solution administration. The radioactivity in: **a** different brain structures and **b** the CSF and blood plasma, five minutes after IN (0.05 ml per nostril) or IP (0.1 ml) 30 mg/ml [^{3}H] GUO administration. Data are expressed as the mean ± SE. $N = 6$ (brain structures and plasma) or 3–4 (CSF) animals per group. *$P < 0.05$ compared to IN groups

60 mg/ml of IN [^{3}H] GUO solution (Supplementary Table 3A). This selectivity of the increase in ADO levels was confirmed in the time curve (Supplementary Table 3B). A significant decrease in XAN levels was observed from 60 to 180 min following 30 mg/ml of IN [^{3}H] GUO solution (Supplementary Table 3B). Blood plasma GUO and HYP levels were not possible to be detected due to its value not reaching the HPLC inferior detection limits (data not shown). This inability seems to be related specifically to the use of TFA 7 % in purines extraction protocol, as GUO plasma levels could be observed using different extraction compounds [7, 16, 25, 26]. Notably, we chose TFA 7 % based on a pilot experiment which we tested different chemicals to homogenize brain tissue and blood samples to obtain the highest radioactivity extraction derived from [^{3}H] GUO (TCA 10 %, TFA 7 %, ACN, methanol, perchloric acid; data not shown).

Comparison between IN and IP [^{3}H] GUO solution administration

Five minutes after administration of 30 mg/ml IP (0.1 ml) and IN (0.05 ml per nostril) [^{3}H] GUO solution, the radioactivity was detected in brain structures (Fig. 3a). The radioactivity levels detected in the olfactory bulb were higher compared to other brain structures after IN but not IP administration. The radioactivity levels in the parietal cortex, hippocampus, and cerebellum were significantly higher after IP than IN administration.

HPLC analyses confirmed no significant change in endogenous purine levels in brain structures after IN [^{3}H] GUO solution administration, while IP [^{3}H] GUO solution administration caused a small number of significant increases, i.e., UA in the olfactory bulb, INO in the hippocampus, and GUO and INO in the cerebellum (Supplementary Table 4).

CSF was collected from six animals of each naïve, IN, and IP GUO groups; however, only four, three, and three CSF samples were obtained without blood contamination, respectively. The radioactivity levels in the CSF were not significantly different between the two administrations (Fig. 3b), while in blood plasma it was significantly higher after IP than IN administration. In both protocols, the radioactivity levels in

blood plasma were significantly higher than in the CSF. Significantly higher ADO levels were observed in blood plasma 5 min after IP, but not IN [^{3}H] GUO solution injection compared to naïve rats. The concentrations of other analyzed purines showed no significant differences between the groups in blood plasma (Table 1). In contrast, IN [^{3}H] GUO solution administration significantly increased the levels of GUO, XAN, UA, INO, and HYP in the CSF, especially GUO, INO, and HYP levels that were 2.5, 2.1, and 4.5 times compared to naïve animals. The concentrations of other analyzed purines in the CSF showed no significant differences between the groups (Table 1).

Evaluation of the neuroprotective potential of IN GUO administration

All animals presented a symmetry rate of approximately 90 % in the cylinder test before surgery (data not shown). Ischemic insult reduced the symmetry to approximately 25 %. IN GUO treatments improved the symmetry rate (Fig. 4, Supplementary Table 5).

Brain TTC staining indicated that permanent focal ischemia induced a lesion in the cerebral cortex of the left

Table 1 Levels of endogenous purine and metabolites in the CSF or blood plasma, measured in naïve animals and in animals at 5 min after IN (0.05 ml per nostril) or IP (0.1 ml) [^{3}H] GUO administration. Levels of endogenous purines and metabolites in blood plasma (A) and CSF (B): comparison between IN and IP [3H] GUO administration

