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EFEITO DA GUANOSINA CONTRA DANOS AGUDOS E CRÔNICOS CAUSADOS POR TRAUMA CRÂNIO ENCEFÁLICO: UMA NOVA PERSPECTIVA ENVOLVENDO O SISTEMA PURINÉRGICO

Santa Maria, RS, Brasil

2017

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM) como requisito parcial para obtenção do grau de **Doutor em Bioquímica Toxicológica.**

Orientador: Prof. Dr. Félix Alexandre Antunes Soares

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Santa Maria, RS
2017

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“Se vi mais longe, foi porque me apoiei em ombros de gigantes.”

Sir Isaac Newton

RESUMO

EFEITO DA GUANOSINA CONTRA DANOS AGUDOS E CRÔNICOS CAUSADOS POR TRAUMA CRÂNIO ENCEFÁLICO: UMA NOVA PERSPECTIVA ENVOLVENDO O SISTEMA PURINÉRGICO

Autor: Fernando Dobrachinski
Orientador: Félix Alexandre Antunes Soares

O traumatismo cranioencefálico (TCE) é uma patologia multifatorial associada a graves alterações fisiológicas que resultam em um impacto socioeconômico desfavorável a nível mundial. Sua fisiopatologia compreende a excitotoxicidade, disfunção mitocondrial e processos inflamatórios que conduzem a morte de células do sistema nervoso central. Consequentemente, essas alterações convergem a manifestações clínicas, como déficits comportamentais e o aparecimento de processos neurodegenerativos. Em modelos experimentais de TCE muitas drogas promissoras reportaram falhas quando testadas clinicamente, muito devido aos seus efeitos localizados em um único sistema. Desde modo, busca-se estudar novas alternativas terapêuticas como a Guanosina (GUO), um nucleosídeo purinérgico endógeno. Atualmente, tem sido dedicada atenção aos seus efeitos neuroprotetores, uma vez que reduz eventos neurotóxicos e degenerativos em diversos modelos experimentais *in vivo* e *in vitro*. Nesse contexto, o objetivo desta tese foi avaliar os possíveis efeitos da GUO sobre as alterações agudas e crônicas causadas pelo TCE e em modelo de excitotoxicidade em roedores. O composto foi investigado em diferentes modelos experimentais e os resultados divididos em um artigo científico e dois manuscritos. Os resultados do artigo 1 demonstraram a ação neuroprotetora da GUO contra os efeitos agudos observados na cascata secundária 3h pós trauma. O desequilíbrio na atividade mitocondrial e no sistema redox caracterizados no modelo utilizado, foram reduzidos pela GUO nas diferentes estruturas cerebrais. Ao mesmo tempo, podemos notar a eficácia da GUO na manutenção da homeostase do Ca^{2+}, também alterado pós o TCE. Este efeito evidencia a íntima relação entre a restauração da atividade mitocondrial e a manutenção do sistema glutamatérgico produzidos pela GUO. No manuscrito 1, o estudo focou-se em avaliar a relação das alterações comportamentais e morfológicas desencadeadas pelo TCE a longo prazo (durante 21 dias), bem como o potencial de ação da GUO. Em relação as comorbidades referenciadas neste modelo experimental, observamos um aumento no comportamento do tipo ansioso dos animais, acompanhado de prejuízos na capacidade cognitiva. Isso corrobora com as alterações encontradas na expressão de proteínas relacionadas aos processos de plasticidade e reparação sináptica no hipocampo. O dano tecidual caracterizado pelo aumento da morte celular, astrogliose e gliose são confirmados 21 dias após o TCE. O tratamento crônico com a GUO conferiu neuroproteção contra estes parâmetros, entretanto este efeito foi impedido pela administração de DPCPX (antagonista do receptor de adenosina A_1). Isso demonstra que a GUO pode modular direta ou indiretamente o sistema adenosinérgico em situações de neuroproteção. No manuscrito 2, o estudo focou-se em avaliar possíveis alvos da ação farmacológica da GUO em um modelo de hiperexcitabilidade. Através de ferramentas eletrofisiológicas pode-se confirmar o seu efeito protetor contra as alterações na transmissão sináptica basal, intimamente associada a ação astrocitária. Percebeu-se neste mesmo sentido que as ondas de cálcio (Ca^{2+}) e seu equilíbrio são essenciais para sua atividade, e que este equilíbrio não é exclusivamente dependente de um canal de Ca^{2+} ou de potássio (K^+). Observou-se ainda que a GUO estabeleceu seu efeito neuprotetor provavelmente através da regulação dos níveis de adenosina e da modulação de receptores do tipo P2 ($P2Y_1$) e não por uma ligação direta com os receptores adenosinérgicos. Desta forma, esta tese contribuiu para caracterizar o efeito neuroprotetor da GUO contra os danos agudos e crônicos causados pelo TCE bem como seus possíveis mecanismos de ação envolvidos.

Palavras-chave: Traumatismo crânio encefálico. Excitotoxicidade. Guanosina. Sistema Purinérgico. Avaliação Comportamental. Eletrofisiologia.

ABSTRACT

EFFECT OF GUANOSINE AGAINST ACUTE AND CHRONIC DAMAGE CAUSED BY TRAUMATIC BRAIN INJURY: A NEW PERSPECTIVE INVOLVING THE PURINERGIC SYSTEM

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Traumatic brain injury (TBI) is a multipathology associated to severe physiological changes that result in an unfavorable socioeconomic impact in a worldwide. Its pathophysiology comprises excitotoxicity, mitochondrial dysfunction and inflammatory processes leading to cells death in the central nervous system. Consequently, cognitive changes converge to clinical manifestations, such as behavioral deficits and the appearance of neurodegenerative processes. In experimental models of TBI many promising drugs reported failures when clinically tested, largely because of their effects on a single system. Thus, it is sought to study new therapeutic alternatives such as Guanosine (GUO), an endogenous purinergic nucleoside. At present, attention has been paid to its neuroprotective effects, since it reduces neurotoxic and degenerative events in several experimental models *in vivo* and *in vitro*. In this context, the objective of this thesis was to evaluate the possible effects of GUO on the acute and chronic alterations caused by TBI and in a model of excitotoxicity in rodents. The action of purinergic nucleoside was investigated in different experimental models and the results divided into one scientific paper and two manuscripts. The results of article 1 demonstrated the neuroprotective action of GUO against the acute effects observed in the secondary cascade 3h post-trauma. The imbalance in mitochondrial activity and the redox system in TBI model, were reduced by GUO in cerebral structures. At the same time, we can note the effectiveness of GUO in the maintenance of Ca^{2+} homeostasis modified after TBI. This effect reveals the relationship between the restoration of mitochondrial activity and the maintenance of the glutamatergic system produced by GUO. In manuscript 1, the study focuses on assessing the relationship of behavioral and morphological chronic changes triggered by TBI (for 21 days), as well as the potential action of GUO. Regarding the comorbidities referenced in this experimental model, we observed an increase in the anxiety-like behavior of animals, accompanied by impairments in cognitive function. Its corroborates with the alterations found in the expression of proteins related to the processes of plasticity and synaptic repair in the hippocampus. In this way, hippocampus damage is characterized by an increase of neuron cell death, astrogliosis and reactive microgliosis 21 days after TBI. Chronic treatment with GUO conferred neuroprotection against these parameters, but the A1 adenosine receptor antagonist blocked this effect. In manuscript 2, the study focused on evaluating possible targets of the pharmacological action of GUO in a model of excitotoxicity. Across electrophysiological tools, it can be confirmed the protective effect of GUO against excitotoxicity, through the astrocytic function. In this context, it was found that the homeostasis of calcium (Ca^{2+}) is essential for its activity and this neuroprotection effect is not only dependent on the Ca^{2+} or potassium (K^{+}) channel activity. Moreover, it was observed that GUO established its protective effect by regulating adenosine levels and modulating P2 (P2Y1) receptors rather than a direct binding with adenosinergic receptors. In this way, this thesis characterized the neuroprotective effect of GUO against acute and chronic damages caused by TBI as well as the possible mechanisms of action involved.

Palavras-chave: Traumatic Brain Injury. Excitotoxicity. Guanosine. Purinergic System. Behavioral Evaluation. Electrophysiology.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

A1	Receptor de adenosina tipo 1
A2A	Receptor de adenosina tipo 2
A2B	Receptor de adenosina tipo 2B
A3	Receptor de adenosina tipo 3
AMPc	Monofosfato de adenosina cíclica
ADP	Adenosina-5’-difosfato
AMP	Adenosina-5´-monofosfato
ATP	Adenosina-5’-trifosfato
Akt	Proteína cinase B
BDNF	Fator neurotrófico derivado do cérebro
Ca^{2+}	Cálcio
CREB	Proteína de ligação ao elemento de resposta de AMPc
DOG	Privação de oxigenio e glicose
ERs	Espécies reativas de oxigênio
GLAST	Transportador de glutamato e aspartato em ratos
GLT-1	Transportador de glutamato do tipo em 1 ratos
GDP	guanosina-5’-difosfato
GMP	Guanosina-5’-monofosfato
GTP	Guanosina-5’-trifosfato
GUO	Guanosina
i.p.	Intraperitoneal
iNOS	Óxido nítrico sintase induzida
LTP	Potencialização a longo prazo
mGluR	Receptor metabotrópico glutamatérgico
Na^{+}	Sódio
K^{+}	Potássio
NGF	Fator de crescimento do nervo
NMDA	N-Metil-D-Aspartato
NMDAR	Receptor N-metil-D-aspartato
P1	Receptor purinérgico do tipo P1
P2	Receptor purinérgico do tipo P2

P2Y	Receptor metabotrópico purinérgico do tipo P2 acoplado à proteína G
P2X	Receptor ionotrópico purinérgico do tipo P2
PF	Percussão de fluído
PIC	Pressão intracranial
PI3K	Fosfatidilinositol-3-quinase
SNC	Sistema nervoso central
TCE	Traumatismo cranioencefálico

SUMÁRIO

1. INTRODUÇÃO

1.1 TRAUMATISMO CRANIOENCEFÁLICO

1.1.1. História e definição

O Traumatismo cranioencefálico (TCE) acompanha a humanidade desde as suas origens, e com a evolução tornou-se evidente o aumento progressivo de vítimas de traumas mecânicos por diversas causas externas. A partir do ano de 1682 que o traumatismo craniano (nomenclatura antiga) começou a ser descrito como importante fator de óbito em suas vítimas, até atingir os elevados índices atuais de morbidade e mortalidade (MELO et al. 2004).

Em relação a sua definição, o TCE é considerado como uma alteração da função cerebral, manifestada como confusão, alteração do nível de consciência, convulsão, coma ou déficit neurológico sensitivo ou motor que resulta da aplicação de uma força, penetrante ou não, sobre o crânio (BRUNS et al. 2003).

1.1.2. Cenário epidemiológico

Estudos epidemiológicos são indispensáveis para o entendimento do TCE com importante finalidade em investigar sobre sua incidência e prevalência na população, apresentando estimativas completas para orientar a prevenção, identificar as melhores práticas terapêuticas e planejar futuros tratamentos que tenham menores custos e sejam mais efetivos (BARKER-COLLO et al. 2009, KUFFLER 2012).

De outro modo, o TCE tem sido denominado mundialmente como uma “epidemia silenciosa” devido ao conhecimento limitado da sociedade sobre essa doença, em virtude da falta de estudos epidemiológicos abrangentes em alguns países e a ausência de registros de muitos casos de TCE nos departamentos de emergências e hospitais.

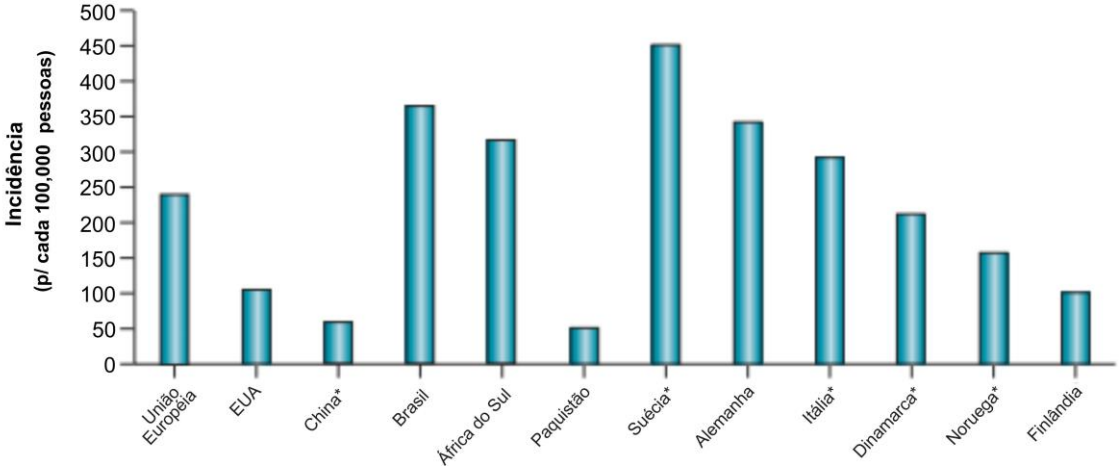
Segundo Andelic, (2013) uma proporção considerável de pessoas idosas e/ou indivíduos que sofrem TCE leve (cerca de 75 %) no seu ambiente familiar são menos propensos a buscar atendimento hospitalar (ANDELIC 2013). Vítimas de TCE moderado e grave, cerca de 25%, são frequentemente mais estudadas por conta dos critérios claros para definição de caso para tais ferimentos. Por outro lado, as lesões leves são mais suscetíveis a erros de diagnóstico ou negligência por profissionais clínicos. Sendo assim, ambos os fatores podem introduzir vieses substanciais nesse cenário epidemiológico.

Alguns dados fornecidos pelo Centro de Controle e Prevenção de Doenças alertam para a dimensão do TCE nos EUA, indicando sua incidência anual de 1,7 milhões de pessoas, das quais cerca de 52.000 morrem, 275.000 necessitam de tratamento hospitalar intensivo e 1,365 milhões são tratadas e liberadas dos departamentos de emergência (PEARSON et al. 2012). Segundo Ma *et al.*, (2014) a cada 7 segundos ocorre um TCE nos EUA e a cada 5 minutos uma pessoa morre e outra fica incapacitada (MA et al. 2014).

Em razão disso, 5,3 milhões de pessoas vivem com algum tipo de sequela, trazendo consigo um alto índice de morbidade e expressivo impacto socioeconômico (HYDER et al. 2007), gerando uma despesa anual de 76,5 bilhões de dólares para os EUA (CORONADO et al. 2011, PEARSON et al. 2012). As principais causas que constituem esse panorama alarmante compreendem as quedas (35%), seguida dos acidentes com veículos automotores (17%), acidentes de trabalho e/ou prática de esportes (16%), assaltos (10%), além de outras causas desconhecidas (22%) (PEARSON et al. 2012).

No Brasil estudos abrangentes abordando o tema são raros, devido a uma incorreta política pública de coleta de dados epidemiológicos. Na grande maioria, os dados acabam especificando uma micro região, o que dificulta o seu entendimento como um todo, mas alguns estudos disponíveis indicam que 74% dos TCEs ocorrem devido a acidentes de trânsito (OLIVEIRA et al. 2012). Em comparação aos EUA, a incidência do TCE no Brasil é cerca de 2,5 a 3 vezes maior na população (Figura 1) e constitui a principal causa de morte em pessoas com menos de 45 anos de idade de ambos os gêneros (MASET et al. 1993, CORONADO et al. 2011, ROOZENBEEK et al. 2013). Mesmo assim as poucas pesquisas que encontramos em nossos país relatam que mais de 50% das mortes ocorridas na infância e adolescência são decorrentes do traumatismo encefálico, números que apontam mais especificamente 25% de óbitos na faixa de 10 – 14 anos e 75% de 15 – 19 anos (CARVALHO et al. 2007). Agravando ainda mais esse quadro epidemiológico, um estudo indica que o TCE poderá tornar-se a terceira maior doença global em 2020 (LOPEZ et al. 1998).

Figura 1. Estimativas da incidência global de trauma crânio encefálico. * Média dos resultados de dois estudos.



Fonte: Adaptado de (ROOZENBEEK et al. 2013)

1.1.3. Classificação do TCE

De acordo com Roozenbeeck *et al*., (2013), o TCE é uma patologia dinâmica, tanto no seu aspecto temporal como na coexistência com vários tipos de lesões. Para melhor compreensão podemos classificá-lo quanto: ao mecanismo físico da lesão (trauma penetrante ou fechado), à distribuição da lesão (lesões focais ou difusas) e à gravidade clínica (lesões leves, moderadas ou graves) (ROOZENBEEK et al. 2013).

1.1.3.1. Classificação quanto ao mecanismo físico da lesão

Com relação aos mecanismos físicos de lesão no TCE, esses podem ser classificados em traumas:

- Penetrantes: Aqueles em que o crânio é atingido ou atinge um objeto (impacto com carga), muitas vezes causando destruição local e exposição do conteúdo craniano ao meio e, dependendo da energia cinética transmitida ao tecido, lesões difusas devastadoras (SAATMAN et al. 2008, LI et al. 2011).

- Fechado: Nesse caso, não é necessário o impacto do crânio com estruturas externas. Comumente acontecem em vítimas de acidentes de trânsito, onde a diferença de densidade do crânio em relação as estruturas cerebrais, geram cisalhamento, tensão e compressão sobre o tecido cerebral, devido às bruscas forças de aceleração e desaceleração da cabeça no momento do acidente (GENNARELLI 1993, GENNARELLI et al. 2005, ANDRADE et al. 2009).

1.1.3.2. Classificação quanto à distribuição da lesão

- Lesão Focal: Nesses casos de TCE, a lesão é causada mais frequentemente pelo trauma com contato direto a uma porção do encéfalo, resultando em contusões, lacerações, hemorragias e extensa morte celular local, geralmente é um quadro grave que necessita de tratamento cirúrgico de urgência, bastante comum em vítimas de acidentes por quedas (SILVER et al. 2005, ROOZENBEEK et al. 2013).

- Lesão Difusa (ou multifocal): Nesses casos de TCE, a lesão acomete o cérebro como um todo através de forças cinéticas que levam à movimentação, rotação e aceleração/desaceleração do encéfalo dentro da caixa craniana, bastante frequente em vítimas de acidente de trânsito. Dentre as lesões difusas, estão a concussão e lesão axonal difusa (ANDRADE et al. 2009).

Ainda existe a possibilidade do TCE com lesões mistas, onde lesões focais e difusas estão presentes no mesmo quadro clínico (SILVER et al. 2005, XIONG et al. 2013).

1.1.4. Tratamentos utilizados na clínica

A atual gestão médica de pacientes acometidos pelo TCE inclui principalmente atendimento pré-hospitalar especializado, cuidados clínicos intensivos e reabilitação a longo prazo, mas carece de gerenciamento clínico efetivo comprovado com agentes neuroprotetores para limitar lesões secundárias ou melhorar a reparação (XIONG et al. 2010). O enorme fardo do TCE, no entanto, suporta claramente a necessidade de tais agentes ou abordagens neuroprotetoras. No entanto, traduzir benefícios promissores pré-clínicos para o cenário clínico tem-se revelado difícil.

Muitos estudos pré-clínicos tem testado a eficácia terapêutica de fármacos em modelos de TBI em animais, tendo como alvo os mecanismos de lesão secundária, incluindo bloqueadores dos canais de cálcio, corticosteróides, inibidores de aminoácidos excitatórios, antagonistas do receptor NMDA, antioxidantes, sulfato de magnésio e fatores de crescimento (NARAYAN et al. 2002). Muitos ensaios clínicos de fase II mostraram efeitos favoráveis, incluindo superóxido dismutase conjugada com polietileno glicol (PEG-SOD), hipotermia moderada, nimodopina e triamcinolona (NARAYAN et al. 2002). Infelizmente, todos os compostos ou abordagens que foram testados até agora em ensaios de fase III não conseguiram mostrar claramente a eficácia (DOPPENBERG et al. 2004).

Os ensaios de fase III clínicos decepcionantes podem ser devidos à heterogeneidade da

população de pacientes com TCE e à variabilidade nas abordagens de tratamento. No entanto, há muitos aspectos que precisam ser considerados antes e durante os ensaios clínicos. Em primeiro lugar, antes da tradução de um agente para ensaio clínico, as evidências pré-clínicas devem ser suficientemente fortes, com base em experiências múltiplas, preferencialmente em vários modelos, e incluir vias e doses de administração ótimas, doses únicas versus doses múltiplas, dose em bolus versus infusão contínua e janelas terapêuticas.

A eficácia dos tratamentos neuroprotetores existentes para o TCE permanece incerta. Por exemplo, o manitol é por vezes eficaz na redução do inchaço cerebral após o dano mecanico. No entanto, a sua eficácia no tratamento de pacientes diagnosticados com TCE grave permanece incerto. Há evidências de que a administração excessiva de manitol pode ser prejudicial, pois o manitol passa a barreira cérebro sangue, cujo qual pode aumentar a pressão dentro do crânio e piorar o inchaço do encéfalo (WAKAI et al. 2007). Um pequeno benefício surge quando o tratamento com manitol é guiado pela medição da pressão intracraniana (PIC) em comparação com o tratamento padrão. Não há dados suficientes sobre a eficácia da administração pré-hospitalar de manitol (WAKAI et al. 2007).

Uma metanálise atualizada apóia achados prévios de que a terapia hipotérmica constitui um tratamento benéfico para esta patologia em circunstâncias específicas. Entretendo, mais evidências devem ser coletadas para que esta terapia se torne disponível. Neste sentido, os profissionais necessitam de muito cuidado ao considerar a administração de hipotermia para o tratamento desta doença (PETERSON et al. 2008).

Evidencias suportam que altos níveis de pressão intracraniana (PIC) ainda é a causa mais frequente de morte e incapacidade após TCE grave. A PCI elevada é geralmente definida acima de 15 a 20 mmHg, quando medida dentro de qualquer espaço intracraniano (subdural, intraventricular, extradural ou compartimentos intraparenquimatosos). Mortalidade e morbidade após grave TCE têm sido fortemente relacionados a PCI elevada (MARMAROU 1992). A causa deste fenômeno é associada à um aumento do volume cerebral à custa de um ou mais componentes intracranianos. Grandes lesões e um aumento no conteúdo de água no cérebro (edema) e no volume sangüíneo cerebral contribuem para aumento da PCI no TCE (SAHUQUILLO et al. 2006). No entanto, não há evidências para apoiar o uso rotineiro de craniotomia descompressiva (DC) para melhorar a mortalidade e a qualidade de vida em adultos com acometidos por este quadro patológico (SAHUQUILLO et al. 2006). Os resultados de ensaios não randomizados e ensaios controlados envolvendo adultos sugerem que DC pode ser uma opção útil quando o tratamento médico máximo não conseguiu controlar a PIC. Além disso, é necessário um estudo controlado com DC em pacientes de TCE grave para que

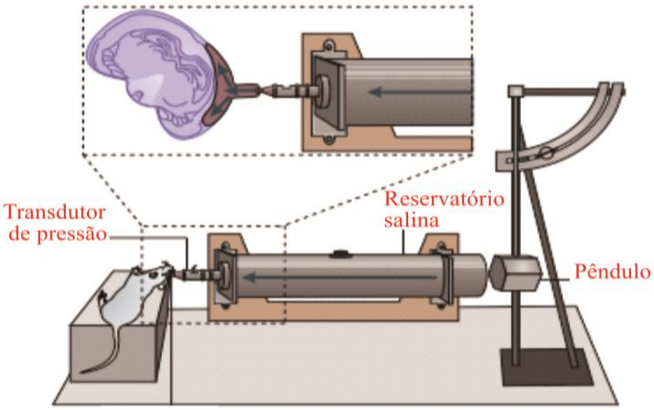
possamos ter uma avaliação mais correta sobre a eficácia deste procedimento em adultos (XIONG et al. 2010).

1.1.5. Modelos experimentais de TCE

Estudos pré-clínicos através de modelos experimentais de TCE em roedores são de grande importância para o entendimento dessa doença, pois produzem muitas características que mimetizam a condição humana e fornecem informações válidas para o desenvolvimento de novos tratamentos e condutas para essa condição clínica. Para estes fins, muitos modelos experimentais de TCE são amplamente utilizados atualmente, dentre eles podemos destacar o percussão de fluido (Figura 2), considerado um dos modelos clinicamente mais relevantes, pois mimetiza em roedores as condições comumente observadas em pacientes com TCE, incluindo:

Alterações fisiológicas: Imediatamente após o TCE, a auto-regulação cerebral torna-se prejudicada, episódios de apnéia, cianose, diminuição na PaO_2 e aumento da $PaCO_2$ são observadas. A pressão intracraniana (PIC) aumenta nos primeiros minutos após o TCE, acompanhada por uma redução no fluxo sanguíneo e aumento da demanda metabólica (glicose até 150 %) (THOMPSON et al. 2005a, THOMPSON et al. 2005c).

Figura 2. Modelo de percussão de fluido **(PF)** envolve a trepanação craniana (linha média, sagital ou lateral) para a realização da craniectomia onde um jato de fluido em alta velocidade promoverá a lesão sobre a dura-máter do animal.



Fonte: Adaptado de (XIONG et al. 2013).

Alterações histológicas: Conforme a gravidade do TCE fica evidente a perda de massa encefálica diretamente no local da lesão (córtex cerebral), bem como contusões em estruturas

cerebrais internas (hipocampo, tálamo, estriado e amígdala) conhecidas por apresentar alterações morfológicas e morte celular (THOMPSON et al. 2005a).

Alterações comportamentais: Comprometimento motor, sensorial e cognitivo. Vários testes comportamentais avaliando esses parâmetros são utilizados para determinar o potencial clínico de vários tratamentos que oferecem intervenções terapêuticas no TCE.

Fisiopatologia: Através do dano focal com características difusas provocadas no momento do insulto, a fisiopatologia do TCE é extremamente semelhante a aquela observada em pacientes como discutida a seguir (MAAS et al. 2008, WALKER et al. 2013).

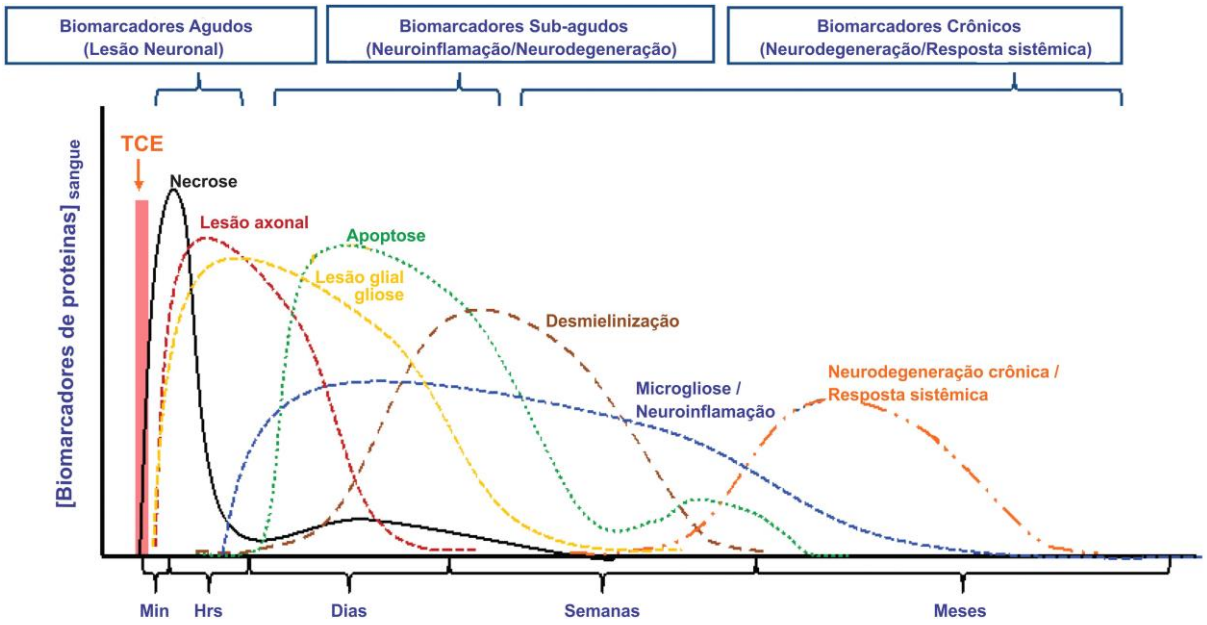
1.1.6. Fisiopatologia

A fisiopatologia do TCE é classificada de acordo com a progressão da lesão, basicamente dividida em dois estágios, denominados lesão primária e secundária.

Este primeiro estágio do traumatismo é caracterizado pelo dano mecânico que ocorre no momento do impacto causando deformação e compressão dos tecidos dentro de segundos. As lesões mais frequentes incluem fraturas ósseas, danos axonais, meníngeos, vasculares e morte celular imediata (PITKANEN et al. 2006). Dessa forma, a lesão primária não é passível de tratamento, apenas prevenção a partir de políticas socioeducativas de incentivo ao uso do cinto de segurança, capacetes, combate à violência urbana, entre outros (FALAVIGNA et al. 2012, XIONG et al. 2013).

Por outro lado, a lesão secundária abrange múltiplos processos fisiopatológicos que ocorrem dentre minutos a anos subsequentemente a lesão primária e inclui vários mecanismos tais como: excitotoxicidade, disfunção mitocondrial, dano oxidativo e inflamação, os quais podem configurar um quadro de neurotoxicidade (WERNER et al. 2007, KUFFLER 2012). Em seguida, o cérebro também inicia mecanismos de “auto-reparo" do tecido lesado ao longo de dias a meses, período no qual é caracterizado por remodelação de circuitos sinápticos, proliferação de axônios, plasticidade sináptica, gliose, neurogênese e angiogênese (Figura 3) (HUNT et al. 2013).

Figura 3. Janela temporal da fisiopatologia do traumatismo cranioencefálico.



Adaptado de (BRAMLETT et al. 2015).

A propagação da lesão secundária ocorre de forma crescente e prolongada, estabelecendo uma janela temporal que permite intervenções neuroprotetoras. O principal objetivo de algumas dessas medidas consiste no reforço benéfico dos mecanismos de "auto-reparo", seguido da prevenção ou redução na progressão dessa lesão, gerando um “ambiente” propício para uma recuperação sem maiores sequelas para o paciente.

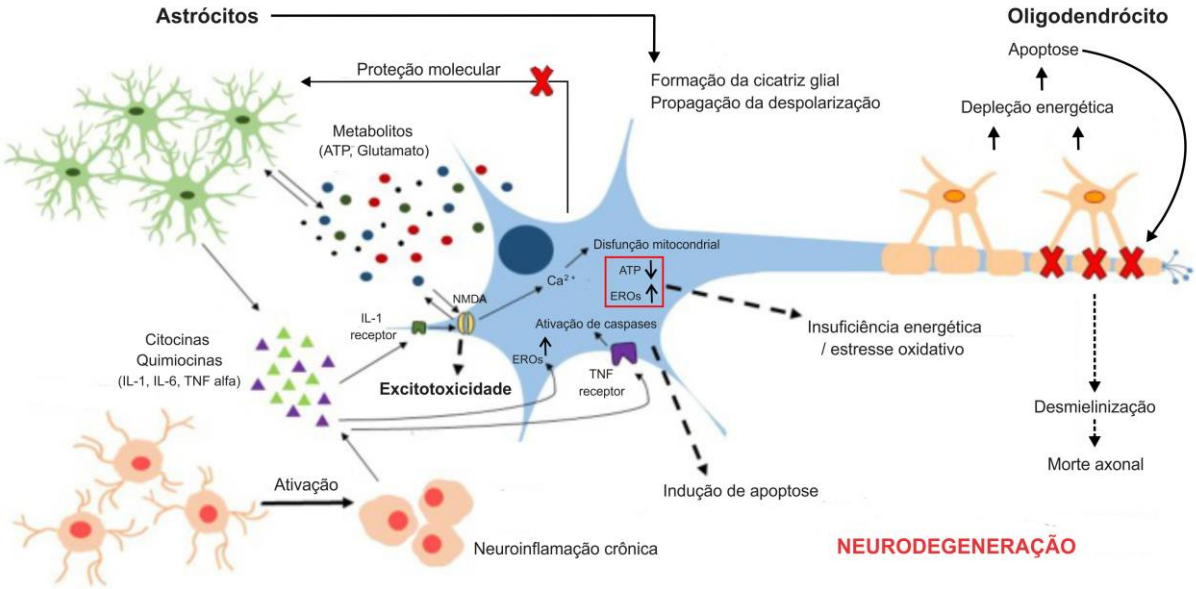
Um dos obstáculos desses estudos clínicos se deve ao caráter heterogêneo do TCE, devido à dinâmica da lesão (localização, gravidade, tipo de lesão) e características pessoais do paciente (idade, gênero, genes, condições pré-existentes), que acabam produzindo diferentes prognósticos e desfechos clínicos (HUNT et al. 2013).

1.1.7. Processo envolvidos na cascata secundária pós traumática

A lesão secundária é iniciada imediatamente após o trauma cerebral convergindo a danos celulares adicionais aos observados na lesão primária. Durante esta segunda fase, o dano é atribuído principalmente à liberação excessiva de neurotransmissores, desequilíbrio de cálcio, estresse oxidativo, disfunção mitocondrial, ativação de genes pró-apoptóticos, processos inflamatórios, alterações morfológicas e em padrões comportamentais (Figura 4) (ZIPFEL et

al. 2000, KROEMER et al. 2007, MAAS et al. 2008).

Figura 4. Mecanismos patofisiológicos envolvidos na cascata secundária pós trauma.



Adaptado de (SAJJA et al. 2016).

O glutamato é um aminoácido que tem como função no sistema nervoso central (SNC) atuar como neurotransmissor excitatório. Através desta atividade esta molécula desempenha um papel essencial em processos de plasticidade cerebral, tais como a aprendizagem/memória, desenvolvimento e envelhecimento (DANBOLT 2001, SEGOVIA et al. 2001). Nessas condições a concentração de glutamato varia em torno de 1-2 μM na fenda sináptica, 10 μM no líquor, 2mM no citoplasma das células astrocitárias, 10 mM no citoplasma de células neuronais e 100 mM nas vesículas sinápticas (DANBOLT 2001, NEDERGAARD et al. 2002).

Em condições patológicas como observadas no TCE, a transferência de energia sequencial para os tecidos cerebrais gerada no momento do impacto do crânio provoca deformação mecânica nos axônios com consequente despolarização das membranas celulares e uma liberação maciça de neurotransmissores, dentre eles o glutamato (PALMER et al. 1993, GLOBUS et al. 1995, BULLOCK et al. 1998).

O glutamato também liberado através de membranas danificadas e passa da circulação sanguínea para o local da lesão devido a danos na barreira hematoencefálica. Rapidamente a concentração desse neurotransmissor aumenta no meio extracelular acima dos níveis fisiológicos estabelecendo um processo patológico denominado de excitotoxicidade, termo criado para definir a morte neuronal causada pela administração exógena de altas concentrações

de glutamato ou compostos com ação agonista em receptores glutamatérgicos (OLNEY 1990).

Neste sentido, a hiperestimulação do sistema glutamatérgico leva a consequências iniciais que envolvem aumento intracelular de íons, tais como sódio (Na^{+}), cálcio (Ca^{+2}), depleção dos níveis de adenosina trifosfato (ATP) e danos neuronais (NICHOLLS 2008). A manutenção da captação glutamatérgica é um processo crucial para manter as concentrações desse aminoácido no meio extracelular abaixo dos níveis tóxicos. Isto é realizado através dos transportadores de aminoácidos excitatórios (transportador de aspartato de glutamato [GLAST] e transportador de glutamato 1 [GLT-1]) presentes principalmente nos astrócitos. Estas proteínas transportadoras são moduladas pelo estado redox da célula (TROTTI et al. 1998). Consequentemente o desequilíbrio na produção de espécies reativas (ERs) e sobrecarga de Ca^{+2} nas mitocôndrias pode resultar em uma "reação em cadeia", levando ao comprometimento da captação de glutamato e morte celular (DANBOLT 2001, DANBOLT et al. 2016). Além disso alguns estudos sugerem que ainda ocorra uma reversão dos transportadores de glutamato na membrana nestas condições, que passam a direcionar o glutamato do citoplasma das células gliais para o meio extracelular (DANBOLT 2001, DANBOLT et al. 2016).

As mitocôndrias, importantes para bioenergética celular, são sensíveis a alterações no estado fisiológico das células e desempenham um papel crítico na lesão secundária que ocorre após o TCE (FINKEL 2001, HUNOT et al. 2001). Em particular, as mitocôndrias acabam sofrendo danos estruturais e funcionais consideráveis pós trauma (SULLIVAN et al. 1998, ROBERTSON 2004, XIONG et al. 2005, SINGH et al. 2006). A sobrevivência neuronal é intimamente ligada à homeostase mitocondrial. Consequentemente, a disfunção nesta organela parece estar envolvida na neurotoxicidade induzida por aminoácidos excitatórios (WHITE et al. 1996, NICHOLLS et al. 1998, STOUT et al. 1998, KROEMER et al. 2000, JIANG et al. 2001, KROEMER et al. 2007). Com o intuito de manter o equilíbrio do Ca^{2+} intracelular, a mitocôndria sequestra grandes quantidades desse íon no seu interior. Entretanto, o excesso de Ca^{2+} resulta na perda do potencial de membrana mitocondrial e diminuição no transporte de elétrons, favorecendo a excessiva produção de ERs seguida de um colapso bioenergético e morte celular (XIONG et al. 1997, SULLIVAN et al. 1999, ROBERTSON 2004, SULLIVAN et al. 2005).

Embora os índices de mortalidade associados ao TCE tenham reduzido substancialmente nos últimos anos, os casos de incapacidades permanentes ainda são alarmantes em muitos sobreviventes (Shukla *et al*., 2011). Muitas sequelas, tais como, problemas comportamentais, emocionais e incapacidades de origem cognitiva e física acabam prejudicando as atividades diárias dessa população caracterizada principalmente por jovens que

até então eram produtivos para a sociedade (Dikmen *et al.*, 2001; Pitkanen e Mcintosh, 2006). Consequentemente, quadros patológicos encontrados no início da cascata celular estão associados a transtornos de curto e longo prazo (COPE et al. 2012), podendo conduzir ou acelerar processos neurodegenerativos e o aparecimento de outras doenças.

Alterações neuroquímicas frequentemente relacionadas ao surgimento de comprometimentos comportamentais após o TCE, ocorrem também através da resposta inflamatória e morte celular (MCINTOSH et al. 1998). A lesão traumática moderada-grave induz mecanismos de lesão secundária causando perda de células neuronais e ativação microglial que continuam durante semanas a meses após a lesão em diferentes regiões do encéfalo (BYRNES et al. 2012, KABADI et al. 2014a). Os modelos experimentais de TCE caracterizam a morte celular em diferentes regiões do SNC, as quais afetam a excitabilidade neuronal e causam déficits na potencialização a longo prazo (LTP) (WITGEN et al. 2005, SCHWARZBACH et al. 2006), sugerindo que as mudanças na plasticidade sináptica contribuem para os déficits cognitivos.

Neste mesmo contexto, observamos que o aumento da excitação neuronal e da redução da oferta metabólica induz a formação de cicatrizes gliais e agravamento da neuroinflamação através da ativação de células da micróglia. A limitação da ativação microglial pós-traumática e cicatrização glial em estudos com animais, caracteriza-se por reduzir não apenas a neurodegeneração progressiva mas também os déficits cognitivos (SHULTZ et al. 2012, WALKER et al. 2013, KABADI et al. 2014a).

Apesar do progresso no conhecimento das lesões secundárias induzidas pelo TCE, mais de 30 estudos clínicos de fase III não apresentaram resultados favoráveis inclusive os que demonstraram drogas terapêuticas efetivas neste modelo experimental. Em parte, muitos ensaios falharam porque o agente proposto para tratamento modulava apenas um único mecanismo da lesão secundária e a terapia por combinação de medicamentos não foi eficaz. Devido à natureza heterogênea do TCE em humanos, a busca por novos tratamentos farmacológicos na pesquisa pré-clínica está direcionada a substâncias com maior capacidade de modular diversas vias neurotóxicas (múltiplos alvos), maximizando assim as chances de se desenvolver uma intervenção terapêutica bem sucedida (LOANE et al. 2010, KABADI et al. 2014a).

1.2 SISTEMA PURINÉRGICO

As purinas constituem uma classe de moléculas orgânicas essenciais para as células e são derivadas das bases nitrogenadas adenina e guanina, incluindo os nucleotídeos com um ou mais fosfatos, como adenosina-5'-trifosfato (ATP), adenosina-5'-difosfato (ADP), adenosina-5´-monofosfato (AMP), guanosina-5'-trifosfato (GTP), guanosina-5'-difosfato (GDP), guanosina-5'- monofosfato (GMP), os nucleosídeos adenosina, inosina e guanosina (GUO), as bases nitrogenadas adenina, guanina, hipoxantina e seus metabólitos. Os nucleotídeos do sistema purinérgico são constituintes essenciais de todas as células vivas e foram identificados primeiramente como componentes estruturais dos ácidos nucleicos. Atualmente, é bem estabelecido que os nucleotídeos e os nucleosídeos exercem funções importantes no metabolismo energético, biossíntese de macromoléculas, constituição de coenzimas e atuam na sinalização extracelular em diversos processos fisiológicos (DUNWIDDIE et al. 2001, BURNSTOCK 2007).

O papel extracelular das purinas na sinalização celular foi inicialmente reconhecido pelos efeitos das purinas derivadas da adenina em diferentes tipos celulares do organismo. No cérebro, além de agirem como neurotransmissores e neuromoduladores, o ATP e a adenosina também atuam em processos de plasticidade como aprendizado e memória, neuroreparação, neurogênese e, agem como agentes neuroprotetores contra estímulos nocivos (CICCARELLI et al. 1999, FREDHOLM et al. 2005, FIELDS et al. 2006, SCHMIDT et al. 2007).

Os efeitos extracelulares das purinas derivadas da adenina são mediados pelos receptores purinérgicos do tipo P1 ou P2. Os receptores pertencentes à família P2 são receptores de ATP e ADP, e são subdivididos em P2X [receptores ionotrópicos, ou seja, canais iônicos permeáveis à Ca^{2+}, Na^{+} e Potássio (K+)] e P2Y (receptores metabotrópicos acoplados às proteínas G) (FIELDS et al. 2006, BURNSTOCK 2007, BURNSTOCK 2008). Os receptores da família P1 são receptores metabotrópicos para adenosina, e são divididos em quatro subtipos: A1, A2A, A2B e A3 (FREDHOLM et al. 2005, FIELDS et al. 2006). Os receptores A1 são acoplados à proteína Gi enquanto os receptores A2A são acoplados à proteína Gs, sendo expressos em todo o SNC. Os receptores A2B também são acoplados à proteína Gs, mas são pouco expressos no cérebro, enquanto os receptores A3 são abundantes no coração e pouco expressos no SNC (PALMER et al. 1995, FREDHOLM et al. 2005, BURNSTOCK 2008). É conhecida a existência de muitos subtipos de receptores dentro da família purinérgica (P1, P2X e P2Y), muitos dos quais podem interagir e formar homodímeros e heterodímeros, dependendo do estímulo. Estes receptores estão amplamente distribuídos por todo o SNC, participando na

transmissão sináptica e mediando interações entre neurônio-glia e glia-glia (RODRIGUES et al. 2005, BURNSTOCK 2008, JARVIS et al. 2009, COPPI et al. 2012).

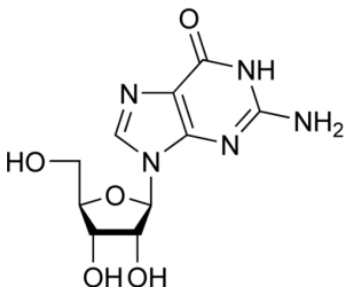
Tamanha é a importância deste sistema, que a regulação da transmissão purinérgica é apontada como um dos mecanismos essenciais na obtenção de efeito neuroprotetor em diversas patologias (FREDHOLM et al. 2005, FIELDS et al. 2006, BURNSTOCK 2008, GOMES et al. 2011).

1.2.1 Guanosina (GUO)

Nos últimos anos vários estudos destacam o fato de que os nucleotídeos e nucleosídeos derivados de guanina têm um papel extracelular importante, relevante tanto em condições fisiológicas quanto patológicas (SCHMIDT et al. 2007, BETTIO et al. 2016a, LANZNASTER et al. 2016a). Tanto neurônios quanto células gliais podem liberar estas moléculas em situação fisiológica, além disso, a quantidade de derivados de guanina liberados por astrócitos em condições basais e após alguns tipos de estímulos (hipóxia/isquemia e convulsões) é maior que a de derivados da adenina (CICCARELLI et al. 1999).

Recentemente, a atenção tem sido dedicada aos efeitos neuroprotetores da GUO, um nucleosídeo purinérgico endógeno que compreende uma base nitrogenada de guanina ligada a um anel de ribose (Figura 5).

Figura 5. Estrutura química da Guanosina.



A GUO pode ser formada em neurônios, astrócitos e no meio extracelular através da ação de ectonucleotidases que catalisam a transformação de GTP a GDP e a GMP, a continua ação destas enzimas podem convergir neste ponto a formação da GUO (SCHMIDT et al. 2007). No SNC os astrócitos são a principal fonte de GUO, em condições fisiológicas ou patológicas liberam esse nucleosídeo ao meio extracelular. Este por sua vez atua como uma molécula de

comunicação extracelular promovendo seus efeitos neuro/glioprotetores (BETTIO et al. 2016a, LANZNASTER et al. 2016a). Além disso, evidências indicam que a GUO também pode ser liberada por neurônios após o processo de despolarização (FREDHOLM et al. 2005).

Este nucleosídeo purinérgico é caracterizado por apresentar efeitos protetores interessantes em diversos tipos de neuropatologias (SCHMIDT et al. 2007, BETTIO et al. 2016a). Estudos indicam que os efeitos extracelulares da GUO podem ser observados através do seu papel neurotrófico, ou seja, através do estímulo do crescimento axonal *in vitro* e em outros tipos celulares. A GUO também promove a proliferação de astrócitos, a interação entre neurônio-astrócito, estimula a síntese e liberação de fatores tróficos como o fator de crescimento do nervo (Parks, #138), aumenta a fosforilação do CREB-cAMP e a expressão do fator neurotrófico derivado do cérebro (BDNF) (RATHBONE et al. 1992, CICCARELLI et al. 2000, CICCARELLI et al. 2001, DECKER et al. 2007, SCHMIDT et al. 2007, GIULIANI et al. 2012).

Os modelos experimentais que estabelecem condições excitotóxicas destacam o efeito da GUO através da modulação do sistema glutamatérgico, mais especificamente relacionado a atividade dos transportadores de glutamato astrocitários. Isso implica na redução da concentração de glutamato na fenda sináptica, subsequente diminuição de eventos neurotóxicos e neurodegenerativos presentes em diversas patologias do SNC (SCHMIDT et al. 2007, BETTIO et al. 2016a, LANZNASTER et al. 2016a).

A manutenção do equilíbrio glutamatérgico exercida pela GUO é bem caracterizada em modelos de convulsão (induzidas por ácido quinolínico e α-dendrotoxina), sendo que sua administração protege das alterações comportamentais presentes nas crises convulsivas e reduz a captação de glutamato (LARA et al. 2001, TAVARES et al. 2002, DE OLIVEIRA et al. 2004). Além disso, a GUO também foi avaliada em modelos experimentais de doenças neurodegenerativas (doença de Alzheimer e de Parkinson). Neste caso, seu efeito foi associado a manutenção da homeostase glutamatérgica, promoção da sobrevivência celular de neurônios, redução de processos inflamatórios, diminuição de ERs e modulação de proteínas envolvidas nos mecanismos de apoptose (LI et al. 2014, LANZNASTER et al. 2016b).

A grande maioria dos estudos que relatam o papel neuroprotetor da GUO, envolvem eventos isquêmicos (THAUERER et al. 2012). Em relação a modelos *in vivo*, o tratamento com a GUO foi capaz de prevenir o prejuízo cognitivo e diminuir o volume de lesão no cérebro de roedores (CHANG et al. 2008, RATHBONE et al. 2011). Neste contexto, este nucleosídeo purinérgico bloqueou as respostas inflamatórias reduzindo o número de células microgliais bem como as citocinas pró-inflamatórias e aumentou o nível das citocinas anti-inflamatórias no

cérebro, o que acarretou na redução da morte neuronal e melhora do comportamento exploratório (HANSEL et al. 2015). Em modelos de isquemia/reperfusão *in vitro* os efeitos da droga são caracterizados por modular a captação de glutamato, estresse oxidativo, disfunção mitocondrial, bloquear respostas inflamatórias (DAL-CIM et al. 2011, MOLZ et al. 2011, DAL-CIM et al. 2013).

Alguns mecanismos são apontados como possível alvo da GUO em processos patológicos, dentre os quais podemos destacar principalmente a regulação da atividade de canais iônicos (canais de Ca^{2+} e K^{+}) e a modulação direta ou indireta da transmissão purinérgica, mais especificamente em relação aos receptores da adenosina e seus mecanismos intracelulares (DAL-CIM et al. 2013, KOVACS et al. 2015, ALMEIDA et al. 2017). Entretanto, esta ação ainda não está bem delineada e seu mecanismo de ação não é totalmente compreendido. Com o intuito de investigar o mecanismo pelo qual a GUO modula o sistema glutamatérgico, estudos foram realizados em modelos de privação de oxigênio e glicose (DOG). Os resultados demonstraram que a mesma diminuiu a liberação de glutamato em fatias de hipocampo submetidas à excitotoxicidade glutamatérgica, através da ativação da via fosfatidilinositol-3-quinase (PI3K)/proteína quinase B (AKT) (DAL-CIM et al. 2011, MOLZ et al. 2011, DAL-CIM et al. 2013).

Deste modo considerando o elevado grau de limitação que o TCE ocasiona ao paciente, a compreensão dos mecanismos de toxicidade cerebral torna-se importante para o desenvolvimento de novas estratégicas terapêuticas. As propriedades neuroprotetoras da GUO devem ser investigadas como uma potencial medida terapêutica contra os complexos danos causados após o trauma.

2. OBJETIVOS

2.1 Objetivo Geral

Esta tese teve como objetivo determinar os efeitos da GUO bem como seu mecanismo de ação frente as consequências crônicas e agudas causadas pelo dano secundário após o TCE.

2.2 Objetivos Específicos

Considerando os aspectos mencionados, os objetivos específicos desta tese compreendem:

- Avaliar o possível efeito neuroprotetor da GUO em eventos agudos causados pelo modelo de TCE em ratos;
- Investigar uma possível relação entre o sistema glutamatérgico e a funcionalidade mitocondrial;
- Avaliar o efeito do tratamento crônico com a GUO nas alterações secundárias observadas após o TCE;
- Determinar a influência do sistema adenosinérgico no tratamento crônico com a GUO no modelo de TCE em ratos;
- Compreender o mecanismo neuroprotetor da GUO em modelos de excitabilidade glutamatérgica;

3. DESENVOLVIMENTO

O desenvolvimento desta tese está apresentado sob a forma de um artigo científico e dois manuscritos. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no próprios artigos ou manuscritos, os quais estão estruturados de acordo com as normas de cada revista onde foram publicados ou submetidos, respectivamente. Em anexo a esta tese encontram-se as autorizações da editora para reprodução dos artigos científicos, bem como a aprovação do projeto de pesquisa pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal de Santa Maria.

3.1 Artigo 1

A regulação da função mitocondrial e do sistema glutamatérgico são os alvos do efeito da Guanosina no trauma crânio encefálico

REGULATION OF MITOCHONDRIAL FUNCTION AND GLUTAMATERGIC SYSTEM ARE THE TARGET OF GUANOSINE EFFECT IN TRAUMATIC BRAIN INJURY

Fernando Dobrachinski, Rogério da Rosa Gerbatin, Gláubia Sartori, Naiani Ferreira Marques, Ana Paula Zemolin, Luiz Fernando Almeida Silva, Jeferson Franco, Luiz Fernando Royes, Michele Rechia Fighera, Félix Alexandre Antunes Soares

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Original Article

Regulation of Mitochondrial Function and Glutamatergic System Are the Target of Guanosine Effect in Traumatic Brain Injury

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Abstract

Traumatic brain injury (TBI) is a highly complex multi-factorial disorder. Experimental trauma involves primary and secondary injury cascades that underlie delayed neuronal dysfunction and death. Mitochondrial dysfunction and glutamatergic excitotoxicity are the hallmark mechanisms of damage. Accordingly, a successful pharmacological intervention requires a multi-faceted approach. Guanosine (GUO) is known for its neuromodulator effects in various models of brain pathology, specifically those that involve the glutamatergic system. The aim of the study was to investigate the GUO effects against mitochondrial damage in hippocampus and cortex of rats subjected to TBI, as well as the relationship of this effect with the glutamatergic system. Adult male Wistar rats were subjected to a unilateral moderate fluid percussion brain injury (FPI) and treated 15 min later with GUO (7.5 mg/kg) or vehicle (saline 0.9%). Analyses were performed in hippocampus and cortex 3 h post-trauma and revealed significant mitochondrial dysfunction, characterized by a disrupted membrane potential, unbalanced redox system, decreased mitochondrial viability, and complex I inhibition. Further, disruption of Ca^{2+} homeostasis and increased mitochondrial swelling was also noted. Our results showed that mitochondrial dysfunction contributed to decreased glutamate uptake and levels of glial glutamate transporters (glutamate transporter 1 and glutamate aspartate transporter), which leads to excitotoxicity. GUO treatment ameliorated mitochondrial damage and glutamatergic dyshomeostasis. Thus, GUO might provide a new efficacious strategy for the treatment acute physiological alterations secondary to TBI.