Nucleoside and metabolites	Näive	IN [3H] GUO	IP [3H] GUO
A. Blood plasma			
GUO	n.d.	n.d.	n.d.
XAN	0.90 ± 0.17	0.82 ± 0.11	0.70 ± 0.11
UA	10.46 ± 0.78	11.49 ± 2.79	11.15 ± 2.48
ADO	0.56 ± 0.15	1.05 ± 0.25	1.55 ± 0.26*
INO	3.60 ± 0.26	3.43 ± 0.61	3.12 ± 0.46
HYP	n.d.	n.d.	n.d.
B. CSF			
GUO	0.10 ± 0.02	0.25 ± 0.04*	0.20 ± 0.04
XAN	2.41 ± 0.07	3.28 ± 0.26*	2.04 ± 0.09
UA	2.39 ± 0.08	3.24 ± 0.21**	1.91 ± 0.03*
ADO	0.18 ± 0.02	0.14 ± 0.03	0.17 ± 0.02
INO	0.67 ± 0.09	1.43 ± 0.26*	0.44 ± 0.04
HYP	2.31 ± 0.33	10.38 ± 2.01*	3.06 ± 0.46

GUO guanosine, *XAN* xanthine, *UA* uric acid, *ADO* adenosine, *INO* inosine, *HYP* hypoxanthine. $N=6$ (plasma) or 3–4 (CSF) animals per group.*$P<0.05$ compared to respective control group, $^{\#}P<0.05$ compared both to respective control and IP groups. Values are expressed as mean ± SE in nmol/ml

*$P<0.05$ compared to the naïve group, **$P<0.05$ compared to the naïve and IP [3H]GUO groups

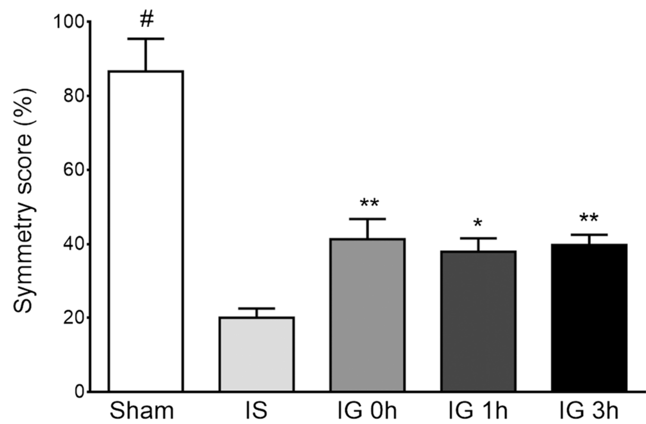

Fig. 4 Performance in the cylinder test 48 h after ischemia. Symmetry score of rats receiving IN treatment of saline (IS, $N=21$) or GUO through a protocol starting immediately (IG 0 h, $N=16$), 1 h (GUO 1 h, $N=9$) or 3 h (GUO 3 h, $N=19$) after ischemia induction. The symmetry score index and the schedule of administration for each treatment are described in material and methods. $^{\#}P<0.0001$ compared to IS, IG 0 h, IG 1 h, IG 3 h groups; *$P<0.05$ compared to IS group; **$P<0.001$ compared to IS group. Data are expressed as the mean ± SE

hemispher measured at 48 h after surgery (Fig. 5). IN GUO administration decreased the infarct volume only when the treatment started immediately after ischemia induction (IG 0 h; Fig. 5a-b, Supplementary Table 6). No statistical correlation between the symmetry score and the infarct volume was found (Pearson's $R^2=0.016$, $P=0.41$; Fig. 5c).

We further evaluated functional mitochondrial integrity in cortical penumbra area of animals that received IN GUO treatment starting 3 h after ischemia onset since this experimental group presents a more interesting therapeutic time window compared to the others. The volume and morphological complexity analyses together with the Mitotracker™ incorporation (mass and potential mitochondrial measurement) through flow cytometry indicated that permanent focal ischemia induced injury to neural cells in the penumbra area of the parietal cortex of the ipsilateral, but not of the contralateral hemisphere. Notably, IN GUO treatment starting 3 h after ischemic insult (IG 3 h) significantly reduced mitochondrial dysfunction when compared to the IS group (Fig. 6a, Supplementary Table 7). We observed a statistical correlation between functional behavior recovery and mitochondrial dysfunction in the penumbra area (Pearson's $R^2=0.39$, $P=0.006$; Fig. 6b).