Keywords: fluid percussion injury; glutamate; glutamate transporters; mitochondria redox system; purinergic nucleoside

Introduction

Traumatic brain injury (TBI) is a major cause of mortality and morbidity, which constitutes a considerable health and socioeconomic problem throughout the world.[1–3] Patients with moderate and severe TBI exhibit notable alterations in cerebral blood flow and intracranial pressure.[1,3] If these sequelae are not treated properly, they may exacerbate brain damage, culminating in neurocognitive deficits and increased risk of death.[1,3]

Two major pathophysiological processes contribute to brain injury post-trauma: the primary injury, in which damage is caused as a direct result of the mechanical impact; and the secondary injury, which is initiated immediately after brain trauma attributed to further cellular damage from the effects of primary injuries. The latter continues to develop over a period of hours or days.[4] During this second stage, the damage is primarily attributed to neurotransmitter release, calcium overload, free-radical–mediated damage, proapoptotic gene activation, and mitochondrial dysfunction.[5,6]

As a principal excitatory neurotransmitter, glutamate plays an essential role in brain plastic processes, such as learning/memory, development, and aging.[7] On the other hand, overstimulation of the glutamatergic system may lead to a excitotoxicity.[8] Glutamate uptake is a crucial process for maintaining extracellular glutamate

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concentrations below toxic levels. This is achieved through specific excitatory amino acid transporters (glutamate aspartate transporter [GLAST] and glutamate transporter 1 [GLT-1]) that are mainly present in astrocytes. These transporter proteins are modulated by the cell redox status[9]; thus, increased reactive oxygen species (ROS) production and calcium overload in mitochondria may result in glutamate uptake impairment and cellular death.[10]

Mitochondria, an important organelle for cellular bioenergetics, are sensitive to changes in the physiological state of cells and play a critical role in secondary injury post-trauma.[11] In particular, mitochondria are responsible for the functional and structural damage post-TBI.[12–14] Dysfunctions in this organelle post-TBI have been linked to the impairment of brain mitochondrial electron transfer chain and energy transduction attributed to the overloading of mitochondrion-associated calcium,[12] oxidative damage, and disruption of synaptic homeostasis,[15] ultimately ensuing with cell death.[16]

Several therapeutic interventions that target mitochondria have shown promising results by reducing overall neuronal tissue damage as well as enhancing neurological outcome post-TBI.[17–19] In addition, pharmacological agents that also focused on the glutamatergic system have been shown to be neuroprotective.[20,21] Neurodegenerative and neurotoxic models have demonstrated that guanosine (GUO) plays an important role in the central nervous system.[22] This concept led us to posit that it may act through these signaling pathways, attenuating excitotoxicity and mitochondrial damage. GUO has been implicated in neuroprotection through modulation of the glutamatergic system, counteracting glutamate excitotoxicity *in vitro*[23–25] and *in vivo*.[22,26] Among these actions, GUO has shown efficacy both in a stroke model[27] and acute spinal cord injury in rats.[28]

Given these observations and the lack of efficacious trauma treatments, the aim of this study was to investigate whether GUO might protect mitochondrial damage induced by TBI in hippocampus and cortex of rats and to verify the relationship of this effect with modulation of glutamatergic transmission.

Methods

Chemical reagents

2′,7′-dichlorofluorescein diacetate (DCFH-DA), methyltetrazolium bromide (MTT), nicotinamide adenine dinucleotide (NADH), 2,6 dichloroindophenol (DCIP), safranine-O, *o*-phthalaldehyde (OPT), *N*-ethylmaleymide, and GUO were purchased from Sigma-Aldrich (St. Louis, MO). [^{3}H]-glutamic acid was purchased from Amersham Biosciences (Amersham, UK). All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

Animals

Male adult Wistar rats (280–320 g) were obtained from a local breeding colony. The animals were kept in a separate animal room, on a 12-h light/dark cycle, in an air-conditioned room (22 ± 2°C, 45–65% humidity). Commercial diet (GUABI, RS, Brazil) and tap water were supplied *ad libitum*. All the animals were acclimatized to laboratory conditions for 1 week before start the experiment. This study was carried out in strict accord with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Santa Maria, Brazil (Permit Number: 153/2014).

Fluid percussion brain injury and experimental design

The fluid percussion brain injury (FPI) model was carried out as previously described.[29] In brief, animals were anesthetized with a single intraperitoneal (i.p.) injection of Equithesin (6 mL/kg), a mixture containing sodium pentobarbital (58 mg/kg), chloral hydrate (60 mg/kg), magnesium sulfate (127.2 mg/kg), propylene glycol (42.8%), and absolute ethanol (11.6%), and placed in a rodent stereotaxic apparatus. A 3-mm-diameter burr hole was drilled on the right convexity, 2 mm posterior to the bregma and 3 mm lateral to the midline, assuring the dura mater remained intact. A plastic injury cannula was placed over the craniotomy with dental cement. When dental cement had hardened, the cannula was filled with chloramphenicol, closed with a proper plastic cap, and the animal removed from the stereotaxic device and returned to its home cage. After 24 h, animals were anesthetized (isoflurane 1%) and the injury cannula was attached to the fluid percussion device. During surgery and FPI, body temperature (37°C) was monitored rectally and maintained with a heating pad and an overhead incandescent bulb. The TBI was produced by a fluid percussion device developed in our laboratory. A brief (10–15 ms) transient pressure fluid pulse (1.46 ± 0.09 atmospheres) impact was applied against the exposed dura causing apnea (30–70 sec), unconsciousness (7–10 min) measured through the righting reflex restoration,[30] and mortality post-TBI was 27.78%. Based on the data, we can classify the FPI protocol as a moderate TBI.[30,31] Pressure pulses were measured extracranially by a transducer (Fluid Control hydraulic automation; Belo Horizonte, MG, Brazil) and recorded on a storage oscilloscope (Tektronix TDS 210; Tektronix, Beaverton, OR).

Sham-operated and sham-GUO animals underwent an identical procedure, with the exception of FPI. The animals were randomly divided into four groups: Sham-saline; Sham-GUO; TBI-saline; and TBI-GUO. The GUO dose was chosen based on previous *in vivo* studies of excitotoxicity, which demonstrated the optimal effect of GUO at 7.5 mg/kg.[26,32] The animals received a single dose of guanosine (7.5 mg/kg dissolved in 0.9% saline) or saline 15 min post-TBI (Fig. 1) by i.p. route. After 3 h, the animals were euthanized by decapitation, the brain was removed, and structures (hippocampus and cerebral cortex) were separated. Samples were used for mitochondrial isolation and for slices preparation, as described below.

Mitochondrial isolation

Mitochondria were isolated by conventional differential centrifugation as described elsewhere, with some modifications.[30,31] At the end of the TBI protocol described above, the brain was rapidly removed to an ice-cold "isolation buffer" (0.32 M of sucrose, 1 mM of ethylene diamine tetraacetic acid (EDTA), 1 mM of ethylene glycol tetraacetic acid, and 10 mM of Tris-HCl, at pH 7.4) kept at 4°C throughout the isolation procedure, and brain structures (hippocampus and cortex) were separated. Tissue was manually homogenized during two cycles of 10 sec in a Teflon glass potter. The homogenate was centrifuged at 1330*g* for 5 min in a Hitachi Himac SCR20B RPR 20-2 rotor (Hitachi, Tokyo, Japan). The

FIG. 1. Representative illustration of experimental procedure. TBI, traumatic brain injury.

supernatant was carefully removed and centrifuged at 21,200*g* for 10 min. The pellets obtained were resuspended in buffer isolation with 15% Percoll. The discontinuous density gradient was prepared manually by layering 3-mL fractions of the resuspended pellet on two pre-formed layers consisting of 3.5 mL of 23% Percoll above 3.5 mL of 40% Percoll. Tubes were centrifuged for 5 min at 30,700*g* with slow brake deceleration. The material equilibrating near the interface between the 23% and 40% Percoll layers was gently diluted 1:4 with isolation buffer and then centrifuged at 16,700*g* for 10 min. A firm pellet was obtained and gently resuspended in the isolation buffer and supplemented with 0.2 mg/mL of fatty-acid–free bovine serum albumin (BSA). After centrifugation at 6900*g* for 10 min, the supernatant was rapidly decanted and the pellet resuspended in the same buffer using a fine Teflon pestle.

Mitochondrial function and redox state

Estimation of reactive oxygen species production. Mitochondrial generation of ROS was determined spectrofluorimetrically with the DCFH-DA assay.[33] Briefly, mitochondrial samples of brain structures (150 μg of protein/mL) were incubated with "isolation buffer III" and the respiratory substrates, glutamate/malate (5 mM) and succinate (5 mM). The reaction was started with the DCFH-DA (1-μM) addition, and the medium was kept at constant stirring during the assay period. Fluorescence analysis was performed at 488 nm for excitation and 525 nm for emission, with slit widths of 3 nm.

Mitochondrial membrane potential determination

Mitochondrial membrane potential ($\Delta\Psi m$) determination was estimated by fluorescence changes in safranine-O assayed.[34] Briefly, mitochondrial samples of brain structures (250 μg of protein/mL) were incubated with "isolation buffer III", safranine-O (10 μM), and the respiratory substrates, glutamate/malate (5 mM) and succinate (5 mM). In the end-of-period assay, 2,4 dinitrophenol (0.1 mM) was added to analyze the maximal mitochondrial uncoupling. Fluorescence analysis was performed at 495 nm for excitation and 586 nm for emission, with slit widths of 3 nm. $\Delta\Psi_m$ is presented as arbitrary fluorescence units per second (AFU/s).

Glutathione reduced and oxidized levels

Mitochondrial reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were measured by the fluorimetric method, with some modifications.[35] Mitochondria samples (500 μg of protein/mL) were resuspended in sodium-phosphate buffer and phosphoric acid (H_3PO_4) 4.5%, and were centrifuged at 100,000*g* for 30 min (HITACHI CP 80WX Ultracentrifuge; Hitachi). For GSH determination, the supernatant was added to phosphate buffer and OPT (1 mg/mL). Fluorescence was measured at 420 nm for emission and 350 nm for excitation, with slit widths of 3 nm. For GSSG determination, the supernatant resulting from the centrifugation was added to *N*-ethylmaleymide (40 mM) and incubated at room temperature for 30 min. After the incubation, it was added to NaOH solution (100 mM) and OPT. GSH and GSSG levels were determined from comparisons with a linear GSH or GSSG standard curve, respectively. Results are expressed as GSH/GSSG ratio.

Manganese superoxide dismutase activity

Aliquots (100-μL) of isolated mitochondria were added to a medium containing sodium bicarbonate-carbonate buffer (50 mM; pH 10.2), EDTA (2 mM), KCN (1 mM), and adrenaline (0.4 mM). The kinetic analysis of superoxide dismutase (SOD) was started after adrenaline addition, and the color reaction was measured at 480 nm.[36]

Dehydrogenase activity

Dehydrogenase activity assay was carried out according to Bernas and Dobrucki, with minor modifications.[37] Samples were incubated in buffer containing glutamate/succinate (5 mM each) and MTT (0.5 mg/mL) for 30 min at 37°C, and MTT reduction reaction was stopped by the addition of 1 mL of dimethyl sulfoxide. Formazan levels were determined spectrophotometrically, reported as the difference of absorbance between 570 and 630 nm. Individual samples were expressed as a percent of the mean control value in the experiment.

Mitochondrial complex I assay

The activity of complex I (NADH dehydrogenase) was performed according to Bottje and colleagues.[38] Briefly, activity was measured by oxidation of NADH. Mitochondria samples were added to a solution containing 35 mM of potassium phosphate buffer (pH 7.4) and 1.3 mM of DCIP in a final volume of 1 mL. The reaction was initiated with the addition of 0.15 mM of NADH. Absorbance at 600 nm was monitored for 3 min to follow the rate of oxidation of NADH, and activity was determined using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Mitochondrial complex II assay

Activity of complex II (succinate: ubiquinone oxidoreductase) was determined through reduction of DCIP by succinate.[39] The reaction mixture consisted of 50 mM of potassium phosphate buffer (pH 7.0), 1 mM of KCN, 0.05 mM of DCIP, 20 μM of rotenone, 16 mM of succinate, and 0.1 mg prot/mL of mitochondria. Absorbance changes were followed at 600 nm, using an extinction coefficient of 19.1 mM^{-1} cm^{-1} for DCIP.

Swelling

Measurement of mitochondrial swelling was performed in a RF-5301 Shimadzu spectrofluorometer (Shimadzu Corporation, Kyoto, Japan) at 600 nm (slit 1.5 nm for excitation and emission).[40] Briefly, mitochondrial samples (100 μg of protein/mL) were incubated with reaction buffer (250 mM of sucrose and 10 mM of Tris-HCl, pH 7.4), inorganic phosphate, $CaCl_2$ (0.1 mM), and with the respiratory substrates, glutamate/malate (5 mM) and succinate (5 mM). Data for mitochondrial swelling are expressed as arbitrary absorbance units per second (AU/s).

Functionality of glutamatergic system

[^{3}H]-glutamate uptake assay. After decapitation, the hippocampus and cortex were immediately dissected on ice (4°C). The

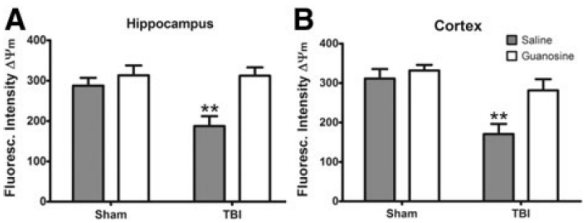

FIG. 2. Effect of GUO treatment (7.5 mg/kg intraperitoneally) on mitochondrial membrane potential ($\Delta\Psi m$) in hippocampus and cortex of rats submitted to TBI. Mitochondrial membrane potential (**A** and **B**) is expressed by fluorescence intensity of $\Delta\Psi$m. Data are expressed as mean ± standard error of the mean ($n = 5$) and were analyzed by two-way analysis of variance, followed by Newman-Keuls' test, when appropriate. Differences were considered significant (**$p < 0.01$; ***$p < 0.001$) when compared to the sham group. GUO, guanosine; TBI, traumatic brain injury.

slices (0.4-mm thickness) were rapidly obtained using a McIlwain Tissue Chopper and immersed at 4°C in Hank's balanced salt solution (HBSS) buffer (pH 7.2). Subsequently, slices were preincubated with HBSS at 37°C for 15 min. Briefly, the incubation was started by the addition of 0.33 μCi/mL of [^{3}H]-glutamate. The reaction was stopped after 7 (cortex) or 5 min (hippocampus) with two ice-cold washes using 1 mL of cold HBSS. After washing, 0.5 N of NaOH were immediately added to the slices and they were stored overnight. Sodium-independent uptake was determined using choline chloride instead of NaCl, which was subtracted from the total uptake to obtain the Na^+-dependent uptake.[23] Incorporated radioactivity was determined with a Packard

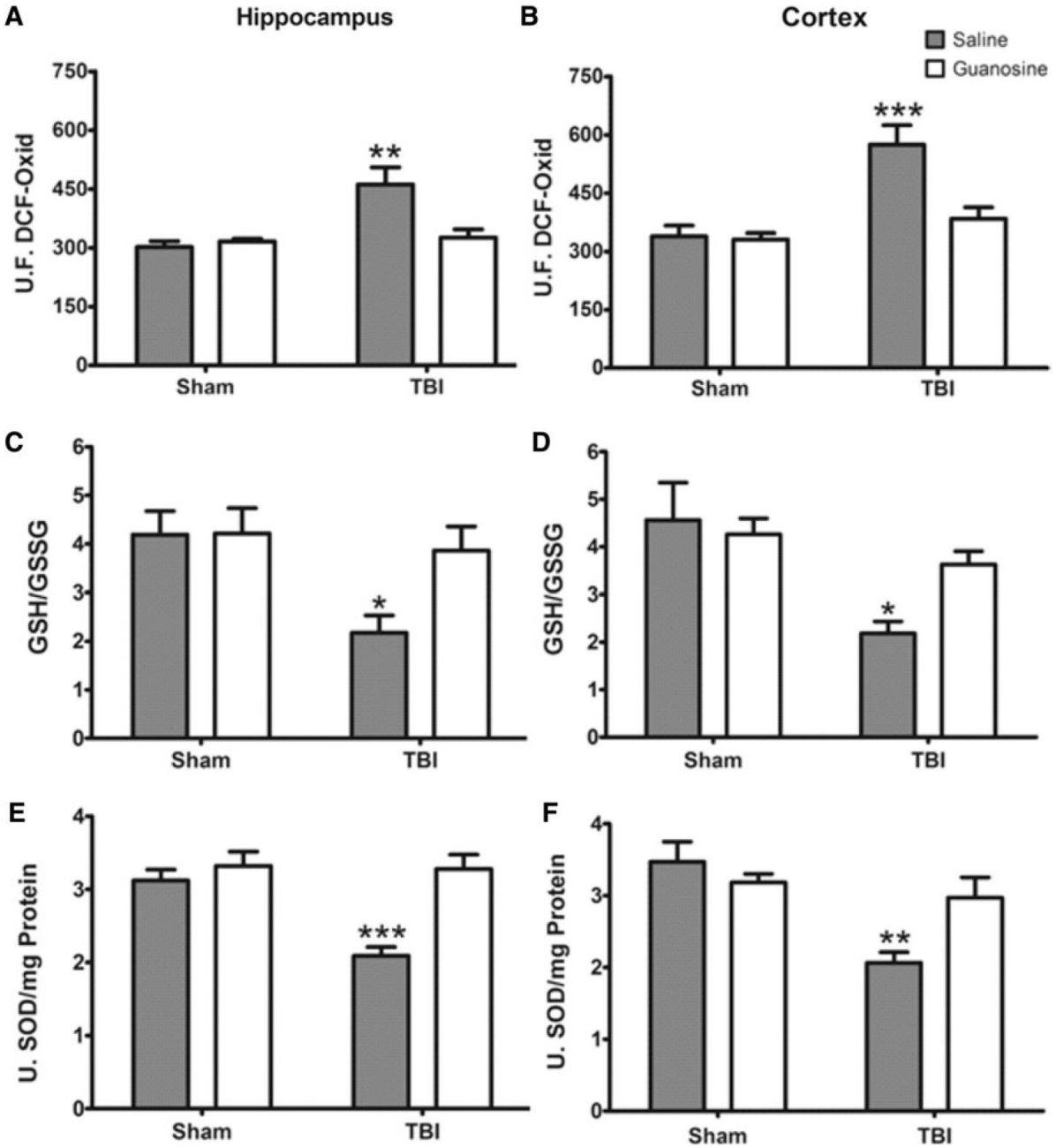

FIG. 3. Effect of GUO treatment (7.5 mg/kg intraperitoneally) on mitochondrial redox status in hippocampus and cortex of rats submitted to TBI. ROS levels (**A** and **B**) are expressed as U.F. DCF-Oxid; GSH/GSSG levels (**C** and **D**) are expressed as GSH/GSSG ratio; MnSOD activity (**E** and **F**) is expressed by units of MnSOD/mg of protein. Data are expressed as mean ± standard error of the mean ($n = 5$) and were analyzed by two-way analysis of variance, followed by Newman–Keuls' test, when appropriate. Differences were considered significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) when compared to the sham group. GSH, reduced glutathione; GSSG, oxidized glutathione; GUO, guanosine; MnSOD, manganese superoxide dismutase; ROS, reactive oxygen species; TBI, traumatic brain injury; U.F. DCF-Oxid, fluorescence units of 2′,7′-dichlorofluorescin oxidation; U. SOD, units of superoxide dismutase.

scintillator (Microbeta[2]; PerkinElmer, Waltham, MA). Protein content was determined using BSA as a standard.[41]

Expression of glutamate transporters

Western blotting was performed according to Franco and colleagues.[42] The hippocampus and cortex were homogenized at 4°C in a buffer (pH 7.0) containing 50 mM of Tris, 1 mM of EDTA, 0.1 mM of phenylmethyl sulfonyl fluoride, 20 mM of Na_3VO_4, 100 mM of sodium fluoride, and protease inhibitor cocktail (Sigma-Aldrich). The homogenates were centrifuged at 1000*g* for 10 min at 4°C, and the supernatants were collected. After total protein determination,[41] using BSA as a standard, β-mercaptoethanol was added to samples in a final concentration of 8%. Then, samples were frozen at −80°C for further analysis. Proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Then, membranes were incubated with specific primary antibodies of glutamate transporters anti-rabbit GLAST

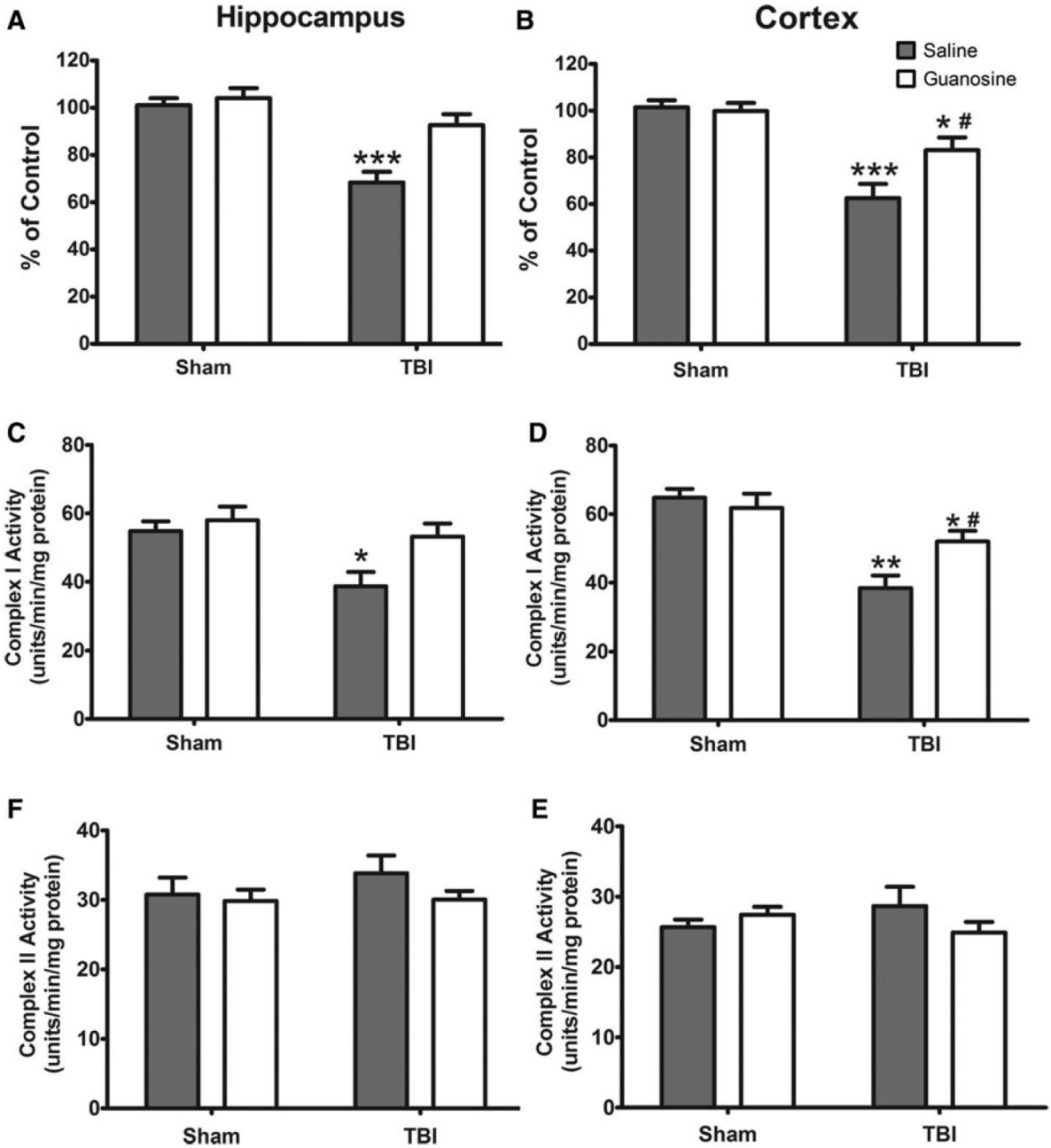

FIG. 4. Effect of GUO treatment (7.5 mg/kg intraperitoneally) on dehydrogenase and mitochondrial complex I and II activities in hippocampus and cortex of rats submitted to TBI. Dehydrogenase activity (**A** and **B**) are expressed by % of control; mitochondrial complex I (**C** and **D**) and II (**E** and **F**) activities are expressed by units/min/mg of protein. Data are expressed as mean ± standard error of the mean ($n = 5$) and were analyzed by two-way analysis of variance, followed by Newman-Keuls' test, when appropriate. Differences were considered significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) when compared to the sham group and ($^{\#}p < 0.05$) as compared to the TBI group. GUO, guanosine; TBI, traumatic brain injury.

(1:1000; Abcam, Cambridge, MA), anti-mouse GLT-1 (1:1000; Thermo Fisher Scientific, Waltham, MA), and β-actin (1:5000; Sigma-Aldrich) for the determination of specific protein targets. Nitrocellulose membranes were provided by GE Healthcare (São Paulo, Brazil). Anti-rabbit immunoglobulin G (IgG) and anti-mouse IgG produced in goat, used as secondary antibody, were purchased from Sigma-Aldrich (São Paulo, Brazil). Densitometric analyses were performed by Quantity One Bio-Rad software (Bio-Rad Laboratories, Hercules, CA), and data were normalized to β-actin as protein control and represented as the percentage relative to sham group.

Protein determination

Protein was measured by the Coomassie blue method, according to Bradford using, BSA as a standard (1 mg/mL).[41]

Statistical analysis

Ex vivo data were analyzed using a two-way analysis (GUO $\times$TBI) of variance, followed by post-hoc comparisons using Newman-Keuls' multiple test, when appropriate. Main effects are presented only when the second-order interaction was not significant. Results are expressed as the mean $\pm$ standard error of the mean. Differences between groups were considered statistically significant when $p<0.05$.

Results

Estimation of mitochondrial membrane potential in mitochondria

$\Delta\Psi_m$ showed a significant TBI and GUO interaction in hippocampus ($F_{(1.16)}=6.14$; $p<0.01$; Fig. 2A) and cortex ($F_{(1,16)}=13.03$; $p<0.01$; Fig. 2B), corroborating the increased ROS generation. TBI caused a significant decrease in $\Delta\Psi_m$. Moreover, the GUO treatment restored the $\Delta\Psi_m$ to levels indistinguishable from the sham-operated animals in both cerebral structures. This disruption in mitochondrial function is consistent with changes in redox status.

Mitochondrial redox state

Optimal mitochondrial function is vital for maintain homeostasis in neural cellular metabolism. Our results corroborate with these hypothesis; the analysis of DCFH levels revealed a significant TBI and GUO interaction in hippocampus ($F_{(1.16)}=8.01$; $p<0.01$; Fig. 3A) and cortex ($F_{(1,16)}=7.54$; $p<0.001$; Fig. 3B) of rats. The TBI group showed a significant increase in ROS production in both brain regions in comparison to the sham group, and treatment with GUO protected against this effect.

Altered GSH levels, and of manganese superoxide dismutase (MnSOD), activity has been implicated as potential contributors to oxidative damage and alteration in mitochondrial redox system post-trauma. Evaluation of GSH/GSSG levels demonstrated a significant main effect of TBI in hippocampus ($F_{(1.16)}=6.34$; $p<0.05$; Fig. 3C) and cortex ($F_{(1.16)}=10.33$; $p<0.05$; Fig. 3D). The results showed a reduction in GSH levels caused by TBI; treatment with GUO ameliorated this effect in both structures. Moreover, analysis of MnSOD activity showed a significant interaction between TBI and GUO in hippocampus ($F_{(1.16)}=8.64$; $p<0.01$; Fig. 3E) and cortex ($F_{(1.16)}=7.25$; $p<0.01$; Fig. 3F) of rats. Trauma caused an inhibition of MnSOD activity and GUO treatment protected against this effect in all structures, showing indistinguishable activity levels from the sham group.

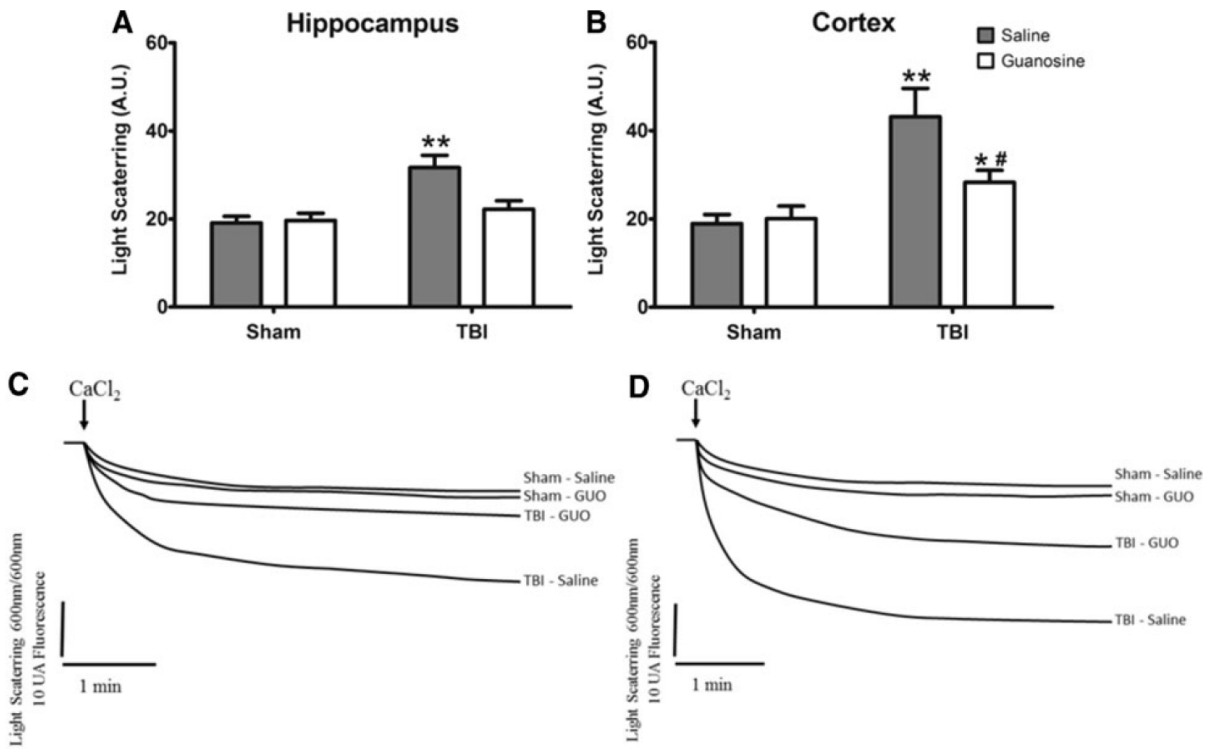

FIG. 5. Effect of GUO treatment (7.5 mg/kg intraperitoneally) on mitochondrial swelling in hippocampus and cortex of rats submitted to TBI. Mitochondrial swelling (**A** and **B**) is expressed as arbitrary absorbance units per second (AU); representative data of mitochondrial swelling (**C** and **D**). Data are expressed as mean $\pm$ standard error of the mean ($n=5$) and were analyzed by two-way analysis of variance, followed by Newman-Keuls' test, when appropriate. Differences were considered significant (*$p<0.05$; **$p<0.01$) when compared to the sham group and ($^{\#}p<0.05$) as compared to the TBI group. GUO, guanosine; TBI, traumatic brain injury.

Dehydrogenase activity and mitochondrial complex I and II assays

The previous results showed increased ROS production associated with a loss of membrane potential that might reduce electron flow and adversely affect mitochondrial function post-TBI. Analysis of dehydrogenase activity displayed a significant TBI and GUO interaction in hippocampus ($F_{(1.16)}=6.50$; $p<0.001$; Fig. 4A) whereas in cortex only a main effect of TBI ($F_{(1.16)}=37.52$; $p<0.001$; Fig. 4B). The results showed decreased mitochondrial dehydrogenase activity in both brain structures of the TBI group. Treatment with GUO maintained normal enzyme activity in the hippocampus in comparison to the sham group. In contrast, it only partially restored the dehydrogenase activity in the cortex.

Analysis of mitochondrial complex I activity revealed a significant main effect of TBI in hippocampus ($F_{(1.16)}=6.78$; $p<0.01$; Fig. 4C) and cortex ($F_{(1.16)}=5.32$; $p<0.01$; Fig. 4D). The complex I activity was inhibited by TBI in both structures and GUO treatment fully restored the activity in the hippocampus and partially in the cortex. The complex II activity was not inhibited in either hippocampus (Fig. 4E) or cortex (Fig. 4F).

Mitochondrial swelling

Analysis of maximal calcium-buffering capacity revealed a significant interaction between TBI and GUO in hippocampus ($F_{(1.16)}=6.10$; $p<0.01$; Fig. 5A) whereas in cortex there was a significant main effect of TBI ($F_{(1.16)}=17.19$; $p<0.01$; Fig. 5B). Mitochondrial swelling was noted in the hippocampus and cortex of the TBI group, with GUO treatment preventing this effect in the hippocampus. In the cortex, it blunted the mitochondrial swelling in relation to TBI, but could not restore it to sham levels. These data confirm the hypothesis that excessive Ca^{+2} uptake into mitochondria is intimately related to functional impairment of this organelle. The alterations found on disruption of Ca^{2+} homeostasis associated with mitochondrial dysfunction led us to evaluate the glutamatergic system functionality.

Glutamatergic system evaluation

Analysis of glutamate uptake showed a significant TBI and GUO interaction in hippocampus ($F_{(1.16)}=6.37$; $p<0.01$; Fig. 6A), whereas in cortex there was a significant main effect of TBI ($F_{(1.16)}=38.46$; $p<0.001$; Fig. 6B). TBI led to decreased glutamate uptake in the cerebral structures of rats when compared to the sham group. Treatment with GUO fully reversed this alteration in the hippocampus and partially in the cortex.

Analysis of GLT-1 levels showed a main effect of TBI in hippocampus ($F_{(1.16)}=16.56$; $p<0.01$; Fig. 6C) and cortex ($F_{(1.16)}=32.93$; $p<0.001$; Fig. 6D) of rats. TBI led to a reduction in GLT-1 levels in both cerebral structures when compared to the sham group. GUO fully reversed this reduction in the hippocampus and partially restored this effect in the cortex. The results on

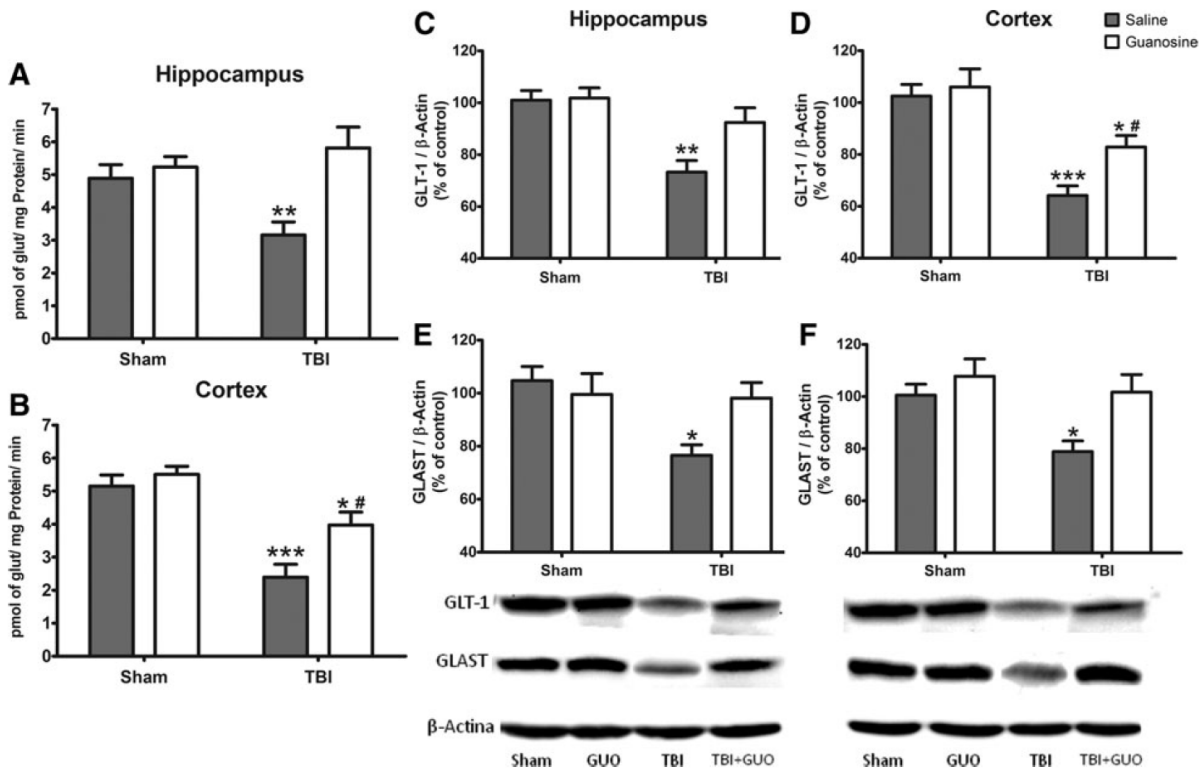

FIG. 6. Effect of GUO treatment (7.5 mg/kg intraperitoneally) on glutamatergic system functionality in hippocampus and cortex of rats submitted to TBI. Glutamate uptake (**A** and **B**) was expressed by pmol of glutamate/mg of protein/min; glial glutamate transporters levels (GLT-1; **C** and **D** and GLAST; **E** and **F**) are expressed by % of control. Data are expressed as mean ± standard error of the mean ($n=5$) and were analyzed by two-way analysis of variance, followed by Newman-Keuls' test, when appropriate. Differences were considered significant (*$p<0.05$; **$p<0.01$; ***$p<0.001$) when compared to the sham group and ($^{\#}p<0.05$) as compared to the TBI group. GLAST, glutamate aspartate transporter; GLT-1, glutamate transporter 1; GUO, guanosine; TBI, traumatic brain injury.

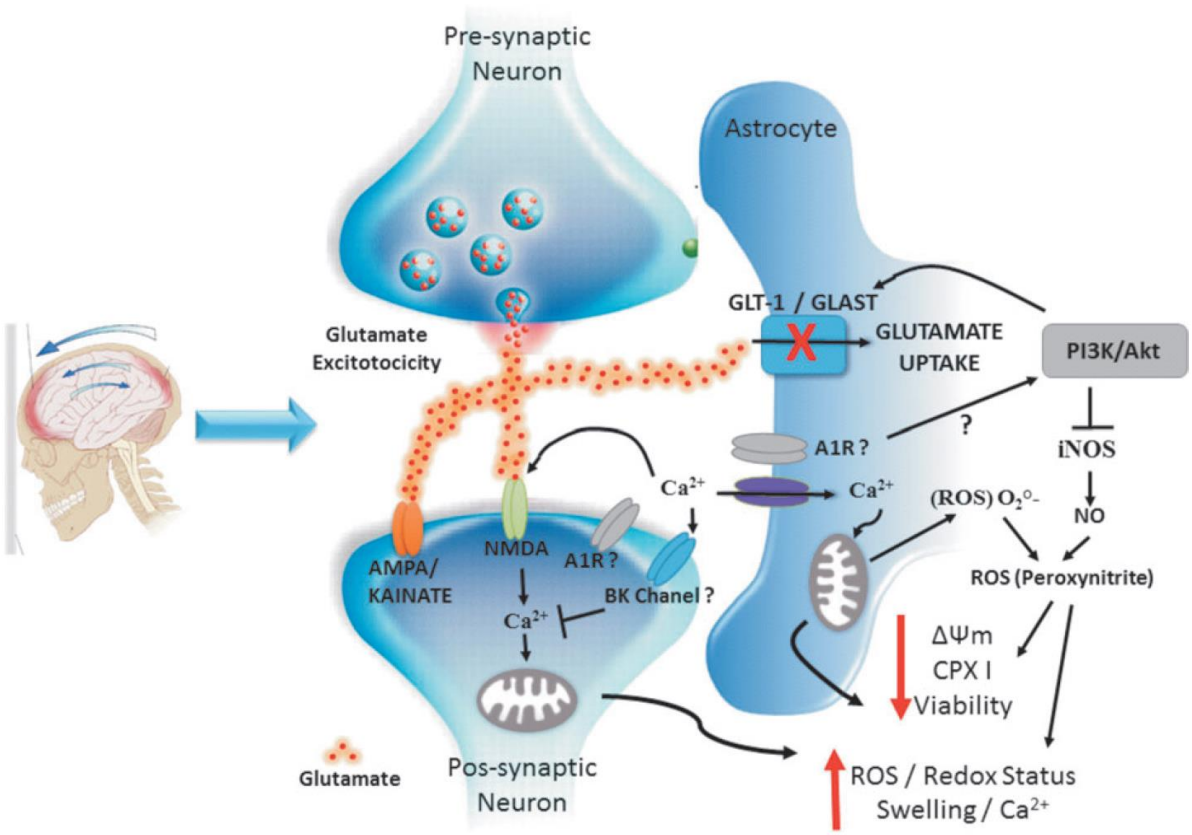

FIG. 7. Schematic illustration of GUO effects against neurotoxicity and mitochondrial impairment induced by TBI in rats. GUO was able to block the injuries caused by the trauma. The signal (''?'') indicates a possible mechanism of action involved. A1R, adenosine A1 receptor; Akt, protein kinase B; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BK, Ca^{2+}-activated K^{+} channels; CPX I, complexin I; GLAST, glutamate aspartate transporter; GLT-1, glutamate transporter 1; GUO, guanosine; iNOS, inducible nitric oxide synthase; NMDA, *N*-methyl-D-aspartate receptor; NO, nitric oxide; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; TBI, traumatic brain injury.

GLAST levels demonstrated a significant TBI and GUO interaction in hippocampus ($F_{(1.16)}=5.67$; $p<0.05$; Fig. 6E) and cortex ($F_{(1.16)}=5.46$; $p<0.05$; Fig. 6F). The data revealed diminished expression of GLAST caused by TBI in both cerebral structures and protective effect by GUO treatment.

Discussion

TBI is a major public health concern and a leading cause of disability worldwide.[2] Though some of the pathophysiological processes associated with TBI have been deciphered,[1,13] there are limitations to the therapeutic strategies available for counteracting its neuropathological sequelae. Here, we show, for the first time, that GUO, an endogenous guanine nucleoside, can mitigate the secondary damage associated with *in vivo* TBI.[25,26,43,44] Specifically, we demonstrate that GUO treatment post-TBI restores glutamatergic function and the maintenance of mitochondrial activity.

Several factors may be responsible for the association between mitochondrial dysfunction and glutamatergic excitotoxicity in the secondary cascade of trauma. Post-TBI, high levels of glutamate are observed, resulting in the excessive activation of *N*-methyl-D-aspartate receptor (NMDA).[5,45,46] Consequently, mitochondrial exposure to excessive glutamate levels rapidly depolarizes and increases ROS production.[13,47–49] This dysfunction appears to be dependent upon the sequestration of Ca^{+2} by mitochondria,[47,48,50] which sequester and release Ca^{+2} through the mitochondrial membrane. Our results showed that the large increase in ROS 3 h post-TBI was correlated with a decreased ΔΨm. GUO treatment maintained the ΔΨm and decreased ROS production in both the hippocampus and cortex. Corroborating these observations, Dal-Cim and colleagues have previously demonstrated that GUO prevented the disruption of mitochondria membrane potential induced by oxygen glucose deprivation (OGD) in hippocampal slices,[43] as well as protected neuroblastoma cells from oxidative damage induced by impairment of mitochondrial activity.[44]

Oxidative stress is one of the first markers of trauma being routinely observed 30 min to 1 h post-injury and is corroborated by other mitochondrial dysfunction markers 3 h post-TBI.[13,15] In agreement, we observed a significant decrease in the GSH/GSSG ratio and inhibition of MnSOD activity in animals submitted to TBI; treatment with GUO maintained GSH levels and enzyme activity indistinguishable from the sham-operated animals. These events can lead to an impaired mitochondrial redox system and oxidative phosphorylation.[12,13]

Inhibition of the mitochondrial respiratory complexes[13,51] and decreased electron flow in the mitochondrial electron transport system were observed in various models of trauma.[13,15,52]

Consistent with these observations, we noted a decrease in mitochondrial viability accompanied by inhibition of complex I activity in animals submitted to trauma. Notably, GUO treatment fully prevented these effects in the hippocampus and partially restored mitochondrial enzyme activity in the cortex. Studies have reported that mitochondrial complex I is a selective target for peroxynitrite-mediated oxidative damage[13,53] besides contributing to the inhibition of the mitochondrial redox system.

Evidences indicate that inducible nitric oxide synthase (iNOS) isoform catalyze substantial synthesis of nitric oxide (NO) in TBI,[54,55] thus reacting with superoxide ions (O2•–) and leading to formation of peroxynitrite ($ONOO^-$), which inhibits neuronal respiration.[13,15,56] Consequently, excessive NO formation may activate NMDA receptors that stimulate Ca^{2+} entry and induce neuronal death (through the mitochondrial damage), further increasing glutamate excitotoxicity.[55,57] The neuroprotective effect of GUO on these parameters may be associated with decreased NO production. Analysis of slices under OGD conditions and glutamate-induced cell death are consistent with this hypothesis, showing a reduction in iNOS levels by GUO through activation of the phosphatidylinositol-3 kinase (PI3K) pathway.[43,58,59]

Ca^{2+} homeostasis represents one of the key mechanisms of TBI, linking the glutamatergic excitotoxicity and mitochondrial dysfunction.[49,51,60] This organelle precisely regulates intracellular homeostasis by sequestering and releasing Ca^{2+}, using several mechanisms that can establish a Ca^{2+} cycle and threaten cellular survival.[60,61] However, excessive amounts of this ion are detrimental and have been shown to result in significant mitochondrial swelling.[15,50,51] Damage to the bioenergetic integrity of mitochondria led to opening of the mitochondrial permeability transition pore and release of cytocromo c, resulting in a significant neuronal death.[5,40,51] The literature corroborates altered Ca^{2+} homeostasis within 30 min post-injury, followed by mitochondrial demise 3 h later.[13,15] Our results show pronounced alterations in mitochondrial swelling 3 h post-trauma, an effect that is completely reversed in the hippocampus and partially restored in the cortex. These protective effects are likely attributed to the modulation of large conductance of Ca^{2+}-activated K^+ channels (BK) by GUO.[43,59]

Several neuroprotective effects of GUO against excitotoxicity have been documented, likely attributed to the modulation of the glutamatergic system.[24,26] In agreement, we showed that TBI decreased glutamate uptake in hippocampal and cortical slices. Moreover, the levels of GLT-1 and GLAST were reduced post-TBI in both brain structures. We observed that GUO was fully able to recover the glutamate uptake and avoided the reduction in glutamate transport levels in both brain structures. These neuroprotective effects might be linked to modulation of glutamate uptake and glutamate transporter activity, given that blockers of these carriers inhibited the GUO effect.[24] It is reported that glutamate transporters are modulated by the cell redox status, and an increased ROS production may result in reverse activity of GLT-1 and GLAST,[9] which contributes to the glutamate uptake impairment. Up until now, GUO normalized the activity of the glutamatergic system, reduced ROS production and alterations in the redox system, and restored the mitochondrial activity. Further, it promoted Ca^{2+} homeostasis, thus mitigating mitochondrial swelling.

The molecular targets and signaling pathways recruited by GUO to modulate its neuroprotective effects remain under evaluation, with GUO being recently classified as an "orphan neuromodulator" (Ciruela F.; July, 2013). This concept led us to hypothesize various cellular mechanisms for its action. With regard to BK channels, strong evidence shows that the activation of adenosine A_1 receptor (A_1) in neurons may preferentially regulate potassium channel conductance.[62,63] In turn, A_1 can also control NMDA receptors as well as inhibit post-synaptically located voltage-sensitive calcium channels.[63] Thus, we posit that GUO may exert its neuroprotection through modulation of A_1 receptor activity, promoting the restoration of ionic gradient and the regulation of glutamatergic transmission.[22,43,59] Notably, GUO was recently shown to have a modulatory effect on the glutamatergic system, which was mediated by activation of PI3K.[43,58,59] In addition, modulation of glutamate uptake and regulation of glutamate transporters activity/expression are linked to the PI3K pathway,[64,65] which is modulated by A_1.[65]

It is suggested that extracellular GUO may contribute to cell signaling through an indirect mechanism involving the adenosinergic system, given that it led to accumulation of extracellular adenosine.[22,66] Indeed, we cannot exclude the possibility that GUO acts through a selective receptor and secondarily modulates the activity of adenosine receptors, BK channels, and the PI3K pathway.

In conclusion, we noted that after the primary insult (mechanical trauma), changes in excitatory amino acids, increase of oxidative stress, and mitochondrial dysfunction occur, which are believed to be the major factors that contribute to the progressive neuropathology observed in TBI. This excessive activation of NMDA receptors,[7,10,61] depolarization, and ROS production leads to a subsequent influx of Ca^{2+}. At this point, we have a chain reaction where we observe the glutamatergic excitotoxicity acting as a trigger, thus the homeostasis of calcium begins its imbalance leading to mitochondria to a toxic stimulus.

Thus, GUO treatment in a TBI rat model produced a neuroprotective effect attributed to the modulation of the glutamatergic system, optimal maintenance of the redox system, and inhibition of intracellular Ca^{2+} alterations in mitochondria. Although its neuroprotective mechanism has yet to be fully understood, the properties of this purinergic nucleoside merit further consideration of its efficacy in ameliorating neurological injuries associated with TBI and altered glutamatergic function. It was essential to evaluate whether the drug was promising in these early stages of TBI. After these preliminary findings, we also consider it fundamental to analyze the long-term alterations related to a clinical context.

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Author Disclosure Statement

No competing financial interests exist.

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3.2 Manuscrito 1

A modulação do receptor A_1 pela Guanosina sobre alterações comportamentais após traumatismo crânio encefálico

Modulation of A_1 receptor by Guanosine in behavioral alterations after traumatic brain injury

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Modulation of A_1 receptor by Guanosine in behavioral alterations after traumatic brain injury

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ABSTRACT

Traumatic brain injury (TBI) is a major public health concern and a leading cause of disability worldwide. This neuropathology results in a significant impairment due to cognitive deficits particularly in chronic neurodegeneration, learning and memory and mood disorders. However, despite of significant effort, very few therapeutic options exist to prevent or reverse cognitive impairment following TBI. Guanosine (GUO) is known for its neuromodulator effects in various models of neuropathologies, specifically those that involve the glutamatergic system. To examine a relevant clinical aspect after brain trauma, this study investigated the effects of chronical GUO treatment in behavioral, molecular and morphological disturbances caused by TBI. Moreover, the influence of adenosinergic system in these effects was evaluated. The rats showed an increase of anxiety-like behavioral and cognitive dysfunction post-injury. In this context, the decrease of hippocampal proteins expression involved in memory process (BDNF and CREB) confirm these findings 21 days after damage. It corroborates with the reduction of synaptophysin, accompanied by the decrease of neurons cells in CA1-CA3 regions of hippocampus. Furthermore, the increase of damage size coincided with the expressive astrogliosis and activated microglia cells 21 days after trauma. On the other hand, the chronic GUO treatment was able to avoid these damages caused by TBI. The presence of A_1 receptor antagonist blunted the GUO proprieties, while the presence of A_{2A} receptor antagonist did not exert effect. These findings reinforce that GUO treatment avoids both acute and chronic changes post TBI and its mechanism is linked to modulation of adenosinergic A_1 receptor.

Keywords: Fluid percussion injury; purinergic nucleoside; behavioral; A_1 receptor; morphology.

INTRODUCTION

Traumatic brain injury (TBI) constitutes a major cause of mortality and morbidity and its incidence is on the rise (GEAN et al. 2010). In consequence, many TBI patients demonstrates a number of long-term cognitive, social and psychiatric complications such as memory loss, anxiety and depression (MAAS et al. 2008). In this context, it becomes increasing the association between the onset of neurodegenerative diseases and TBI (STOICA et al. 2010).

During the last decade, our understanding of the complex pathophysiology that occurs following TBI has vastly improved. It has been elucidated that the primary mechanical insult initiates a cascade of secondary injury that contribute to cellular and behavioral deficits (MAAS et al. 2008, WALKER et al. 2013). In this second stage, damage is sustained to neuronal cells, resulting in abrupt membrane depolarization and indiscriminate release of glutamate (OSTEEN et al. 2004, JIA et al. 2015). This excessive insult results in glutamate excitotoxicity, accumulation of intracellular calcium, mitochondrial dysfunction, inflammatory events and neuronal cells loss (OSTEEN et al. 2004, WALKER et al. 2013, DOBRACHINSKI et al. 2017).

Hippocampal injury-associated learning and memory deficits are often the hallmarks of brain trauma (SCHMIDT et al. 1999, SUN et al. 2007). Models of TBI have been demonstrated cell loss in the hippocampus, altered neuron excitability and deficits in long-term potentiation (LTP), (WITGEN et al. 2005, SCHWARZBACH et al. 2006) suggesting that changes in synaptic plasticity contribute to cognitive deficits. Moderate-to-severe fluid percussion injury (FPI) in rats induces secondary injury mechanisms causing neuronal cell loss and microglial activation that continue over weeks to months after the injury in hippocampus (BYRNES et al. 2012, KABADI et al. 2014b). The increase excitation and reduction of metabolic supply induces a glial scar formation and an aggravation of neuroinflammation. Limiting posttraumatic microglial activation and glial scarring in animal studies reduces not only the progressive

neurodegeneration, but also cognitive deficits (SHULTZ et al. 2012, WALKER et al. 2013, KABADI et al. 2014b)

It is well known that most successful drugs used to treat TBI in experimental models fault report when tested clinically (LOANE et al. 2010, KABADI et al. 2014b). In fact, many trials have failed because the proposed agent regulates only a single mechanism of injury. In order to limit secondary damage after TBI, multiple studies have been conducted. Thus, investigations for new pharmacological treatments in pre-clinical research focus on multi-targets drugs to maximize the likelihood of developing a successful therapeutic intervention (LOANE et al. 2010). Interestingly, purinergic nucleosides/nucleotides are highlighted for demonstrating a multi-target neuroprotective effects in different models of neurodegenerative diseases (BURNSTOCK et al. 2011).