Histological analysis of the nasal septum was performed to verify the integrity of the mucosa after IN GUO treatment (IG 0 h group). No histological alteration was observed (Supplementary Figure 1).

Discussion

In the present work, we conducted pharmacokinetic evaluation of GUO IN administration to determine when, how, and where GUO could reach the brain. We observed a dose-

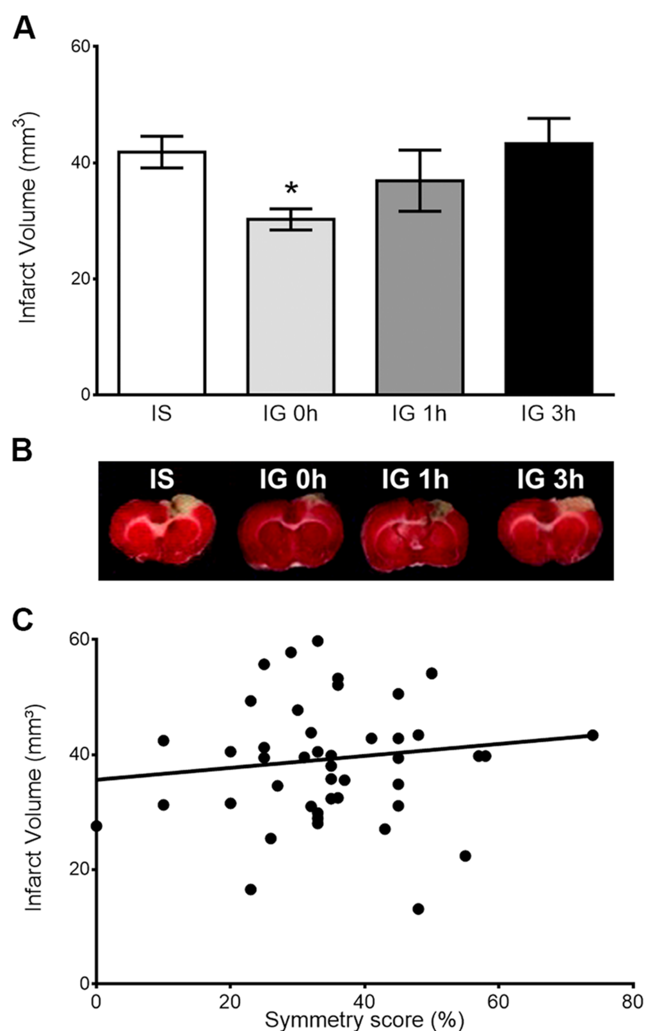

Fig. 5 Parameters of brain infarct measured 48 h after cerebral ischemia. **a** Volume of brain infarct of rats receiving treatment with saline (IS, $N = 14$) or GUO starting immediately (GUO 0 h, $N = 16$), 1 h (GUO 1 h, $N = 9$) or 3 h (GUO 3 h, $N = 12$) following ischemia induction. **b** Representative images of brain TTC staining of experimental groups. **c** Correlation (IS and GUO 0H groups) between cortex infarct volume and forelimb function (symmetry score) measured 48 h after ischemia, Pearson's $R^2 = 0.016$, $P = 0.41$. *$P < 0.05$ compared to IS group

dependent and saturable pattern of GUO exposure in brain structures. Although, to our knowledge, this was the first time that IN GUO administration was investigated, the delivery of nucleoside analogs to the CNS has been demonstrated by several works through IN administration in rodents [34, 35]. Indeed, nucleoside transporters were found in the nasal epithelia, pointing out that the uptake of GUO by their specific transporters can facilitate its delivery to the brain [36]. The pathway by which GUO applied to the nostril reaches the CNS seems to be reached as fast as only 5 min post-injection. This result is in accordance with other studies, which demonstrated that different drugs could reach maximal CNS concentrations immediately after administration [37, 38]. No significant difference was observed in GUO endogenous levels in the brain structures following IN [^{3}H] GUO solution administration in any condition tested in this work. These results could be expected as GUO exposure on each brain structure was lower than the variability of total endogenous purines among naïve animals.