Guanosine (GUO), an endogenous purinergic nucleoside, is known to act as intercellular signaling modulator at the central nervous system (CNS) and modulates glutamatergic transmission. GUO has long been recognized as a neurotropic agent, affording neuroprotection in several *in vitro* and *in vivo* models of injury and neurodegenerative diseases (BETTIO et al. 2016b). Our group demonstrated multi-target neuroprotective effect of GUO against the secondary damage caused by acute TBI in rats (GERBATIN et al. 2016, DOBRACHINSKI et al. 2017). However, the underlying mechanism(s) of its neuroprotection have yet to be understood. Thus, the mechanisms involved in the modulation of adenosinergic system have gained attention as potential alternatives to GUO therapy (DAL-CIM et al. 2013, JACKSON et al. 2013, KOVACS et al. 2015).

Given these observations and the lack of efficacious trauma treatments, the goal of this study was to investigate if chronic treatment with GUO could protect behavioral, molecular and morphological alterations induced by TBI as well as to verify the involvement of adenosinergic system.

MATERIALS AND METHODS

Animals Chemicals Reagents

Male adult Wistar rats (280 - 320g) were obtained from a local breeding colony. The animals were kept in a separate animal room, on a 12h light/dark cycle, in an air-conditioned room (22±2 °C, 45–65% humidity). Commercial diet (GUABI, RS, Brazil) and tap water were supplied ad libitum. All the animals were acclimatized to laboratory conditions for one week before start the experiment. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Santa Maria, Brazil (Permit Number: 153/2014). All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

Drugs and Experimental design

The following drugs were used: Guanosine (GUO) which were dissolved in 0.9% saline and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; A_1 adenosine receptor antagonist) (SCH 58261; A_{2A} adenosine receptor antagonist) (Sigma Chemical Co., USA) dissolved in saline with 0.1% DMSO. Appropriate vehicle-treated groups were also assessed simultaneously. The compounds were administered by intraperitoneal (i.p.) route in a constant volume of 1ml/kg. The chemicals were purchased from Sigma (St. Louis, MO,USA). Trizol® reagent, iScript™ cDNA synthesis Kit (Biorad®) and SYBR® Select Master Mix were purchased from Thermoscientific®. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Sham animals underwent an identical procedure, with the exception of FPI. The animals were randomly divided into different groups: Sham-vehicle; Sham-GUO; TBI-vehicle and TBI-GUO. The GUO dose was chosen based on previous *in vivo* studies of excitotoxicity, which

demonstrated the optimal effect of guanosine at 7.5 mg/kg intraperitoneally (ip) (SOARES et al. 2004, DOBRACHINSKI et al. 2017). The animals received a single dose of GUO or vehicle per day and the first administration was performed 1 hour after TBI. To assess the involvement of adenosinergic system in the neuroprotective effects caused by GUO, the animals were divided in other groups: Sham-vehicle; Sham-DPCPX; TBI-DPCPX; Sham-DPCPX-GUO and TBI-DPCPX-GUO. The animals received a single dose of DPCPX at 1 mg/kg ip (KOPF et al. 1999, PREDIGER et al. 2005) or vehicle per day and the first administration was performed 30 min after TBI. During chronic treatment, DPCPX was always administered 30 min before GUO. The treatment with drugs occurred for 21 days post-injury (Scheme 1). After the all behavioral protocols, one group of animals were perfusioned and other group were euthanized by decapitation. The brain was removed and hippocampus was separated.

Fluid Percussion Brain Injury (FPI)

FPI model was carried out as previously described (D'AMBROSIO et al. 2004). In brief, animals were anesthetized with a single intraperitoneal (i.p.) injection of Equithesin (6 ml/kg), a mixture containing sodium pentobarbital (58 mg/kg), chloral hydrate (60 mg/kg), magnesium sulfate (127.2 mg/kg), propylene glycol (42.8%), and absolute ethanol (11.6%) and placed in a rodent stereotaxic apparatus. A 3-mm-diameter burr hole was drilled on the right convexity, 2 mm posterior to the bregma and 3 mm lateral to the midline, assuring the dura mater remained intact. A plastic injury cannula was placed over the craniotomy with dental cement. When dental cement has hardened, the cannula was filled with chloramphenicol, closed with a proper plastic cap and the animal removed from the stereotaxic device and returned to its homecage. After 24 h, animals were anesthetized (isoflurane 1%) and the injury cannula was attached to the fluid percussion device. During surgery and FPI, body temperature (37ºC) was monitored rectally, and maintained with heating pad and an overhead incandescent bulb. The TBI was produced by a fluid-percussion device developed in our laboratory. A brief (10–15 ms) transient

pressure fluid pulse (1,55 ± 0.09 atm) impact was applied against the exposed dura causing apnea (30-70 seconds), unconsciousness (7-10 min) measured through the righting reflex restoration (THOMPSON et al. 2005b) and mortality after TBI was 26.94%. Based on the data we can classify the FPI protocol as a moderate TBI (THOMPSON et al. 2005b, KABADI et al. 2010). Pressure pulses were measured extracranially by a transducer (Fluid Control hydraulic automation, Belo Horizonte, MG, Brazil) and recorded on a storage oscilloscope (Tektronix TDS 210).

Behavioral tests

Locomotor behavioral tasks

To analyze exploratory and locomotor activities, rats were placed on the center of activity-monitoring chamber. Behavioral testing was conducted in a Plexiglas activity-monitoring chamber 50 × 48 × 50 cm (Insight - Equipamentos, Pesquisa e Ensino, Ribeirão Preto, SP, BRA). Distance, speed and rearing as measures for locomotor and exploratory activity were measured over 4 min during 4 days. The protocol time was chosen to avoid the possible locomotion alteration caused by the drugs used (EL YACOUBI et al. 2000, HALLDNER et al. 2004).

Elevated plus-maze (Mandolesi, #417)

To evaluate anxiety state, rats were exposed to an elevated plus maze (PELLOW et al. 1986). The apparatus consists of two elevated (50 cm high) and open arms (50 cm × 10 cm) positioned opposite to one another and separated by a central platform and two arms of the same dimension, but enclosed by walls (50 cm × 10 cm × 40 cm) forming a cross. The maze is lit by a dim light placed above the central platform and each rat was placed at the center of the maze, facing one of the open arms. The following measures were obtained from the test: (a)

time spent in the open arms relative to the total time spent in the plus-maze, expressed as percentage and (b) number of entries into the open arms An entry was defined as placing all four paws within the boundaries of the arm. A video camera was positioned over the EPM, and the behavior was recorded using a video tracking system for offline analyses by a blind observer to the treatment condition. The sessions lasted for 5 min, and after each trial, the maze was cleaned with an alcohol solution (20%).

Inhibitory avoidance memory test

The step-down inhibitory avoidance task (IA) has been used to evaluated aversive memory (BEKINSCHTEIN et al. 2007). Rats were trained using a 50 × 25 × 25 cm plexiglass box with a 5 cm-high, 8 cm-wide, and 25 cm-long platform on the left end of a series of bronze bars which made up the floor of the box. For the IA training session, rats were gently placed on the platform facing the left rear corner of the training box. When they stepped down and placed their four paws on the grid, a 2s 0.5 mA scrambled foot shock was delivered. The retention trial was performed 24 h after training. Each rat was placed again on the platform, and the transfer latency time (i.e., time took to step down from the platform) was measured in the same way as in the acquisition trial, but foot shock was not delivered and the transfer latency time was recorded to a ceiling of 600 s. The criterion for learning was taken as an increase in the transfer latency time on retention (second) trial as compared to the acquisition (first) trial. So, short transfer latencies indicate poor retention. To avoid confounds by lingering olfactory stimuli, the arena was cleaned with 20% ethanol after each animal was tested.

Object recognition memory test

Training and testing in the object recognition task (OR) were carried out in an open-field arena (50 × 50 × 50 cm) (MELLO-CARPES et al. 2013). The first procedure consisted in the habituation of the animals to the training box. Rats were first habituated to the apparatus

during 10 min of free exploration. The training was conducted by placing individual rats into the field (duration time 5 min), in which two identical objects (objects A1 and A2; duple Lego toys) were positioned in two adjacent corners, 9 cm from the walls. In a short-term memory (STM) test given 1.5h after training, the rats explored the open field for 5 min in the presence of one familiar (A) and one novel (B) object. In a long-term memory (LTM) test given 24 h after training, the same rats explored the field for 5 min in the presence of familiar object A and a novel object C. All objects presented similar textures, colors and sizes, but distinctive shapes. A recognition index calculated for each animal in STM test was expressed by the ratio TB/(TA+TB) [TA= time spent exploring the familiar object A; TB= time spent exploring the novel object B] and in LTM test was expressed by the ratio TC/(TA+TC) [TA= time spent exploring the familiar object A; TC= time spent exploring the novel object C]. Time intervals for testing STM and LTM were chosen on the basis of previous studies characterizing the neurochemical pathways mediating the formation of STM and LTM for object recognition (DE LIMA et al. 2005, MELLO-CARPES et al. 2013). The experiments were performed by an observer blind to the treatment condition of the animals. To avoid confounds by lingering olfactory stimuli and preferences, the objects and the arena were cleaned with 20% ethanol after each animal was tested. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws. Sitting on or turning around the objects were not considered exploratory behaviors.

Ex vivo assays

RNA Isolation

Total RNA was isolated from hippocampus using Trizol® reagent (Invitrogen®) immediately after euthanasia. Conversion of total RNA to cDNA was performed with the iScript™ cDNA synthesis Kit (Biorad®), accordingly to the manufacturer's instructions.

Analysis of mRNA Expression by q-PCR (Quantitative Real-Time PCR)

Gene specific primer sequences were based on published sequences in GenBank Overview (http://www.ncbi.nlm.nih.gov/genbank/) designed with Primer3 program version 0.4.0 (http://frodo.wi.mit.edu/primer3/) and custom made by Invitrogen® (Table 2). Tubulin served as reference gene. Total RNA samples were treated with DNase I (Invitrogen®) to remove genomic DNA contamination in the presence of RNase inhibitor. Reverse transcription (RT) of approximately 1µg total RNA was performed using iScript™ cDNA synthesis Kit (Biorad®), accordingly to the manufacturer's suggested protocol. RT products (cDNAs) were maintained at -20 ºC. Quantitative real-time PCR were performed in 20 µl PCR mixture containing 1 µl RT product (cDNAs) as template and a SYBR® Select Master Mix (Applied Byosistems). PCR mixtures were subjected to PCR at 95ºC for 5 min followed of 40 cycles of 15s at 95ºC, 15 s at annealing temperature appropriated to each primer sequence, and 25s at 72ºC for extension in a Thermocycler StepOne Plus (Applied Biosystems, Foster City, CA, USA). All samples were analyzed as technical quadruplicates with a notemplate control also included. SYBR Green fluorescence was analyzed by StepOne Plus Software version 2.0 (Applied Biosystems, Foster City, CA, USA), and Cq value (ΔCq) for each sample was calculated and reported using ΔΔCq method (LIVAK et al. 2001). Briefly, for each well, a ΔCq value was obtained by the difference in Cq values (ΔCq) between the target gene and the reference gene. The ΔCq mean value obtained from the control group of each gene was used to calculate the ΔΔCq of the respective gene ($2^{-\Delta\Delta Cq}$).

Microscopy sections

For the brain fixation, animals under deep anesthesia (thiopental sodium, 100 mg/kg i.p.) were transcardially perfused with 600 mL of heparinized saline, followed by 600 mL of 4 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4. as previously described (CUNHA et al. 2006). The brains were removed and immersed in the same fixative

overnight and then kept in 30% sucrose in physiological saline (0.9% NaCl) for at least 48h before sectioning. Frozen brains were sectioned (30 µm coronal slices) with a Leica CM1850 cryostat (Leica Microsystems) and store in an anti-freezing solution.

Histochemistry and immunohistochemistry

The slices of hippocampus were mounted on slides coated with 2% gelatin with 0.08% chromalin (chromium and potassium sulfate), allowed to dry at room temperature, and stored at -20°C until use. Neuronal morphology in dorsal hippocampus sections was evaluated by cresyl violet staining of Nissl bodies, as previously described (CUNHA et al. 2006, KASTER et al. 2015). Briefly, sections were incubated for 10 min with cresyl violet (Sigma-Aldrich) solution (0.5% in acetate buffer). Sections were then washed twice with acetate buffer, twice in 100% ethanol, cleared with xylene, and mounted with Vectashield mounting medium (Vector Laboratories). The number of cresyl violet positive neurons in the CA1-CA3 regions was determined using a light microscope. The image was displayed on a computer monitor, and cells with round, obvious nuclei and visible nucleoli were counted. The regions were counted in 6–8 sections per rat using a computerized image analysis system (Carl Zeiss, Germany). Counting was performed by one person who was unaware of the group identities. The values are expressed as the means ± SEM per section.

Immunohistochemistry detection of Iba-1 (a marker of microglia) (Jensen et al., 1997) and of glial fibrillary acidic protein (GFAP) (activated astrocytes) (PEKNY et al. 2005) was performed to evaluated microgliosis and astrogliosis, respectively. The sections were first rinsed for 5 min with PBS (140 mM NaCl, 3 mM KCl, 20 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and then three times for 5 min with Trizma base solution (TBS) (0.05 M containing 150 mM NaCl, pH 7.2) at room temperature. Free floating sections were then permeabilized and blocked with TBS containing 0.2% Triton X-100/ 1% BSA/ 10% goat serum during 45 min, incubated

in the presence of the anti-Iba-1 rabbit polyclonal antibody (1:1000; Wako Chemicals, Richmond, VA) or anti-GFAP mouse monoclonal antibody (1:1000; Abcam, Inc, Cambridge, MA, USA) for 48h at 4°C, rinsed three times for 10 min in TBS, and subsequently incubated with goat anti-mouse or goat anti-rat secondary antibody conjugated with a fluorophore (Alexa Fluor 488; Alexa Fluor 525; Invitrogen) (1:500) for 2 h at room temperature. Fluorescence microscopy was performed using a LEICA (TCS SP5 II). Semi quantitative analysis of mean fluorescence intensities (MFIs) of GFAP and Iba-1 were performed using NIH ImageJ software. Eight images of slides were obtained per hippocampal region. Images were converted into an 8-bit format, and the background subtracted. An intensity threshold was set and kept constant for all images analyzed. MFI per square millimeter area was calculated by dividing the MFI units by the area of outlined regions and was presented as a bar graph. The values are expressed as the means ± SEM per section.

Statistical analysis

The data were analyzed using a two-way analysis of variance, followed by post-hoc comparisons using Newman-Keuls' multiple test, when appropriate. Main effects are presented only when the second-order interaction was not significant. Results are expressed as the mean ± SD of the mean. Differences between groups were considered statistically significant when $p < 0.05$.

In order to verify the normal distribution of data, D'Agostino normality test were utilized to determine the parametric and nonparametric data. The inhibitory avoidance memory test data were analyzed by two-way analysis of variance, followed by post-hoc comparisons using ScheirerRay-Hare's test and expressed as median ± interquartile range. Differences between groups were considered statistically significant when $p < 0.05$.

RESULTS

Evaluation of locomotor activity

Locomotor activity was evaluated to discard any kind of interference in the behavioral evaluation of the animals. In the same way, it was carried out for 4 days to check if the drugs used did not cause locomotor influence. Our results did not show locomotor change in the parameters selected (speed, distance and rearing) among the groups in all days of analysis (supplementary material). The data shown in Table 1 represent the results of the fourth day of locomotor evaluation.

Anxiety-like behavioral assessment

The percentage of time spent in the open arms of the plus maze, an index of anxiety-like behavioral, was significantly reduced in animals following FPI as compared to sham group [($F_{(5.66)} = 9.224$; $p < 0.01$) Fig. 2A]. Treatment with GUO fully reversed this alteration ($p < 0.01$), but in presence of DPCPX this effect was not observed ($p < 0.01$; Fig. 2A). It was not observed significant differences in the number of entries in open arms (Fig 2B). This analysis indicates that trauma injury results in anxiety-like behavior in animals and GUO recovery this parameter through the modulation of A1 receptor.

Learning and memory evaluation

Acquisition memory process did not show differences between the analyzed groups (Fig. 3A). Analysis of aversive memory performed in IA test revealed a significant decrease in memory consolidation of animals submitted to TBI [H(5)= 9.903; $P< 0.001$] (Fig. 3B). The GUO treatment avoid this impairment in memory process [H(5)=13.93; $P< 0.01$] (Fig. 3B). However, the DPCPX administration prevent the GUO effect in animals that suffered TBI [H(5)=10.93; $P< 0.01$] (Fig. 3B). These findings support the hypothesis that GUO recovered and enhanced the aversive memory of rats.

Animals with functional working memory tend to avoid areas that they have just been and explore novel areas or objects. No differences were found in training section of object recognition (Fig. 4A). Analysis of short [($F_{(5.66)}$ =5.112; $p < 0.05$) Fig. 4B] and long-term memory [($F_{(5.66)}$ =11.98; $p < 0.01$) Fig. 4C] revealed a significant interaction between TBI and GUO treatment. Treatment with GUO fully reversed this alteration in both protocols used ($p < 0.01$; Fig. 4B and 4C). Adenosinergic system were correlated with this effect, once DPCPX blocked the GUO effect in the animals in short (p < 0,05; Fig. 4B) and long-term recognition (p < 0,05; Fig. 4C). This results reinforces the importance of adenosinergic system in GUO effect, highlighting the A_1 receptor in memory process.

Ex vivo Analysis

Expression of plasticity's proteins

BDNF and CREB plays an important role in the regulation of functional and structural neuroplasticity. Analysis of this proteins expression showed a significant TBI and GUO interaction in CREB [($F_{(5.24)}$ = 7.473; $p < 0.01$) Fig. 4A] and BDNF expression [($F_{(5.24)}$ = 16.37; $p < 0.01$) Fig. 4B]. TBI led to decreased expression of these essential proteins to memory pathway in hippocampus of rats when compared to the sham group. Treatment with GUO fully reversed this alteration in CREB ($p < 0.01$; Fig. 5A) and BDNF expression ($p < 0.01$; Fig. 5B), but in presence of DPCPX this effect was not observed in respectively proteins expression ($p < 0.05$; Fig. 5A); ($p < 0.001$; Fig. 5B).

In the same sense, synaptophysin (SYN) and growth associated protein 43 (GAP-43) are synaptic proteins colocalized to the presynaptic terminal involved in synaptic remodeling, formation of novel neuronal connections and neurorepair. Analysis of expression of SYN showed a significant TBI and GUO interaction [($F_{(5.24)}$ = 6.814; $p < 0.05$) Fig. 6A]. TBI led to decreased expression of these proteins to memory pathway in hippocampus of rats when

compared to the sham group. Treatment with GUO fully reversed this alteration, but in presence of DPCPX this effect was not observed ($p < 0.05$) Fig. 6A]. However, no differences in GAP-43 expression was found 21 days after traumatic injury (Fig 6B).

Neuronal morphology

We assessed the neuronal loss by histological analysis of cresyl violet stained coronal sections followed for 21 days post-injury. The analysis revealed a significant neuronal loss of CA1-CA3 hippocampus region 21 days after TBI and showed a significant TBI and GUO interaction [($F_{(5.30)}$ =6.913; $p < 0.05$) Fig. 7B]. Treatment with GUO fully reversed this alteration ($p < 0.05$; Fig. 7B), but in presence of DPCPX this effect was not observed ($p < 0.05$; Fig. 7B). This analysis indicates that trauma injury results in a progressive neurodegeneration in the CA1-CA3 regions of hippocampus 21 days post-injury.

Expression of GFAP and IBA-1 (astrocytic scar and microglia activation)

We assessed the glial scar formation through expression of GFAP in hippocampus coronal sections followed for 21 days post-injury. The analysis revealed a significant increase in GFAP fluorescence intensity in CA1-CA3 hippocampus region 21 days after TBI, showed a significant TBI and GUO interaction [($F_{(5.30)}$ = 7,904; $p < 0.01$) Fig. 8B]. Treatment with GUO fully reversed this alteration ($p < 0.01$; Fig. 8B), but in presence of DPCPX this effect was not observed ($p < 0.05$; Fig. 8B). This analysis indicates that trauma injury results in astrocytic scar in the CA1-CA3 regions of hippocampus 21 days post-injury.

We also assessed the microglial activated cells by IBA-1 expression in hippocampus sections followed for 21 days post-injury. The analysis revealed a significant increase in in IBA-1 fluorescence intensity in CA1-CA3 hippocampus region 21 days after TBI, showed a significant TBI and GUO interaction [($F_{(5.30)}$ = 6.639; $p < 0.01$) Fig. 9B]. Treatment with GUO

fully reversed this alteration ($p < 0.01$; Fig. 9B), but in presence of DPCPX this effect was not observed ($p < 0.05$; Fig. 9B). This analysis indicates that trauma injury resulted an increase of inflammatory process in the CA1-CA3 regions of hippocampus 21 days post-injury.

DISCUSSION

Traumatic brain injury (TBI) is an international health concern often resulting in chronic neurological abnormalities, including cognitive deficits and emotional disturbances (BRUNS et al. 2003). Though some of the pathophysiological processes associated with TBI have been deciphered (SINGH et al. 2006, ROOZENBEEK et al. 2013), there are limitations to the therapeutic strategies available for counteracting its neuropathological sequelae. In our previous work, we found that the GUO avoided acute damage caused by the secondary cascade observed after TBI through the modulation of the glutamatergic system and mitochondrial function (GERBATIN et al. 2016, DOBRACHINSKI et al. 2017). This study describes the beneficial effects of GUO administration against the impairment of anxiety-like behavior and cognitive deficits caused by post-traumatic injury. In accordance, GUO treatment prevented the protein expression altered by TBI that are involved in plasticity process as well as maintained the morphology in hippocampus. Our data also show the modulation of adenosinergic system, especially A_1 receptor that are involved in these effects.

Consequences of trauma involves disruptions in ionic, metabolic and physiological homeostasis, all of which culminate into the various cognitive, physical and psychosocial deficits observed in animal models and humans following TBI (WALKER et al. 2013, BRAMLETT et al. 2015). Disruptions in these physiological processes can progress over hours, days and months following brain trauma (MAAS et al. 2008, BRAMLETT et al. 2015). Additionally, it defines the lateral FPI model in rats as one that can be utilized to investigate the pathophysiology of anxiety disorders following TBI (JONES et al. 2008). Here we report the presence of persistent anxiety-like behavior following 14 days post-traumatic injury.

Moreover, our results demonstrated that the GUO treatment avoided this behavioral alteration. This effect can be explained by DPCPX antagonist, which abolished the GUO effect. In accordance, purine-based nucleosides and nucleotides like GUO and GMP also present anxiolytic and antidepressant properties in preclinical tests (ALMEIDA et al. 2016).

The development of novel drugs that focus on the glutamatergic and purinergic systems get featured thought to play a role in anxiety and mood regulation (BURNSTOCK et al. 2011, JUN et al. 2014, ALMEIDA et al. 2016). In this sense, A1 receptors play important roles in the glutamatergic neurons of hippocampus and other limbic structures that are closely related to anxiety behavior, which strengthen their potential as targets for treating anxiety (BURNSTOCK et al. 2011).

The physiologic alterations in the hippocampus after traumatic injury were correlated with dysfunction of hippocampal-dependent learning and memory tasks, including impairment in spatial learning and aversive memory functions (IZQUIERDO et al. 2006, WALKER et al. 2013). In particular, the integrity in the CA1-CA3 and DG sub regions, is essential for performance in the behavioral tasks (IZQUIERDO et al. 2006, MELLO-CARPES et al. 2013, WALKER et al. 2013). Our results showed pronounced alterations in different kinds of learning/memory behavioral after trauma, accompanied by a decrease in CREB and BDNF expression. Interestingly, GUO treatment abolished the alterations in memory tests and the proteins expression involved in plasticity pathways. This is reinforced by the literature data, once it has been demonstrated that the GUO stimulates neural stem cells through activation of cAMP, phosphorylation of cAMP response element-binding protein (CREB) and increases the expression of brain derived neurotrophic factor (BDNF) (SU et al. 2013). The transcriptions factors that mediates the expression of several proteins are involved in the induction and modulation of neuroplasticity (BRAMHAM et al. 2005, BEKINSCHTEIN et al. 2007, BENITO et al. 2010).

It is becoming increasingly clear that alterations in the purinergic system are involved in the development and course of learning/memory disorders (BURNSTOCK 2008, BURNSTOCK et al. 2011). The adenosinergic system is closely related to this protective effect, since the chronic administration of DPCPX blocked the memory consolidation performed by GUO in different behavioral tasks. The modulation of this system by GUO in plasticity process is associated downstream effects in cellular pathways, such the activation of PI3K/Akt and MAPK signaling (TRONSON et al. 2007, BENITO et al. 2010, DAL-CIM et al. 2013). These mechanisms by which GUO exerts beneficial effects turns it a viable treatment for neurological conditions, particularly in TBI.

Memory maintenance or consolidation depends of several different biochemical and molecular variables over the next several hours or days post-trauma, especially in the hippocampus (IZQUIERDO et al. 2006, TRONSON et al. 2007, WALKER et al. 2013). The molecular mechanisms associated with the regenerative capability of the adult brain following injuries (DA SILVA et al. 2013) involve the expression of two pre-synaptic proteins that are related to axonal reorganization and synaptogenesis, growth-associated protein-43 (GAP-43) and synaptophysin (SYN). Our results reinforce this concept, showing a reduction of SYN expression 21 days post-TBI and a recovering by chronic GUO treatment. SYN has been widely considered as a constitutive pre-synaptic protein and used for the identification of axonal nerve terminals and synapses, a role for this protein in synaptogenesis and synaptic reorganization has also been suggested during development and following injury (Bergmann et al., 1997). Otherwise, no differences were found in GAP-43 expression.

Corroborates with the disturbance of memory consolidation and the proteins expression involved in plasticity, the moderate lateral fluid percussion (LFP) injury causes a progressive neuronal cell loss in ipsilateral hippocampus (WITGEN et al. 2005, SCHWARZBACH et al. 2006, BYRNES et al. 2012, KABADI et al. 2014b). The GUO avoided the damage in CA1-

CA3 regions of hippocampus, through the blockade of excitotoxic events observed in the secondary cascade after TBI, such neuronal death (GERBATIN et al. 2016, DOBRACHINSKI et al. 2017), mechanisms of cell proliferation and neurogenesis in the injured brain (RATHBONE et al. 1999, SU et al. 2009).

Despite of the damage observed in neurons cells, reactive astrocytes surrounding the lesion produce inflammatory and cytotoxic mediators that contribute to additional recruitment and activation of microglia (SMITH et al. 1997, LOANE et al. 2016, SAJJA et al. 2016). This movement contributes massively to neuronal excitotoxicity, which further contributes to neurodegeneration process. Otherwise, limiting posttraumatic microglial activation and astrogliosis in animal studies reduces not only the progressive neurodegeneration, but also cognitive deficits (SHULTZ et al. 2012, KABADI et al. 2014b, SAJJA et al. 2016). This concept is supported by our data, the increase of IBA-1 and GFAP expression were observed in CA1-CA3 of hippocampus 21 days post-injury and GUO treatment reduced this parameters. Our group demonstrate the anti-inflammatory effect of GUO in acute trauma model (GERBATIN et al. 2016). This effect might be dependent of modulation of adenosinergic system and the consequent activation of anti-inflammatory signaling cascades (DAL-CIM et al. 2013), considering that DPCPX blunted the GUO effect.

Several neuroprotective effects of purinergic nucleoside against neurotoxicity models and neurodegenerative diseases have been documented, likely due to the modulation of glutamatergic system (BETTIO et al. 2016b, GERBATIN et al. 2016, DOBRACHINSKI et al. 2017). The molecular targets and signaling pathways recruited by GUO to modulate its neuroprotective effects remain under evaluation, with GUO being recently classified as an “orphan neuromodulator” (CIRUELA 2013).

Several studies have proposed a role for adenosine in neurodegenerative and neuropsychiatric disorders, suggesting that the manipulation of this system, mostly through the

activation of A_1R and $A_{2A}R$, may be a potential strategy for the pharmacological treatment of these neurological conditions (CUNHA 2005, BURNSTOCK 2008, BURNSTOCK et al. 2011). Our work corroborates with the literature, showing that GUO at least in part, modulates adenosinergic system (more specifically A1 receptor). The mechanisms underlying this neuroprotector effect by GUO may involve the modulation of A1 or even a putative oligomeric interaction resulting in A1R activation and A2AR blockage (DAL-CIM et al. 2013, BETTIO et al. 2016b) and the consequent activation of the PI3K/Akt and MAPK signaling cascades (DAL-CIM et al. 2011, DAL-CIM et al. 2013). An alternative hypothesis shows that the activation of adenosine A1 receptor in neurons may preferentially regulate potassium channel conductance (such BK channels) (CUNHA 2005, BOISON et al. 2010). In turn, A1 can also control NMDA receptors as well as inhibit post-synaptically located voltage sensitive calcium channels (CUNHA 2005). Thus, we posit that GUO may exert its neuroprotection through modulation of A1 receptor activity, promoting the restoration of ionic gradient and the regulation of glutamatergic transmission (DAL-CIM et al. 2011, DAL-CIM et al. 2013, BETTIO et al. 2016a).

The literature data describes that the glutamatergic system regulation can involve the A_{2A} receptors regulation and the GUO activity (DAL-CIM et al. 2013). In this way, It was also evaluated the participation of A_{2A} in these effects. However, the presence of SCH58261 (A_{2A} receptor antagonist) did not show any changes in relation to the neuroprotective effect of GUO after TBI (data not show).

Strong evidences suggest that extracellular GUO may contribute to cell signaling, through an indirect mechanism involving the adenosinergic system (JACKSON et al. 2013, JACKSON et al. 2014), in which leads to accumulation of extracellular adenosine (JACKSON et al. 2013, BETTIO et al. 2016b). Furthermore, it should be noted that many of the neurotrofic effects of GUO persist in the presence of P1 antagonists (GYSBERS et al. 1992). Thus, itself

is not considered an effective ligand for P1 and P2 receptors (RATHBONE et al. 1991, CICCARELLI et al. 2000), indicating that this nucleoside also acts by distinct mechanisms that are independent of the adenosinergic system.

The possible increase of ADO levels caused by GUO in pathological conditions can be correlated to an increase of ATP/ADP release to glial cells (JACKSON et al. 2013, KOVACS et al. 2015). In this way, the specific modulation of P2 receptors (P2X and P2Y) by GUO cannot be excluded (BALLERINI et al. 2006). Stimulation of specific astrocyte P2 receptors by extracellular ATP/ADP, represents an important component of the physiological and pathological glial responses including neuromodulation, neurotransmission and neurorepair (JAMES et al. 2002, THAUERER et al. 2012). After these evidences the relation of GUO with other purinergic receptors, such P2, cannot be excluded, specific studies for this system appear as possible target of guanosine action.

In summary, we considered fundamental to analyze the long-term alterations observed in brain trauma related to a clinical context, in order to develop the most meaningful outcome measures for successful rehabilitation. The chronic GUO treatment produced a neuroprotective effect in behavioral, molecular and morphological parameters of TBI model, through the modulation of the adenosinergic system.

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Author Disclosure Statement

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FIGURE LEGENDS

Table 1 Evaluation of locomotor activity of GUO effects after traumatic brain injury. The representative results are about fourth day of evaluation. Data are expressed as mean ± S.E.M. (n=10-12 in each group).

Fig 1. Representative illustration of experimental procedure.
In the period of pharmacological intervention, the GUO treatment was started 1h after TBI and performed during the 21 days of the experimental protocol. The modulation of adenosinergic system by GUO was evaluated in other groups through the administration of DPCPX and SCH 58261 30 min before GUO. The work memory was performed by object recognition indicates with short-term memory (STM) and long-term memory (LTM).

Fig 2. Effects of GUO treatment (7,5mg/kg i.p./per day) on anxiolytic-like behavioral in animals submitted to TBI. Time spend in open arms (A) and number of entries in open arms (B) were evaluated in elevated plus maze test (14 days after TBI). The concentration of DPCPX (1mg/kg, i.p) were used in protocol. Data are expressed as mean ± S.E.M. (n = 10-12) and were analyzed by two-way ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***) when compared to the sham group.

Fig 3. Effects of GUO treatment (7,5mg/kg i.p./per day) on aversive memory in animals submitted to TBI. A Training trials required to reach the acquisition criterion (A) and retention in the test session (B) were evaluated in inhibit avoidance test (15 and 16 day respectively after TBI). The concentration of DPCPX (1mg/kg, i.p) were used in protocol. Data are presented as median and interquartile ranges, (n = 10-12) per group and were analyzed by two-way ANOVA, followed by Mann–Whitney test when appropriated. Differences were considered significant $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***) when compared to the sham group.

Fig 4. Effects of GUO treatment (7,5mg/kg i.p./per day) on object recognition in animals submitted to TBI. Training (A), short (B) and long-term memory (C) were evaluated in object recognition test (20-21 days after TBI). The concentration of DPCPX (1mg/kg, i.p) were used in protocol. Data are expressed as mean ± S.E.M. (n = 10-12) and were analyzed by two-way

ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant $p < 0.05$ (*); $p < 0.01$ (**) when compared to the sham group.

Fig 5. Effects of GUO treatment (7,5mg/kg i.p./per day) on expression of BDNF and CREB in animals submitted to TBI. Expression of CREB (A) and BDNF (B) were evaluated in qPCR test (21 days after TBI). The concentration of DPCPX (1mg/kg, i.p) were used in protocol. Data are expressed as mean ± S.E.M. (n = 5) and were analyzed by two-way ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***) when compared to the sham group.

Fig 6. Effects of GUO treatment (7,5mg/kg i.p./per day) on expression of Synaptophysin and GAP43 in animals submitted to TBI. Expression of Synaptophysin (A) and GAP43 (B) were evaluated in qPCR test (21 days after TBI). The concentration of DPCPX (1mg/kg, i.p) were used in protocol. Data are expressed as mean ± S.E.M. (n = 5) and were analyzed by two-way ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant $p < 0.05$ (*) when compared to the sham group.

Fig 7. Effects of GUO treatment (7,5mg/kg i.p./per day) on hippocampus (CA1-CA3) neuronal loss in animals submitted to TBI. Representative images (A) and number of neuronal cells (B) were performed using crezyl violet (21 days after TBI). The concentration of DPCPX (1mg/kg, i.p) were used in protocol. Data are expressed as mean ± S.E.M. (n = 6) and were analyzed by two-way ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant $p < 0.05$ (*); $p < 0.01$ (**) when compared to the sham group.

Fig 8. Effects of GUO treatment (7,5mg/kg i.p./per day) on hippocampus (CA1-CA3) glial scar in animals submitted to TBI. Representative images reactive astrocytes cells (A) and (B); expression of glial scar (C) were performed using fluorescence intensity of GFAP expression (21 days after TBI). The concentration of DPCPX (1mg/kg, i.p) were used in protocol. Data are expressed as mean ± S.E.M. (n = 6) and were analyzed by two-way ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant $p < 0.05$ (*); $p < 0.01$ (**) when compared to the sham group.

Fig 9. Effects of GUO treatment (7,5mg/kg i.p./per day) on hippocampus (CA1-CA3) microglial activation cells in animals submitted to TBI. Representative images of microglial activation cells (A) and (B); expression of microglial activation cells (C) were performed using fluorescence intensity of IBA-1 expression (21 days after TBI). The concentration of DPCPX (1mg/kg, i.p) were used in protocol. Data are expressed as mean ± S.E.M. (n = 6) and were analyzed by two-way ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant $p < 0.05$ (*); $p < 0.01$ (**) when compared to the sham group.

Table 1

	Sham	GUO	TBI	TBI+GUO	TBI + DPCPX	TBI + DPCPX + GUO
Distance (mm)	4986.00 ± 755.70	5412.0 ± 1149.00	4819.00 ± 774.3	5023.0 ± 693.7	4730.00 ± 503.4	4768.00 ± 875.45
Rearing	17 ± 4	15 ± 3	18 ± 4	16 ± 5	17 ± 5	15 ± 4
Speed (mm/s)	30.49 ± 10.70	36.25 ± 8.42	32.42 ± 11.02	31.66 ± 9.35	36.95 ± 11.96	35.32 ± 12.14

Figure 1

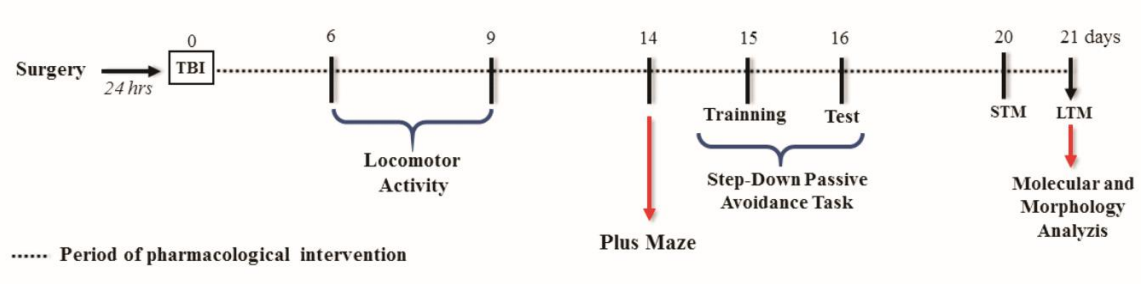


Figure 2

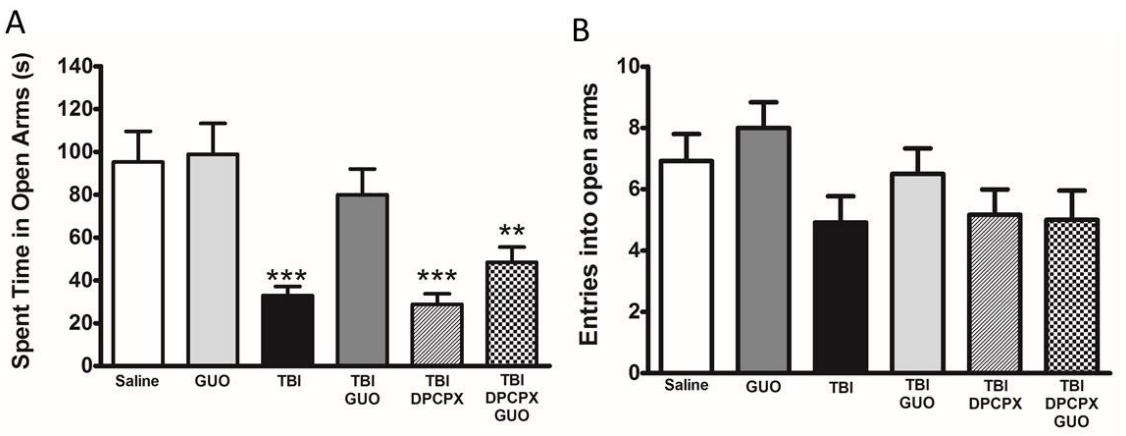


Figure 3

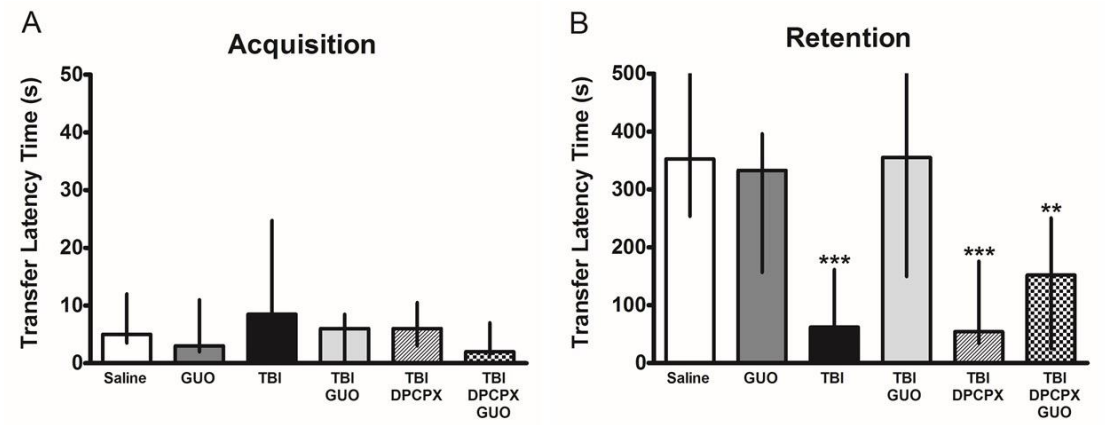
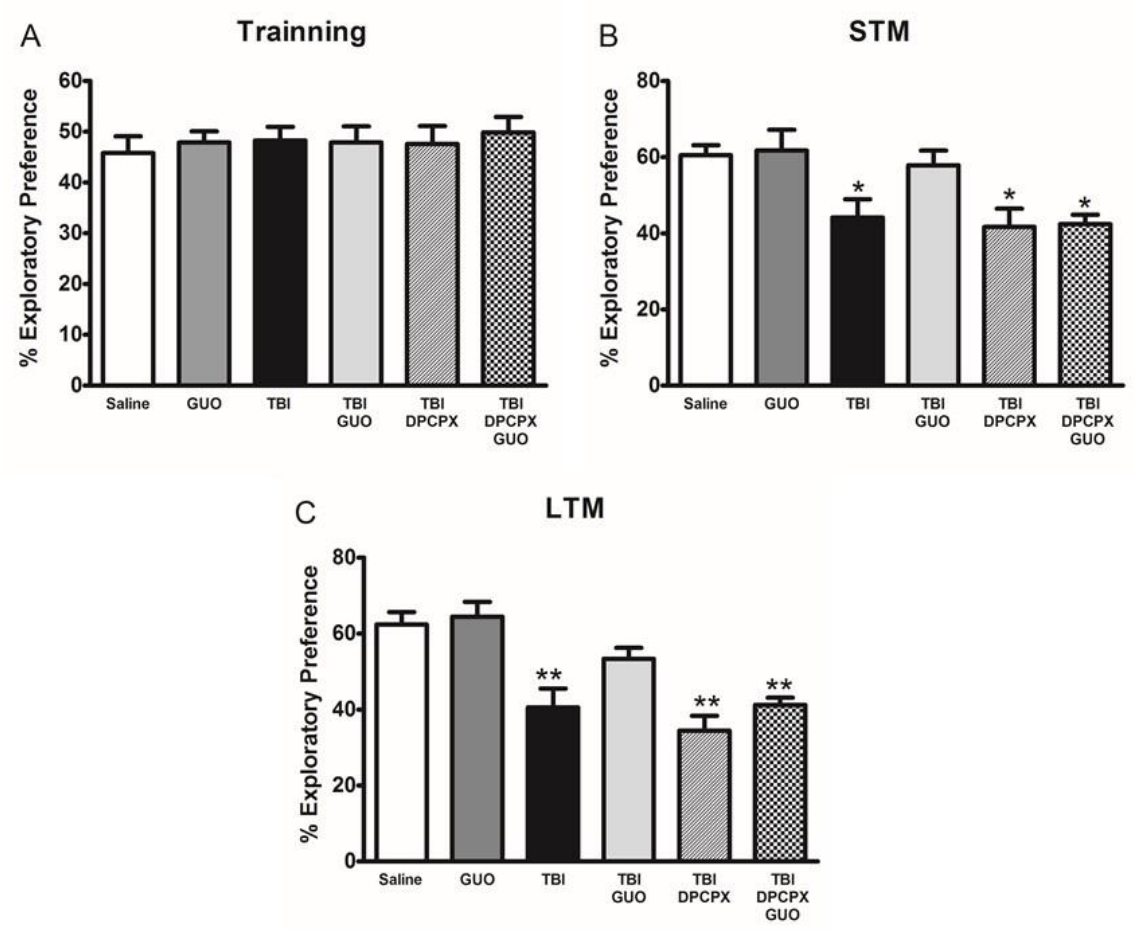


Figure 4



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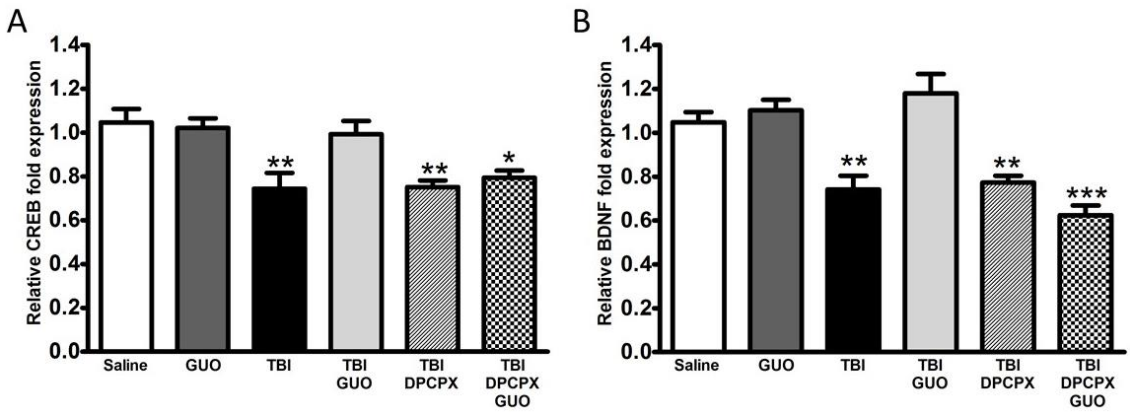


Figure 6

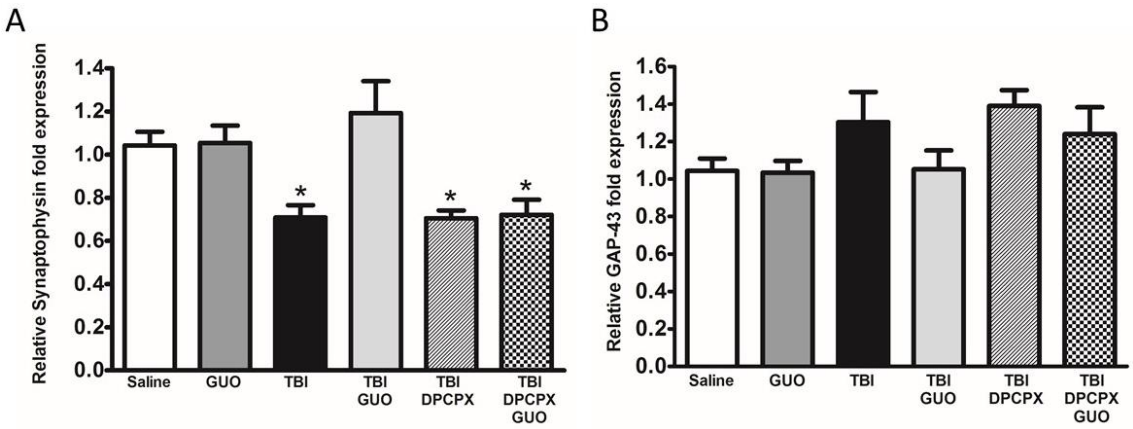


Figure 7

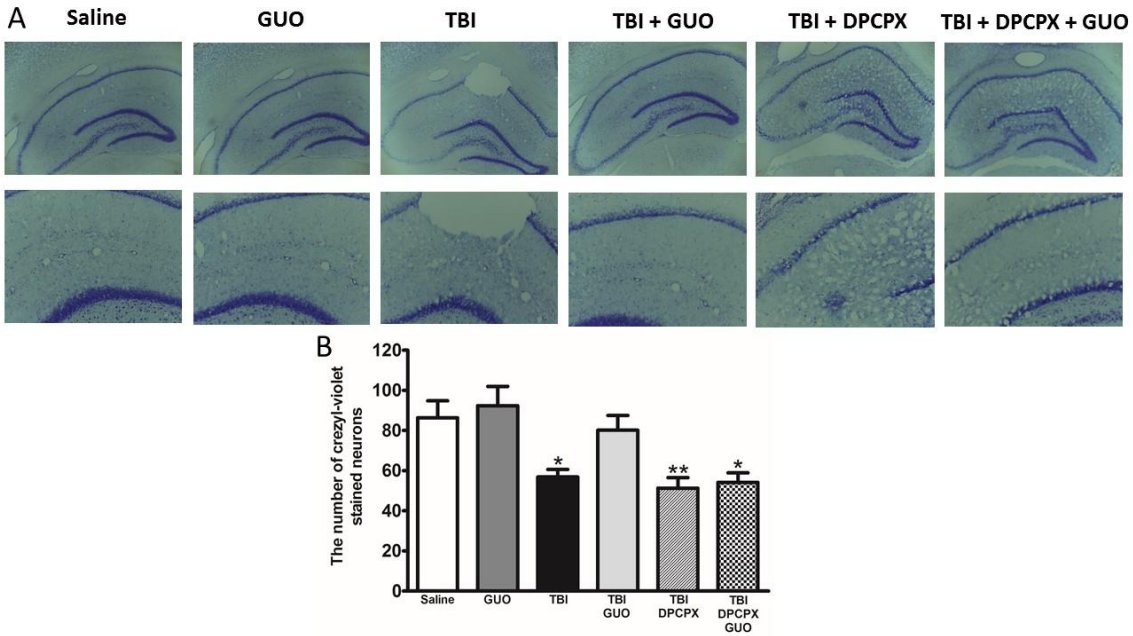


Figure 8

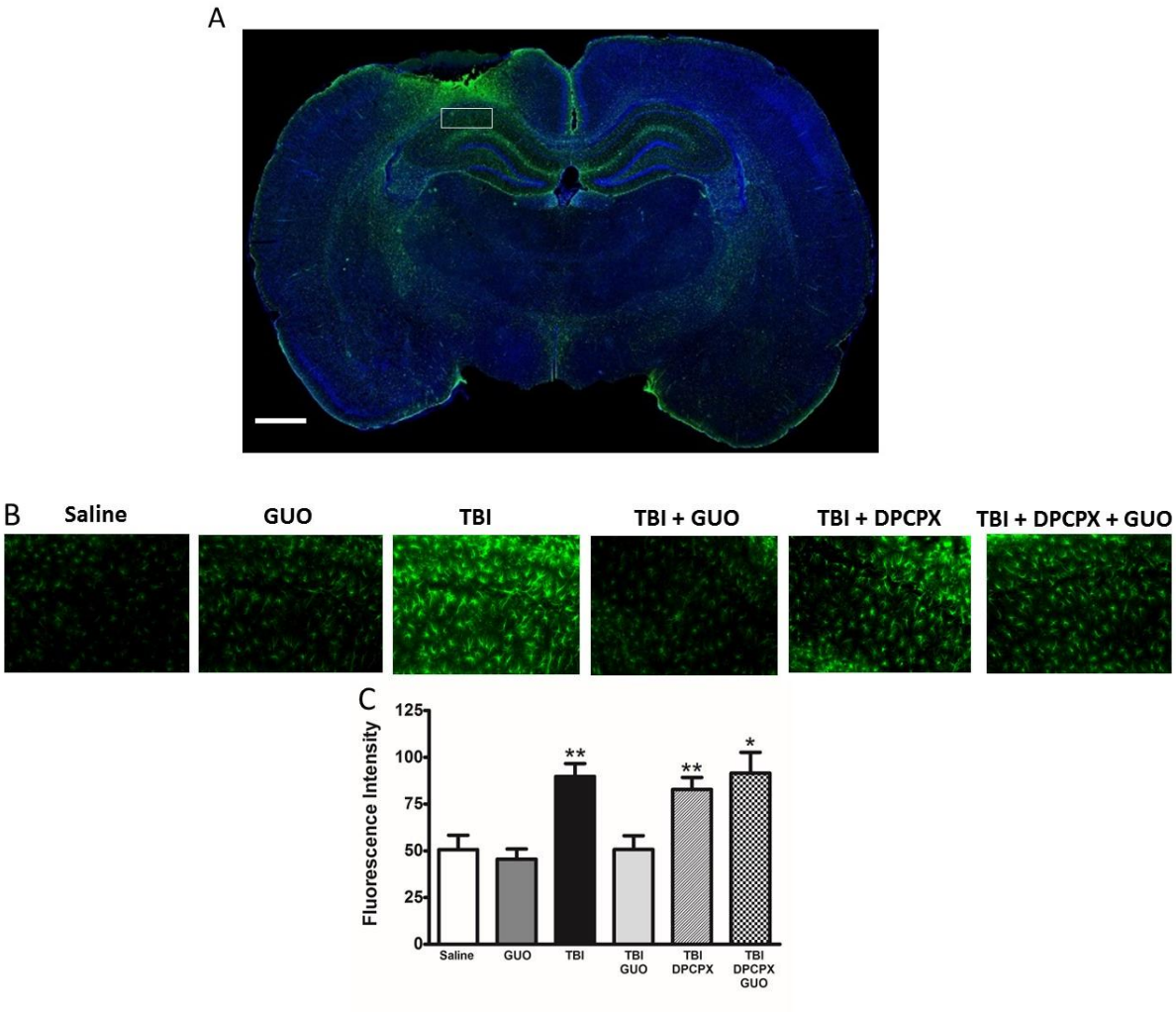
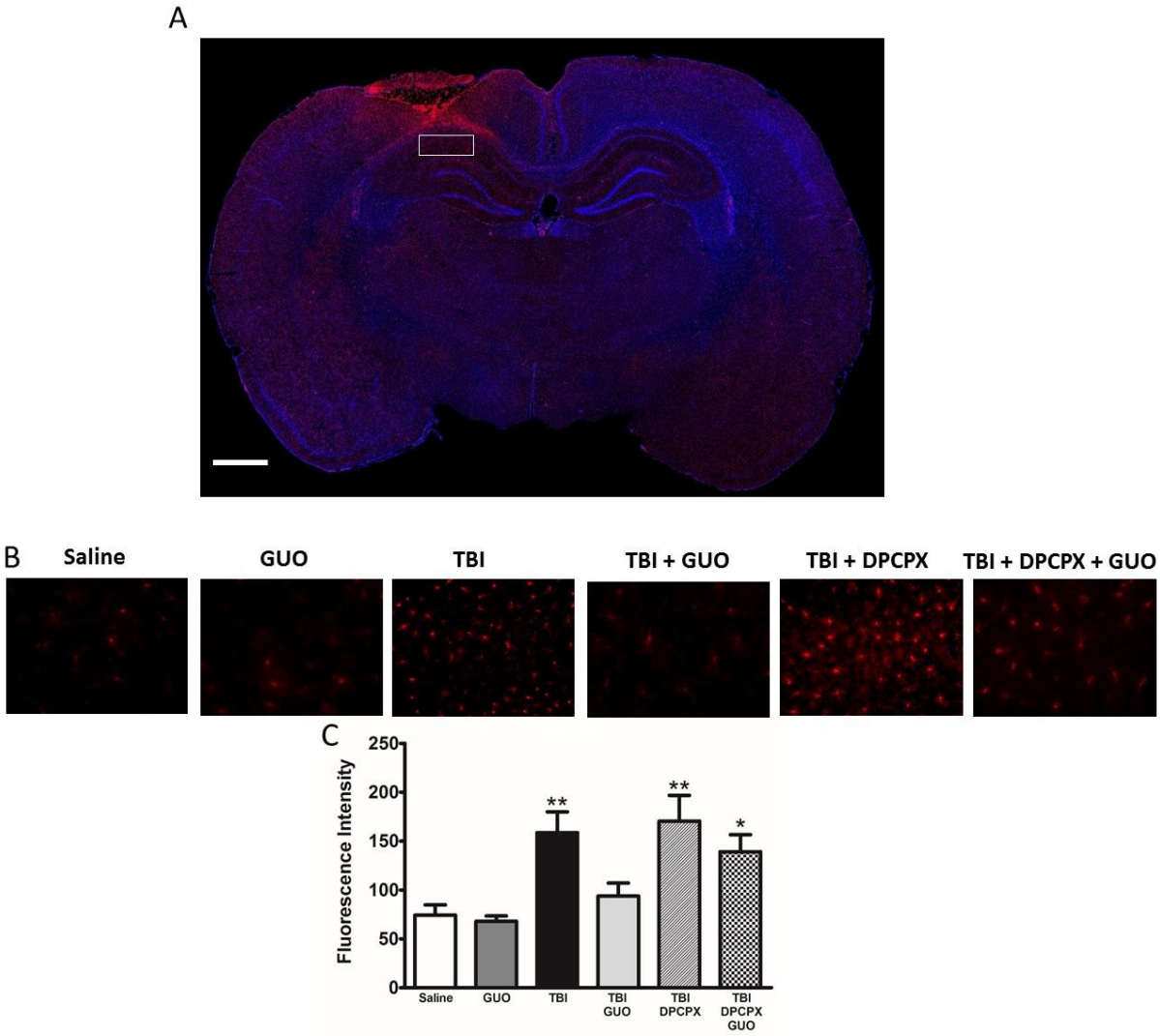