In blood plasma, IN [^{3}H] GUO administration increased ADO levels 15 min after injection of 15–60 mg/ml IN GUO. Previously, we reported that chronic oral GUO treatment was also able to increase ADO plasma levels [7]. Some works in the literature have focused attention on the interplay of extracellular ADO and GUO levels. An experimentally based hypothesis is that GUO caused an increase in extracellular ADO levels by competing with nucleoside transporter [19, 20]. In fact, some of GUO's neuroprotective effects, but not all, are ADO receptor dependent [18–22, 39].

Interestingly, radioactivity amounts were higher in brain structures following IP than IN [^{3}H] GUO solution administration. As expected, significantly, more radioactivity amount was detected in the blood plasma after IP compared with IN [^{3}H] GUO administration; however, the same levels of radioactivity were detected in CSF. Taken together, these data support the hypotheses that GUO reaches CSF after IN administration by a pathway, at least partially, not involving systemic circulation and that brain structures take up purines directly from blood after IP administration. CSF purines analysis indicated that GUO levels were significantly increased after IN [^{3}H] GUO administration. Moreover, GUO direct metabolites, i.e., XAN and UA, as well as INO and HYP, which concentrations can be influenced by GUO, are also significantly increased after IN [^{3}H] GUO administration, demonstrating a fast delivery pathway to the CNS. CSF GUO levels increased after IP [^{3}H] GUO administration but did not reach a statistically significant difference. Except for ADO, no purine levels showed a significant difference compared to endogenous levels in CSF after IP [^{3}H] GUO administration. The increase of CSF ADO levels may be associated with the increased blood plasma ADO levels after IP [^{3}H] GUO injection. Together, these data reinforced the idea that the CSF may be a major source of distribution to the brain following IN administration while the purine levels after IP administration reflect blood levels. This hypothesis is reinforced by works of Jiang's group [19, 20] that showed, using a mixture of unlabeled and labeled [^{3}H] GUO (40 times more [^{3}H] GUO than was used here), that GUO is promptly and markedly metabolic breakdown after systemic administration reflecting in an increase of GUO metabolites in the brain [25, 26].

As we demonstrated that an IN route can be an efficient method for the delivery of GUO to the CNS, we further evaluated the IN GUO treatment neuroprotective potential in a brain ischemia model. Stroke, which mainly causes ischemic events, is the third cause of death worldwide and the main cause of chronic, severe adult disability. The current therapy can restore cerebral blood flow within a narrow time window