A
B
Saline
GUO
TBI
TBI + GUO
TBI + DPCPX
TBI + DPCPX + GUO
C
Fluorescence Intensity
125
100
75
50
25
0
**
**
*
Saline
GUO
TBI
TBI GUO
TBI DPCPX
TBI DPCPX GUO

Figure 9


A
B
Saline
GUO
TBI
TBI + GUO
TBI + DPCPX
TBI + DPCPX + GUO
C
Fluorescence Intensity
250
200
150
100
50
0
**
**
*
Saline
GUO
TBI
TBI GUO
TBI DPCPX
TBI DPCPX GUO

3.3 Manuscrito 3

O efeito neuroprotetor da Guanosina através da modulação do receptor $P2Y_1$

The Neuroprotector effect of Guanosine through the modulation of $P2Y_1$ Receptor

Fernando Dobrachinski; Henrique Silva; Patrícia Sofia Alcylon Morais; Lisiane O. Porciúncula; Rodrigo A. Cunha; Félix Alexandre Antunes Soares

The Neuroprotector effect of Guanosine through the modulation of $P2Y_1$ Receptor

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Abstract

Glutamate is the most important excitatory neurotransmitter of the mammalian central nervous system (CNS), playing an important role in memory, synaptic plasticity and neuronal development. However, glutamate overstimulation is also implicated in excitotoxic process, which involves a several neurodegeneration diseases. Guanosine (GUO) is known for its neuromodulator effects in various models of neuropathologies, specifically those that involve the glutamatergic system. To examine a role of GUO pharmacological mechanisms, this study investigated the effects of GUO in basal synaptic transmission in slices submitted to hiperexcitability model. In this sense, GUO did not modify the basal synaptic transmission. Moreover, the purinergic nucleoside confirms a classical neuroprotector effect by inhibiting the NMDA-induced excitotoxicity in Shaffer collaterals in hippocampus slices. The results confirm that this effect depend of astrocytic function through the modulation of glutamatergic system. The GUO has been demonstrated to modulate adenosinergic system, more specifically A_1 receptors. The purinergic nucleoside was not able to modify the inhibition curve of 2-chloroadenosine and the effect purinergic nucleoside was avoided by GUO, indicating that this nucleoside increase the extracellular levels of adenosine. Interestingly, this results shows that GUO can act indirectly by modulation of adenosinergic system, which was associated to adenosine release. It was also confirmed that the GUO decreased the alterations in basal synaptic transmission caused by adenosine deaminase. In addition, the increase of adenosine levels by GUO can modulates the P2 activity and the presence of MRS 2179 (antagonist at $P2Y_1$ receptors) inhibited the GUO neuroprotection. These results indicate that GUO targets the glutamatergic hiperexcitability related to modulation of $P2Y_1$ receptors.

Keywords: purinergic receptor; adenosine; excitotoxicity; electrophysiology; purinergic nucleoside; NMDA.

Introduction

Glutamate (GLU) is an amino acid, which is considered the main central excitatory neurotransmitter in mammals (ERECINSKA et al. 1990, DANBOLT 2001). In the central nervous system (CNS), GLU plays an essential role in plasticity processes such as learning/memory, motor behavior, development and ageing (CONQUET et al. 1994, IKONOMIDOU et al. 1999, DANBOLT 2001, YAMASAKI et al. 2014).

The homeostasis of glutamatergic system is an essential process in the brain, which your uptake is a crucial process for maintaining extracellular GLU concentrations below toxic levels. This is achieved through specific excitatory amino acid transporters (EAATs) that are mainly present in astrocytes (DANBOLT 2001). On the other hand, overstimulation of the glutamatergic system may lead to a excitotoxicity mechanisms (NICHOLLS 2008) that is potentially involved in several neurodegenerative process such traumatic brain injury, stroke, Parkinson’s and Alzheimer's diseases (AD) (LEWERENZ et al. 2015, KINOSHITA et al. 2016).

Glutamatergic signaling dysfunction refers to the rapid and massive release and inhibited uptake of GLU as a result of energy failure (DANBOLT 2001, DANBOLT et al. 2016). The induction of such excitotoxicity depends on calcium (Ca^{2+}) influx through *N*-methyl-D-aspartate–type glutamate receptors (NMDARs). The accumulation of GLU overactivates a downstream signaling pathways in CNS cells, many of which involve a surge in ions influx, causing the high intracellular Ca^{2+} concentration. This phenomenon is related to a great number of CNS disorders, thus the search for pharmacological strategies against excitotoxic process is of great relevance (LIPTON 2007, HARDINGHAM et al. 2010).

Neurodegenerative and neurotoxic models have demonstrated that guanosine (GUO) plays an important role in the central nervous system (CNS) (BETTIO et al. 2016a). This guanine nucleoside has been implicated in neuroprotection through its ability to modulate

mechanisms involved in excitotoxic events, counteracting glutamate excitotoxicity *in vitro* (FRIZZO et al. 2003, DOBRACHINSKI et al. 2012, DAL-CIM et al. 2013, DAL-CIM et al. 2016) and *in vivo* (BETTIO et al. 2016a, GERBATIN et al. 2016, LANZNASTER et al. 2016b, DOBRACHINSKI et al. 2017). However, the underlying mechanism(s) of its neuroprotection have yet to be understood.

Several studies have been outlined some pharmacological mechanisms for GUO, mainly focusing on oxygen and glucose deprivation (OGD) model (OLESKOVICZ et al. 2008, DAL-CIM et al. 2011, DAL-CIM et al. 2013). The mechanisms underlying this glutamatergic modulation by GUO may involve regulation of purinergic system (more specifically adenosinergic receptors) and activation of calcium-dependent high (big) conductance potassium (BK) channels, which could regulate the PI3K/Akt and MAPK signaling cascades (OLESKOVICZ et al. 2008, MOLZ et al. 2011, DAL-CIM et al. 2013).

Thus, mechanisms that involve a modulation of purinergic system are highlighted as potential alternatives to GUO therapy. Given these observations and unclear concepts of pharmacological pathways for GUO neuroprotection, the purpose of this study was to investigate the GUO mechanism in excitotoxicity model.

Materials and Methods

Animals

Male C57/BL6 mice (8-10 weeks old) were obtained from Charles River (Barcelona, Spain). Mice were housed under controlled temperature (22 ± 2 °C), 66% humidity, subject to a fixed 12-h light/ dark cycle, with free access to food and water. All studies were approved by the Ethics Committee of the Center for Neuroscience and Cell Biology of Coimbra (ORBEA-78/2013) and conducted according to the European Union guidelines described in the Guide for the Care and Use of Laboratory (2010/63/EU).

Drugs

Tetraethylammonium chloride (TEA), [[(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-Amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS 2365), 2'-Deoxy-N^6-methyladenosine 3',5'-bisphosphate tetrasodium salt (MRS 2179), 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH 58261), charybdotoxin (CHA), Nifedipine and Cyclopiazonic acid (CPA) were from Tocris Biosciences (Bristol, UK). Guanosine (GUO), N-Methyl-D-aspartic acid (NMDA), sodium trifluoroacetate (TFA), adenosine deaminase (ADA), L-α-aminoadipic acid (LAA), 2-chloroadenosine (Carvalho, #230) and 8-cyclopentyl-1,3- dipropylxanthine (DPCPX) were from Sigma (St Louis, MO, USA). The drugs were dissolved in aCSF solution to the desired concentration, with adequate controls for the possible influence of dimethylsulfoxide.

Electrophysiological recordings

Following decapitation, the brain was quickly removed and placed in ice-cold, oxygenated (95% O_2 and 5% CO_2) in aCSF solution (in mM: NaCl, 124; KCl, 3; NaH_2PO_4, 1.25; glucose, 10; $NaHCO_3$ 26; $MgSO_4$, 1; and $CaCl_2$, 2) and the hippocampus was dissected. Coronal transversal slices (400 µm) were prepared with a McIlwain chopper and allowed to recover for 60 min at 35°C and for 30 min at room temperature in a Harvard Apparatus resting chamber with gassed aCSF. Individual slices were transferred to a submersion recording chamber (1 mL capacity) and continuously superfused at a rate of 3 mL/min with gassed aCSF kept at 30.5°C. A bipolar concentric electrode was placed on the Schaffer collateral–commissural pathway and stimulated every 20s with rectangular pulses of 0.1ms. The orthodromically-evoked field excitatory postsynaptic potentials (fEPSPs) were recorded

through an extracellular microelectrode pipette filled with 4M NaCl (2–4 MΩ resistance) and placed in the stratum radiatum of the CA1 area. We first constructed an input–output curve to select the intensity of the stimulus to evoke a fEPSP of ~ 60% of maximal amplitude. Recordings were obtained with an ISO-80 amplifier (World Precision Instruments, Hertfordshire, UK) and digitized using a ADC-42 board (Pico Technologies, Pelham, NY, USA). Averages of three consecutive responses were continuously monitored on a personal computer with the LTP 2.01 software (WinLTP Ltd., Bristol, UK) (WOOLLEY et al. 1997). Responses were quantified as the initial slope of the averaged fEPSP and the effect of drugs, added to the aCSF superfusion solution, was estimated by changes of the fEPSP slope compared with baseline. The drugs were added through the aCSF superfusion solution and its effects were normalized as the % of the baseline and quantified as % inhibition in comparison to the normalized PS.

Statistical analysis

The number of determinations (n, corresponding to independent mice) in each experimental condition is indicated in the legend to the figures. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc test. The comparison of two experimental conditions was performed using the unpaired Student's t test, as indicated in each case. Statistical significance was set as $P < 0.05$.

Results

Effect of GUO on basal synaptic transmission

In order to evaluate the action of GUO in the basal synaptic transmission and consequently to choose a concentration to be used, different concentrations of this purinergic compound were tested. No significant changes in basal synaptic transmission caused by GUO

were observed (Fig. 1), thus based on the data of the literature (CICCARELLI et al. 2001, BAU et al. 2005, BALLERINI et al. 2006) and on our results, we chose the concentration of 300µM.

Effect of GUO on NMDA hiperexcitability

Evidences indicate the role of GUO during excitotoxic events associated a different neuropathologies (BETTIO et al. 2016a). In this sense, we observed a high excitability and a decrease in basal synaptic transmission in slices submitted to NMDA 8µM ($F_{(3.16)} = 108.1$; $p < 0.01$; Fig. 2B and 2D). Otherwise, in presence of GUO 300 µM this excitotoxic effect was abolished ($F_{(3.16)} = 28.32$; $p < 0.01$; Fig. 2A and 2C). The positive control confirmed that there is no excitotoxic precondition in slice, since the pharmacological effect of NMDA was the same as that presented in the first part of the protocol (Fig. 2B and 2D).

The role of astrocytes on GUO effect

It is believed that its neurotrophic effect on glutamatergic hyperexcitability, is due to a great contribution or even to a main role mediated by the compound in glial cells (astrocytes). In our NMDA protocol, the slices were incubated with TFA 500µM (metabolic inhibitor of astrocytes) (SWANSON et al. 1994, RAYMOND A. SWANSONFONNUM et al. 1997, CHRISTIAN et al. 2013) during 2h ($F_{(3.16)} = 61.19$; $p < 0.01$; Fig 3A) and 4h ($F_{(3.16)} = 41.42$; $p < 0.01$; Fig 3B) and the GUO neuroprotective effect was inhibited.

In order to confirm astrocytic hypothesis and previous results, we used other pharmacologic tool for the inhibition of the astrocytes functionality. The NMDA protocol was performed at 2h ($F_{(3.16)} = 45.07$; $p < 0.01$; Fig 3D) and 4h ($F_{(3.16)} = 62.14$; $p < 0.01$; Fig 3E) after slices incubation with LAA (metabolic inhibitor of astrocytes) (BRIDGES et al. 1992, FLEMING et al. 2011) and the GUO neuroprotection was also abolished. Thus, our data demonstrate that the glia cells function are essential for the neuroprotective role of this purinergic nucleoside.

Involvement K and Ca channels in GUO pathway

The homeostasis and regulation of Ca^{2+} have been demonstrated fundamental in GUO effect (OLESKOVICZ et al. 2008, DOBRACHINSKI et al. 2017). Consequently, it was observed the inhibition of GUO effect on NMDA model ($F_{(3.16)} = 98.94$; $p < 0.01$; Fig 6A and 6B) through cyclopiazonic acid 30µM (CPA, SERCA inhibitor of the endoplasmic reticulum) and we believed that Ca^{2+} involvement is crucial to the effect of GUO. It is postulated that GUO action is linked to the large conductance of K^+ channels activated by Ca^{2+} (BK channels) or other K^+ channels (DAL-CIM et al. 2011, DAL-CIM et al. 2013).

In the same way, it was observed that the neuroprotective effect of GUO was abolished using a K^+ channel blocker (TEA, 25 mM) ($F_{(3.14)} = 93.14$; $P < 0.01$; Fig. 5B) and more specifically a Ca^{2+} sensitive channel blocker [Charybdotoxin 100nM (CHA)] ($F_{(3.16)} = 69.65$; $P < 0.01$; Fig. 5A). This result reveals that the GUO, in a direct or even an indirect way, could be interacting with these channels in order to establish an ionic balance and exert its neuroprotection.

However, when it was used L-type Ca^{2+} channel blocker [Nifedipine 20µM (NIFE)] ($F_{(3.16)} = 47.60$; $p < 0.01$; Fig. 5C), the effect of GUO was not observed. Based on this information, we evaluated the action of some Ca^{2+}-dependent channels and systems in order to assess if this effect was exclusively dependent on a specific channel or could be related to Ca^{2+} equilibrium. This information reinforces the effect of this purinergic nucleoside, which is closely related to Ca^{2+} metabolism and not directly to BK channels, as the literature reports on OGD model (DAL-CIM et al. 2013).

Importance of adenosinergic system on GUO effect

The neuroprotector effects of GUO was associated to modulation of adenosinergic system, more specifically A_1 receptor. However, GUO and purinergic nucleotides generally

have a very low affinity for binding to adenosine receptors, showing that their action is not linked directly to these receptors. This theory was confirmed by our experiences, demonstrating that GUO did not modify the inhibitory curve performed by 2-chloroadenosine 2 UI/mL (CADO, adenosine receptor agonist) (Fig. 6A) and the action of DPCPX 100nM (selective A_1 adenosine receptor antagonist) in basal synaptic transmission (Fig. 6). However, in presence of adenosine deaminase (ADA) the basal synaptic transmission increased but GUO inhibits this effect ($p < 0.01$; Fig. 7A and 7B). We can argue that this action of the compound may affect the release of adenosine into the extracellular environment, often from ATP or even directly interfere with ADA activity.

Involvement of Purinergic receptors (P1 type) on GUO neuromodulator effect

Evidence indicates the modulation of P2Y receptors as a possible target for GUO(BALLERINI et al. 2006). In the first moment, we confirm the potential neuroprotector effect of $P2Y_1$ receptor agonist 5µM (MRS 2365) against excitotoxicity caused by NMDA model ($F_{(1.16)} = 47.60$; $p < 0.01$; Fig. 8A), whereas the antagonist 10µM (MRS 2179) prevented this neuroprotection ($F_{(1.16)} = 47.60$; $p < 0.01$; Fig. 8B). This hypothesis was pharmacologically confirmed by blocking the effect of the P2Y1 agonist by antagonist ($F_{(1.16)} = 36.14$; $p < 0.01$; Fig. 8C).

Our data demonstrates that the neuroprotective effect of GUO was blocked by $P2Y_1$ receptor antagonist (MRS 2179) in this model ($F_{(3.18)} = 20.25$; $p < 0.01$; Fig. 9B). In adition, the agonist (MRS 2365) showed no additive effect when compared to GUO ($F_{(1.16)} = 25.41$; $p < 0.01$; Fig. 9A). Thus, the purinergic nucleoside could be acting through this receptor P2Y1.

Discussion

This study described for the first time that the GUO is able to avoid the impairment of synaptic basal transmission caused by glutamatergic hiperexcitability process in hippocampus slices. Moreover, this data also provide evidence that the modulation of purinergic receptors (P2), especially $P2Y_1$ receptor, are involved in these effects.

The prevention of glutamate-mediated toxicity was a central therapeutic target in numerous diseases; unfortunately, a long list of drugs with assorted mechanisms of action on glutamate receptors and transporters failed to show efficacy when assessed in clinical trials (LOANE et al. 2010, KABADI et al. 2014a). Several studies have indicated that the GUO plays an important role as an extracellular signaling molecule, activating molecular pathways that lead to neuroprotective effects with relevance for the development and operation of the SNC (BETTIO et al. 2016a, LANZNASTER et al. 2016a). Thus, the electrophysiology records show pronounced alterations in basal synaptic transmission after NMDA exposition, which effect was abolished by GUO.

Indeed, neuroprotective effects of purinergic nucleoside against models of neurotoxicity and neurodegenerative disease have been documented, likely due to the modulation of glutamatergic system (BETTIO et al. 2016a, GERBATIN et al. 2016, LANZNASTER et al. 2016a, DOBRACHINSKI et al. 2017). This hypothesis is associated to the maintenance of mitochondrial activity, redox status and regulation of astrocytic transporters activity and expression in glia cells (DAL-CIM et al. 2013, LANZNASTER et al. 2016b, DOBRACHINSKI et al. 2017). Our data confirm that the pharmacological mechanisms involved in GUO neuroprotection are regulated by astrocytes, since its metabolic inhibition avoided the effect of purinergic nucleoside. Nevertheless, under injury conditions, astrocytes are the main source of purines in the extracellular space and the release of these molecules is associated with the

physiologic or reparative role in the CNS (CICCARELLI et al. 1999, CICCARELLI et al. 2000, CICCARELLI et al. 2001).

The excessive glutamate release and NMDA receptor stimulation begin an excitotoxic cascade (ROSSI et al. 2000). Its involves an increase of intracellular Ca^{2+} levels and promotes the alteration of synaptic process, endoplasmatic reticulum stress, production of reactive oxygen species and mitochondria impairment (ROSSI et al. 2000, DANBOLT 2001, NICHOLLS 2008). Therefore, a putative dependence on Ca^{2+} homeostasis was evaluated in the neuroprotective effect of GUO. The incubation of hippocampal slices with a reversible inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) abolished the neuroprotective effect of GUO, showing a dependence on extracellular Ca^{2+} levels. This hypothesis was corroborated with the literature data while the Ca^{2+}-free medium inhibited the GUO effect in hippocampus slices submitted an OGD (OLESKOVICZ et al. 2008). Moreover, the maintenance of Ca^{2+} homeostasis was essential for the neuroprotection of GUO in a TBI model (DOBRACHINSKI et al. 2017).

Several studies report that role of GUO in neuroprotection involves a modulation of K^+ channels, more specifically BK channels, regulating the homeostasis and contributing to neuroprotection (BENFENATI et al. 2006, DAL-CIM et al. 2011, DAL-CIM et al. 2013). The evaluation of a putative K^+ channels involvement on GUO effect showed that TEA and charybdotoxin, respectively a non-selective K^+ channel blocker and a specific inhibitor of the big conductance Ca^{2+}-activated K^+ channel, prevented GUO-induced neuroprotection against hiperexcitability in hippocampal slices. However, in the presence of Nifedipine (L-type Ca^{2+} channel blocker) the GUO effect was also abolished. Thus, GUO may act via K^+ and Ca^{2+} channels activation and depend upon the extracellular Ca^{2+}. Nevertheless, Ca^{2+} entrance via voltage-gated Ca^{2+} channels, or mobilization of intracellular Ca^{2+} stores, may modulates calcium-dependent K^+ and Ca^{2+} channels, favoring GUO-induced neuroprotection (AKITA et

al. 2000). Otherwise, the possibility of GUO act as regulator of these channels through the modulation of the adenosinergic system cannot be ruled out (AKITA et al. 2000, DAL-CIM et al. 2013).

The modulation of adenosinergic system has emerged as a therapeutic approach for the treatment of various neurological conditions and evidences have been associated this system to the target of GUO-induced neuroprotection (DAL-CIM et al. 2013, KOVACS et al. 2015, BETTIO et al. 2016a). However, GUO itself is not considered an effective ligand for P1 and P2 receptors (RATHBONE et al. 1991, CICCARELLI et al. 2000). This fact corroborates with our results, once the inhibition curve of CADO and the effect of DPCPX on basal synaptic transmission was not modified by GUO. Thus, the purinergic nucleoside does not appear to directly interfere with the binding of adenosinergic modulators. On the other hand, it does not exclude that the GUO could act through this mechanism.

The literature data demonstrated that GUO leads to an accumulation of extracellular adenosine, which is associated to GUO-induced neuroprotection on seizures models (BOISON 2005, JACKSON et al. 2013, KOVACS et al. 2015). Our results corroborate with this hypothesis because in presence of ADA the basal synaptic transmission increase while the presence of GUO inhibit this effect. This observation can be associated to an increase of adenosine extracellular levels through the release of neuron/glial cells and the interference in ADA activity caused by GUO. Interestingly, this results indicate that the modulation of adenosinergic system performed by GUO could be achieved through indirect regulation of these receptors.

In fact, the possible increase of adenosine levels caused by GUO in pathological conditions can be correlated to an increase of ATP/ADP release by glial cells (JACKSON et al. 2013, KOVACS et al. 2015). In this way, the specific modulation of P2 receptors (P2X and P2Y) by GUO cannot be excluded (BALLERINI et al. 2006). Besides, the literature data

demonstrated that the ATP and GUO upregulated the $P2Y_1$ receptor mRNA expression in astrocytes (BALLERINI et al. 2006).

Stimulation of specific astrocyte P2 receptors by extracellularly ATP/ADP represents an important component of the physiological and pathological glial responses, including neuromodulation, neurotransmission and neurorepair (JAMES et al. 2002, THAUERER et al. 2012). In the first moment, we reinforced the potential neuroprotective effect of $P2Y_1$ receptor, once the agonist of this receptor block the NMDA-induced excitotoxicity. The specific effect of this receptor modulation was confirmed by the antagonism of $P2Y_1$. This reinforce the importance of P2 receptors on the neuroprotector effects. Additionally, the neuroprotector effect of GUO in NMDA-induced excitotoxicity was avoided by the $P2Y_1$ antagonist while in the presence of $P2Y_1$ agonist, the purinergic nucleoside did not show additive effects.

Recent data have provided the existence of an association between A_1 and $P2Y_1$, which have been suggested to be involved in the modulation of brain damage and contribute, alone or in combination, to neurodegenerative/regenerative processes (NEARY et al. 2003, FRANKE et al. 2006, TONAZZINI et al. 2007). Ischemia, to which hippocampus is particularly vulnerable, produces a marked increase in glutamate within the brain extracellular space, thereby triggering excitotoxic injuries (ERECINSKA et al. 1990, ROSSI et al. 2000, DANBOLT 2001, NICHOLLS 2008). The presence of glutamate in pathological conditions and its release in neurons and in astrocytes are modulated through A_1 (MASINO et al. 2002) and $P2Y_1$ (RODRIGUES et al. 2005, JOURDAIN et al. 2007).

Considering the new information available and the A1R and P2Y1R involvement in glutamatergic modulation (MENDOZA-FERNANDEZ et al. 2000, MASINO et al. 2002, RODRIGUES et al. 2005), we can speculate that there is an A1R–P2Y1R cross-talk in rat hippocampal glutamatergic synapses and surroundings glia, where these receptors are co-localized (TONAZZINI et al. 2007). This might be one of the mechanisms for the adenine

nucleotide-mediated inhibition of glutamatergic neurotransmitter release. A cross-talk / heteromerization of A_1–$P2Y_1$ could exert a fine-tuning modulation of glutamatergic neurotransmission, providing a switch mechanism by which low and high concentrations of adenosine or GUO could regulate glutamate system.

In summary, the GUO avoided a NMDA-induced excitotoxicity in hippocampus slices through the modulation of $P2Y_1$ receptors. In this sense, this study demonstrates that this effect depends of astrocytic activity, extracellular Ca^{2+} levels and adenosine levels to develop its mechanism of neuroprotection.

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Author Disclosure Statement

The authors declare no conflict of interest and no competing financial interests exist.

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Figure Legends

Fig. 1 Impact of GUO on synaptic transmission in Schaffer collaterals of mouse hippocampal slices. The representative of electrophysiology records on Schaffer collaterals in hippocampus slices (A) and the GUO tested in four different concentrations (3 µM, 30 µM, 100 µM and 300 µM), was devoid of effects on both basal synaptic transmission (B). The data are mean ± SD of n = 4. *P < 0.05 compared to control using a Newman–Keuls post hoc test.

Fig. 2 Effect of guanosine on NMDA-induced excitotoxicity. Bars and representatives indicate the protocol used. Figures (A and B) indicate the representative protocol, while (C and D) are calculated as means. Data were expressed as mean ± SD of n = 5. and analyzed by one-way, followed by Newman-Keuls where appropriate. Differences were considered significant when $p \leq 0.05$. in compare to control group.