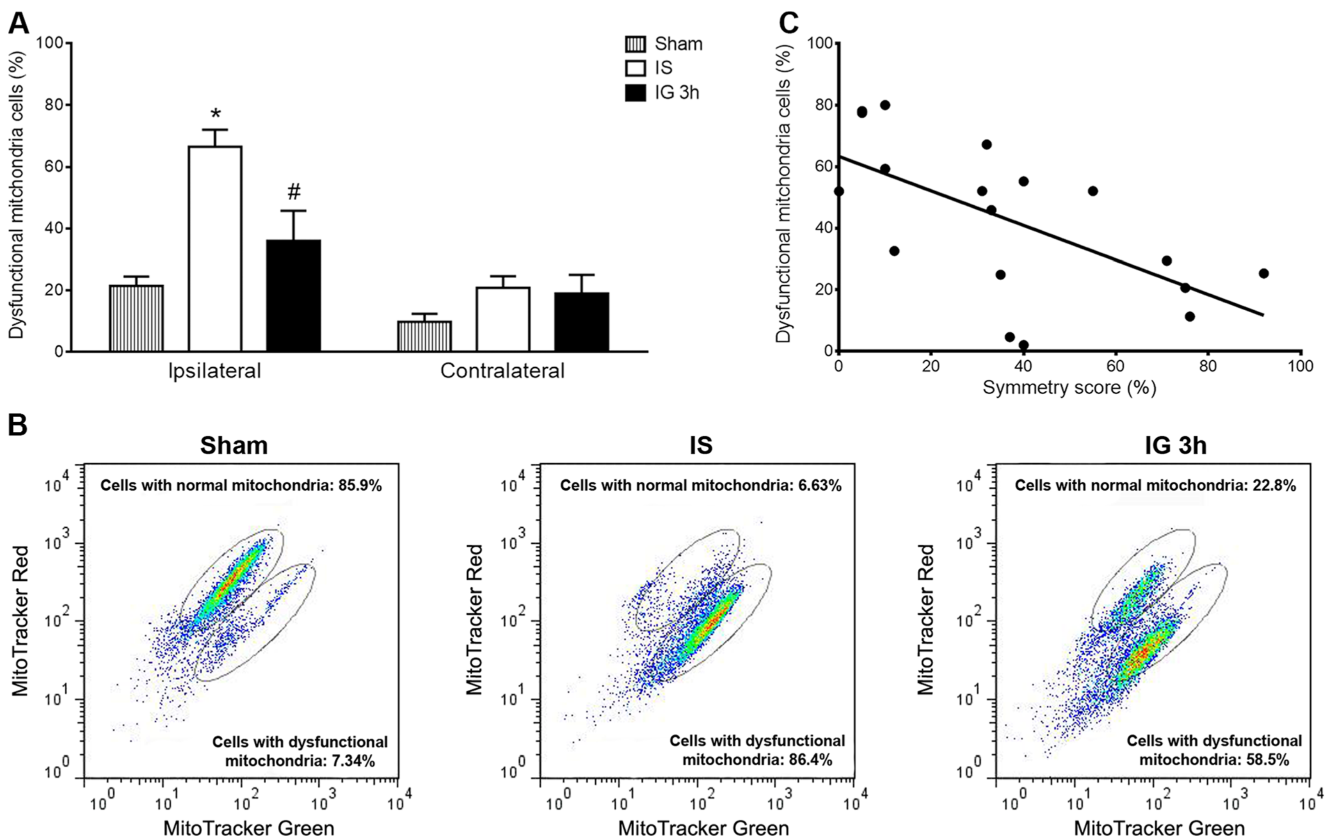

Fig. 6 Dysfunctional mitochondria in the penumbra zone (contralateral and ipsilateral sides) measured 48 h after ischemia induction: **a** percentage of dysfunctional mitochondria in the penumbra area from rats of SHAM ($N = 5$ animals), IS ($N = 7$ animals), and IG 3 h ($N = 7$ animals). The description of each experimental group is detailed in material and methods. **c** Correlation between infarct volume and forelimb function (symmetry score; Pearson's $R^2 = 0.39$, $P = 0.006$)

to prevent damage to the "penumbra" area, which surrounds the infarct core [40]. In order to increase the therapeutic time window, neuroprotection is an alternative or adjunct approach to thrombolysis, targeting the cerebral parenchyma in the acute ischemic phase [41].

The neuroprotective effects of the nucleoside GUO have been demonstrated after systemic administration in ischemia models by our and other groups [11–15]. We recently showed, using the same permanent focal cerebral ischemia model used in the present work, that systemic GUO treatment started immediately after ischemia induction improved functional recovery of behavioral impairment, simultaneously preventing brain oxidative stress, modulating glutamatergic parameters, attenuating changes in the inflammatory system, thus decreasing neuronal degeneration in the penumbra area [11, 12]. Additionally, other groups demonstrated that GUO treatment starting before a transient focal cerebral ischemia induction (middle cerebral arteria occlusion model [MCAO]) decreased brain infarct volume and improved neurological deficits, acting directly in the CNS [13, 15].

The main goal of the present work was to explore the neuroprotective potential of GUO through an alternative administration route, i.e., the IN pathway. In addition to being a non-invasive method, the advantage of the IN route is to be a direct pathway for endogenous drugs to be delivered to the CNS, decreasing the influence of systemic metabolism and the blood brain barrier, as we observed for GUO in our pharmacokinetics data. Moreover, the IN route presents alternative pathways to deliver drugs to the CNS that does not involve blood vessels, which are compromised in stroke [27, 28]. Remarkably, IN GUO treatment that was initiated immediately after ischemia onset prevented behavior impairment and decreased brain infarct volume with a dose almost seven times lower than the effective dose for IP administration [11, 12]. This effective result of IN compared to systemic administration was also observed for other neuroprotective drugs against ischemia damage [29, 30] .

Although the exact GUO mechanism of action is still not fully clear, GUO seems to counteract different steps following the ischemia cascade events [11, 12]. Thus, we tested different therapeutic schedules of treatment, increasing the time of the first dose administered after ischemia induction. We observed that IN GUO treatment starting 1 or 3 h after ischemia induction were efficient in preventing functional behavior impairment induced by ischemia but had no effect on brain infarct volume, evaluated by the TTC technique. Interestingly, no

statistical correlation between these two parameters was observed. In this way, it is important to consider that TTC methodology relies on the degree of dehydrogenase enzymes and cofactors functionality, but the tissue that stains positively is not necessarily health. Together, these results suggest that GUO may be acting in a different damaged region. The area between the pale-ischemic core and the red-colored healthy tissue represents the penumbra zone where brain tissue is damaged but not yet dead, thus comprising the transition zone between the core and the repairable zone that is the anatomical target of protective intervention [42, 43]. In agreement with the idea of neuroprotection action in the penumbra zone, we observed that IN GUO treatment starting 3 h after the insult statistically decreased the number of events containing dysfunctional mitochondria in the penumbra area. To reinforce this idea, we detected a statistical correlation between the mitochondrial status in the penumbra area and the behavioral performance. Our data are in agreement with our previous work, which reported a correlation between oxidative stress parameters in the penumbra area (but not infarct volume) and functional recovery of animals submitted to ischemia treated with systemic GUO [12]. However, the wide time window for therapy with GUO seems to be an outstanding outcome particular to the IN route, as systemic GUO injection was efficient only when administered before or immediately after ischemia onset [11–15].

In summary, we showed in the present work that the IN route is an efficient route to promptly deliver the neuroprotective nucleoside GUO to the CNS. Moreover, we demonstrated that IN GUO prevented behavioral injury in a much lower dose and a wider therapeutic time window compared to systemic administration, an effect related to improvement in tissue viability in the penumbra area. As the search for an effective neuroprotective treatment that can be started later after ischemia onset is currently one of the most challenging goals to treat stroke, we believe that our present data support that IN GUO may be a promise strategy to treat stroke. We expect that our present results will stimulate other groups to explore the neuroprotective potential of IN GUO treatment.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Authors' contributions Author contributions: D.B.R., G.C.M., S.O.L., D.O.S., and M.G. designed research; D.B.R., G.C.M., G.B.M.R., G.H.D., R.F.A., L.F.P., G.H., A.C.M.H., S.O.L., and M.G. performed research; L.F.P., A.C.M.H., D.O.S. and M.G. contributed new reagents/analytic tools; D.B.R., G.C.M., G.B.M.R., R.F.A., L.F.P., G.H., A.C.M.H., S.O.L., D.O.S., and M.G. analyzed data; and D.B.R., G.C.M., S.O.L., D.O.S., and M.G. wrote the paper.

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