Fig. 3 Astrocytes participation on effect of guanosine on NMDA-induced excitotoxicity. (A) indicates 2h of TFA incubation; (B) indicates 4h of TFA incubation; (C) GUO positive control; (D) indicates 2h of LAA incubation and (E) indicates 4h of LAA incubation. Data were expressed as mean ± SD n = 5 and analyzed by one-way, followed by Newman-Keuls where appropriate. Differences were considered significant when $p \leq 0.05$. in compare to control group.

Fig. 4 Involvement of calcium on effect of guanosine on NMDA-induced excitotoxicity. Picture (A) indicates a representative data of CPA presence; (B) indicates CPA interference on GUO effect NMDA-induced excitotoxicity. The data are mean ± SEM. n = 5 $*P < 0.05$ compared to control using a Newman–Keuls post hoc test.

Fig. 5 Involvement of Ca^{2+} and K^{+} channels of guanosine effect on NMDA-induced excitotoxicity. (A) Influence of Charybdotoxin; (B) TEA and (C) Nifedipine of GUO effect NMDA-induced excitotoxicity. The data are mean ± SEM. n = 5 *P < 0.05 compared to control using a Newman–Keuls post hoc test.

Fig. 6 Evaluation of adenosinergic system of guanosine effect on basal synaptic transmission. Picture (A) demonstates influence of GUO on chloradenosine inhibition curve; (B) influence

of GUO in DPCPX effect in synaptic basal transmission; (C) Representative graphic influence of GUO in DPCPX effect on synaptic basal transmission. The data are mean ± SEM of n = 5-6. *P < 0.05 compared to control using a Newman–Keuls post hoc test.

Fig. 7 Evaluation of adenosinergic system of guanosine effect on basal synaptic transmission. Picture (A) representative influence of GUO on ADA effect on synaptic basal transmission and (B) influence of GUO of ADA on synaptic basal transmission. The data are mean ± SEM of n = 5-6. *P < 0.05 compared to control using a Newman–Keuls post hoc test.

Fig. 8 Involvement of P2Y1 receptor on NMDA-induced excitotoxicity. (A) Effect of MRS; (B) MRS on NMDA-induced excitotoxicity and (C) MRS pharmacological block of MRS on NMDA-induced excitotoxicity. The data are mean ± SEM. *P < 0.05 compared to control using a Newman–Keuls post hoc test.

Fig. 9 Involvement of P2Y1 receptor of GUO effect on NMDA-induced excitotoxicity.
(A) Effect of MRS; (B) MRS on NMDA-induced excitotoxicity. The data are mean ± SEM. *P < 0.05 compared to control using a Newman–Keuls post hoc test.

Figures

Figure 1.

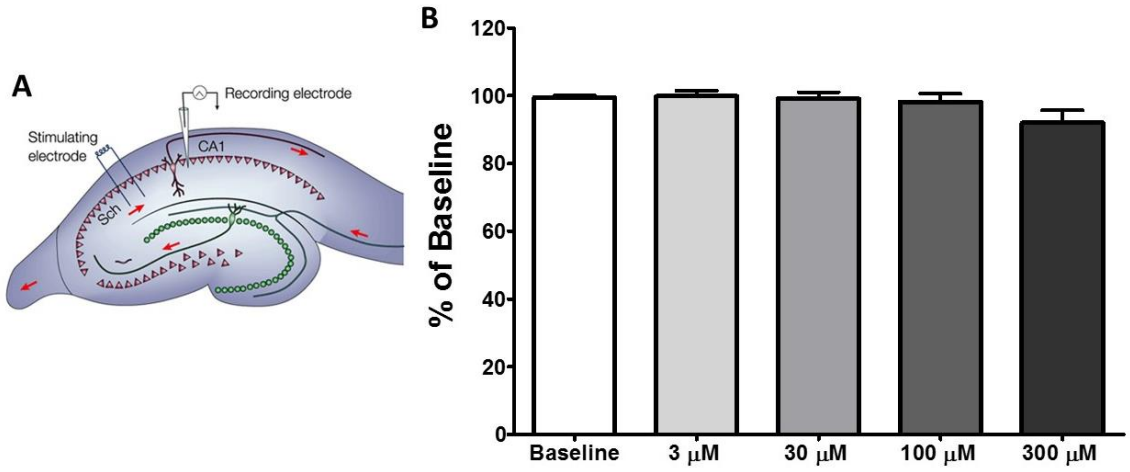


Figure 2.

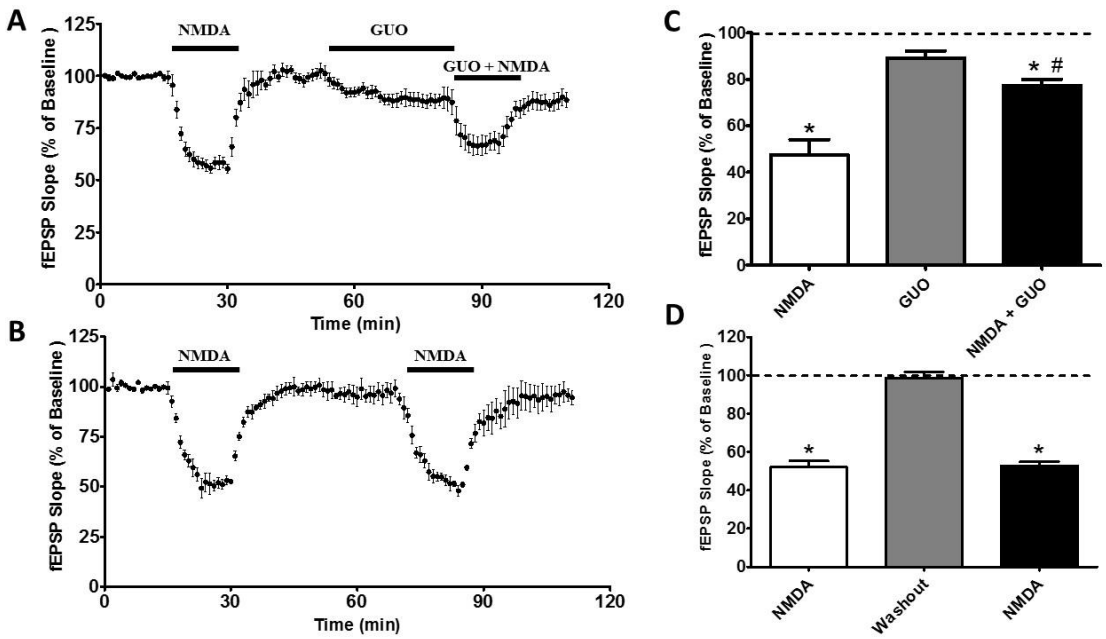


Figure 3.

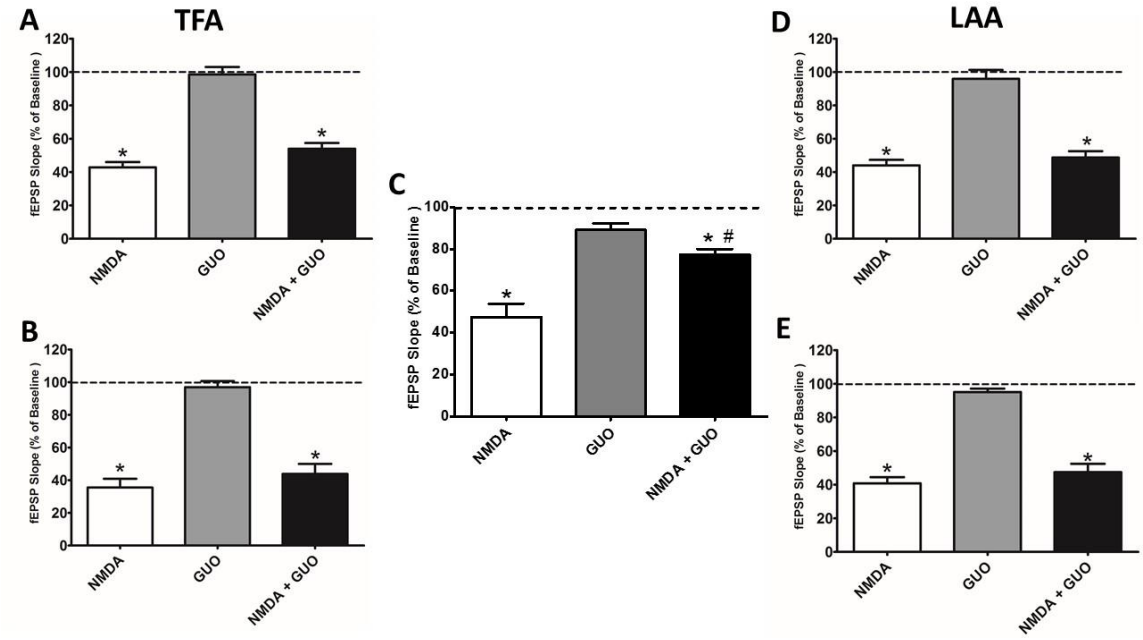
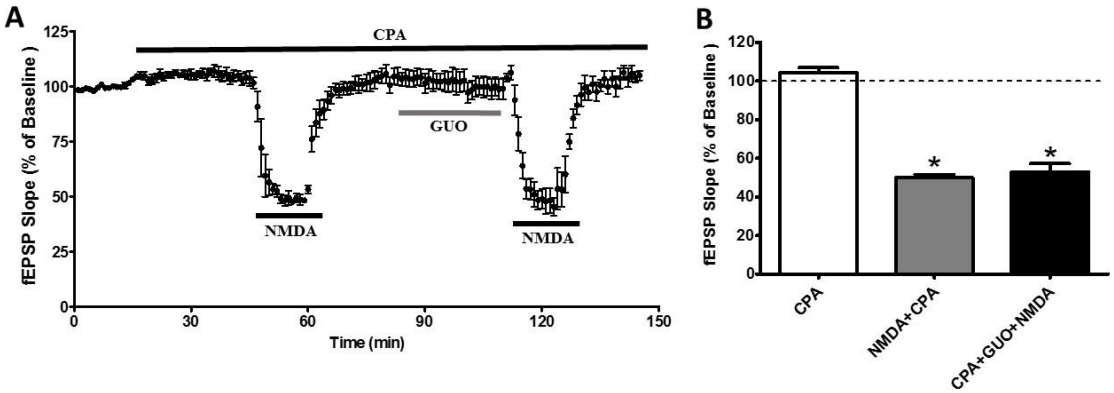


Figure 4.



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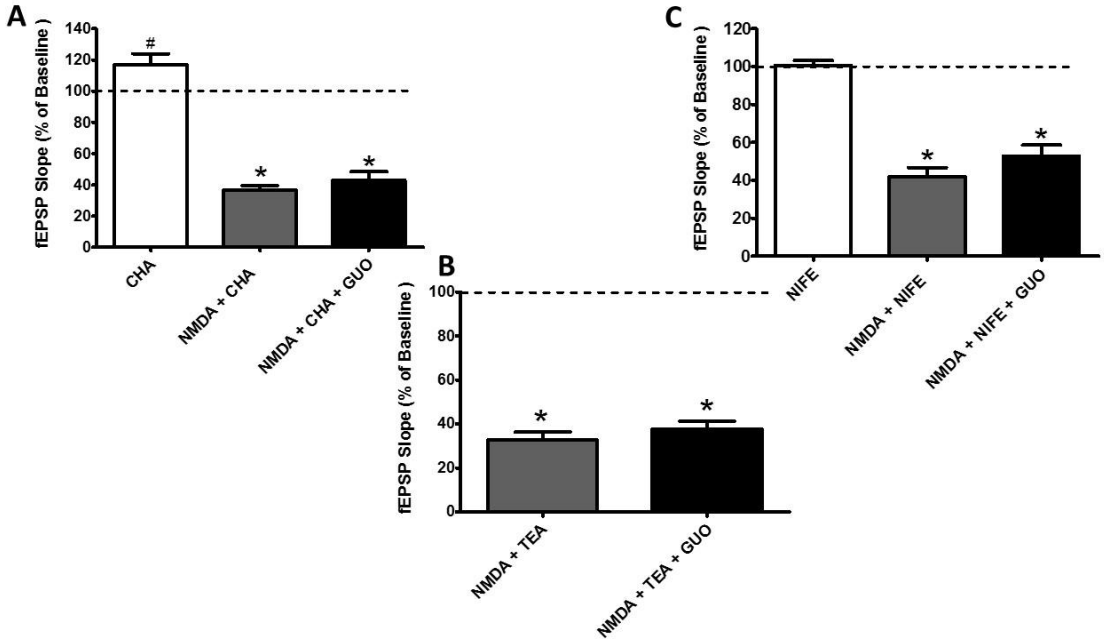

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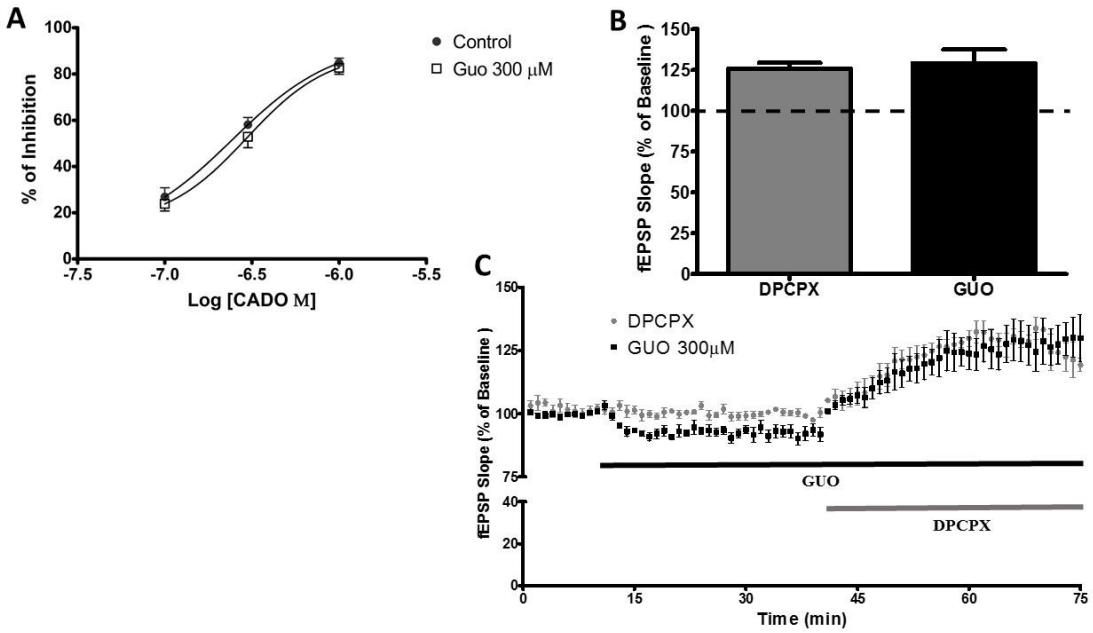

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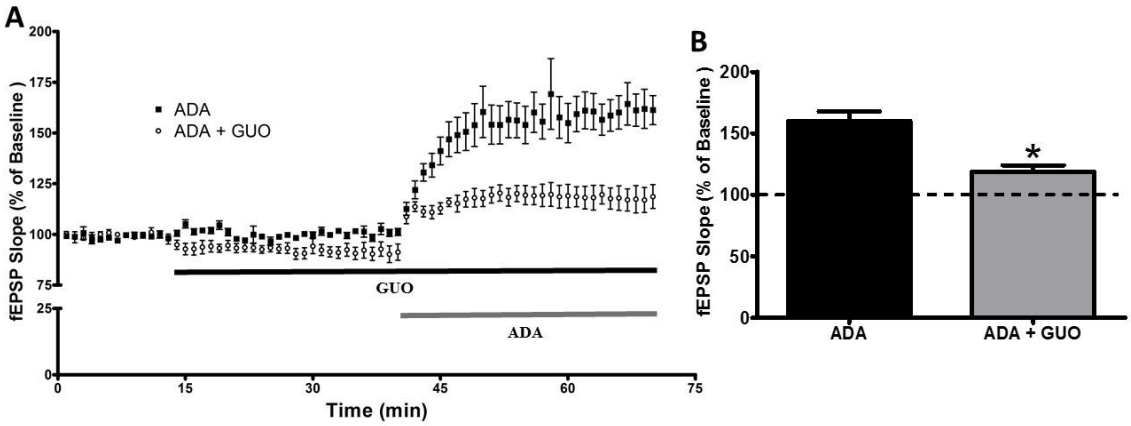


Figure 8.

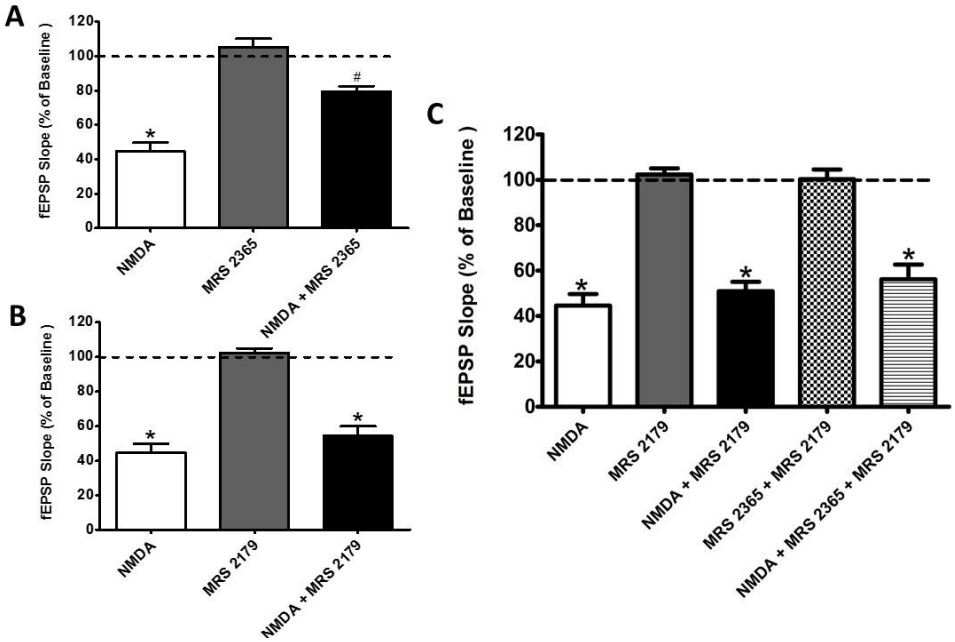
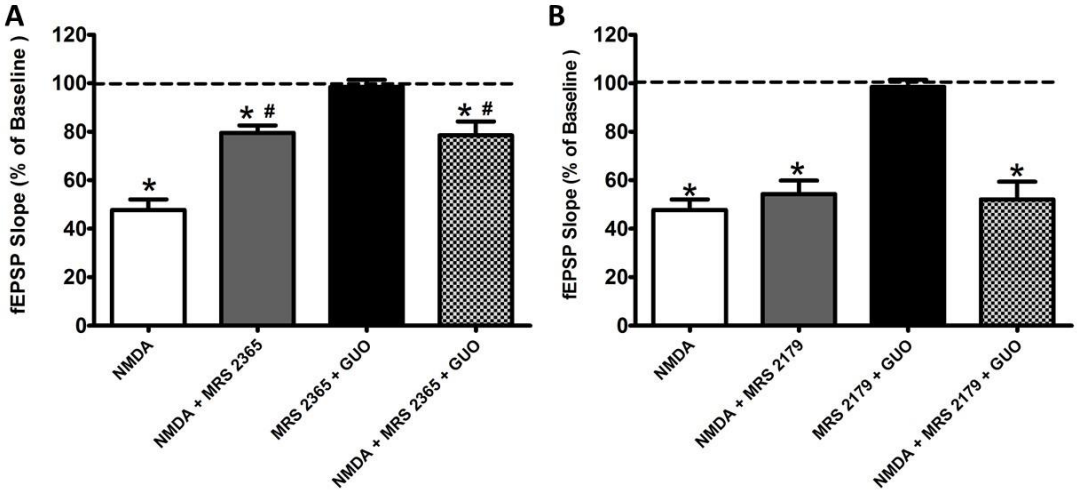


Figure 9.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NMDA
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MRS 2365 + GUO
NMDA + MRS 2365 + GUO
B
fEPSP Slope (% of Baseline)
120
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NMDA + MRS 2179 + GUO

4. DISCUSSÃO

Durante a última década houve um avanço significativo na compreensão da complexidade fisiopatológica que ocorre após o TCE. É estabelecido que o insulto mecânico primário inicia uma cascata de lesões secundárias que contribuem para os déficits celulares e comportamentais (MAAS et al. 2008, WALKER et al. 2013). Em virtude destes aspectos, esta tese investigou uma molécula capaz de modular diferentes alvos de neuroproteção. Os estudos foram direcionados para avaliar os efeitos da GUO contra os danos causados pelo TCE bem como os diferentes alvos moleculares que possam estar envolvidos.

Primeiramente, os experimentos do **artigo 1** foram realizados com o objetivo de investigar o efeito neuroprotetor da GUO contra os danos agudos presentes na cascata secundária após o TCE, traçando sua ação farmacológica inicial. Em seguida, foi avaliado no **manuscrito 1** o efeito crônico da GUO nas alterações causadas pelo TCE a longo prazo. Além dos aspectos clínicos considerados no **manuscrito 1**, ferramentas farmacológicas que destacam o sistema adenosinérgico foram usadas com o objetivo de esclarecer os possíveis mecanismos de ação da GUO. Apesar do empenho para desvendar os mistérios farmacológicos desse "neuromodulador órfão", ainda restam dúvidas sobre seu mecanismo de ação. Com isso, os experimentos do **manuscrito 2**, foram realizados em um modelo experimental excitotóxico *in vitro* para esclarecer e compreender as possíveis rotas de ação da GUO nos processos de neuroproteção.

No **artigo 1**, investigou-se o efeito neuroprotetor da GUO frente aos danos secundários agudos observados após o traumatismo crânio encefálico. Entre as principais alterações observadas estão o desequilíbrio na homeostase do Ca^{2+} e do sistema glutamatérgico e as alterações mitocondriais. Esta organela desempenha uma função vital na bioenergética celular, sendo sensível a alterações do estado fisiológico celular e possui um papel crítico na lesão secundária pós-trauma (SULLIVAN et al. 2005, SINGH et al. 2006). Diversas perturbações na atividade da mitocôndria puderam ser observadas 3h após o TCE. Dentre estes parâmetros, destacamos a diminuição no potencial de membrana mitocondrial ($\Delta\Psi m$), aumento de EROs, inibição de enzimas relacionadas ao sistema redox e de enzimas relacionadas a funcionalidade mitocondrial. A administração da GUO 15 min após o trauma bloqueou estas alterações, principalmente no hipocampo.

Como descrevemos anteriormente, dentre as principais causas agudas da cascata secundária após o trauma está o dano mitocondrial, intimamente correlacionado a excitotoxicidade glutamatérgica, visto que níveis elevados de glutamato na fenda sináptica

resultam na ativação excessiva do receptor NMDA (ZIPFEL et al. 2000, ARUNDINE et al. 2003). Consequentemente, a exposição mitocondrial a exacerbados níveis de glutamato rapidamente despolarizam a organela, aumentando a produção de EROs e afetando o equilíbrio redox (WHITE et al. 1996, SINGH et al. 2006). Essa disfunção depende principalmente do sequestro de Ca^{+2} pelas mitocôndrias, (WHITE et al. 1996, OPII et al. 2007, PANDYA et al. 2013) podendo conduzir a danos na sua integridade bioenergética, abertura do poro de transição de permeabilidade mitocondrial, liberação de citocromo C e morte neuronal (SULLIVAN et al. 2005, VOTYAKOVA et al. 2005).

Neste ponto, está claro que o equilíbrio do Ca^{+2} extra e intracelular é fundamental para a manutenção celular (LIFSHITZ et al. 2004). Essa alteração foi totalmente bloqueada no hipocampo e parcialmente no córtex dos animais tratados com GUO. Este dado torna-se um ponto de regulação interessante, pois a homeostase do Ca^{2+} representa um dos principais mecanismos do TCE, conectando a excitotoxicidade glutamatérgica e a disfunção mitocondrial (LIFSHITZ et al. 2004, SULLIVAN et al. 2005, OPII et al. 2007).

Consequentemente, a avaliação do sistema glutamatérgico se torna essencial a fim de avaliar o efeito neuroprotetor do nucleosídeo purinérgico. Nossas evidências deixam claro a excitotoxicidade glutamatérgica pós-traumática, baseada na diminuição da captação de glutamato e expressão dos transportadores glutamatérgicos presentes nos astrócitos. Corroborando com a literatura, a administração da GUO conseguiu evitar totalmente essas alterações no hipocampo e parcialmente no córtex dos animais que sofreram TCE. Relatos da literatura corroboram com estes resultados, demonstrando que a ação moduladora do nucleosídeo purinérgico está relacionado a regulação glutamatérgica dependente da atividade dos transportadores astrocitários (TROTTI et al. 1998, DAL-CIM et al. 2016). Estes por sua vez são modulados pelo estado redox da célula, assim uma produção aumentada de EROs pode resultar na sua atividade reversa (TROTTI et al. 1998) e contribuir para o aumento de glutamato na fenda sináptica.

A partir destes primeiros achados conclui-se que a administração da GUO teve sucesso contra os danos agudos observados após o trauma, através da regulação tanto da atividade mitocondrial quanto do sistema glutamatérgico, tendo como ligação direta entre estes dois efeitos a manutenção da homeostase do cálcio. Entretanto, os alvos moleculares e vias de sinalização recrutadas pela GUO para modular seus efeitos permanecem sob avaliação.

A “distância” entre a investigação experimental e a prática clínica poderia ser potencialmente reduzida, dando maior ênfase para a avaliação sistemática dos déficits comportamentais nos ensaios pré-clínicos. Um dos objetivos importantes da investigação

laboratorial no modelo de TCE, seria conectar os mecanismos celulares que levam à disfunção e morte celular pós-traumática com as deficiências comportamentais observadas. O uso de ferramentas moleculares e parâmetros comportamentais em conjunto com avaliações histológicas é determinante para a obtenção de resultados mais concisos. Consequentemente, no **manuscrito 1** avaliou-se o efeito da administração crônica da GUO por 21 dias contra os danos causados pelo traumatismo crânio encefálico a longo prazo. Cabe salientar, que o sistema purinérgico tem sido apontado como alvo para os efeitos neuroprotetores deste nucleosídeo purinérgico. Desta maneira, foram utilizadas ferramentas farmacológicas para analisar uma possível relação entre os receptores adenosinérgicos e os efeitos neuroprotetores da GUO.

Os danos agudos demonstrados no **artigo 1** estão intimamente relacionados a alterações consideradas a médio e longo prazo, até mesmo ao aparecimento ou facilitação de doenças degenerativas. Deste modo, no **manuscrito 1** tornou-se vital a avaliação destes parâmetros em relação ao tratamento com GUO. Evidências mostram que os roedores expostos ao TCE apresentam um aumento do comportamento do tipo ansioso bem como depressivo, sendo associados a disfunção cognitiva à curto e longo prazo (JONES et al. 2008, SHULTZ et al. 2012, WALKER et al. 2013). Associa-se isto a alterações no hipocampo após lesão traumática, estabelecendo a disfunção na cognição, incluindo diferentes tipos de estímulos de consolidação do aprendizado (IZQUIERDO et al. 2006, WALKER et al. 2013). Em particular, a integridade das sub-regiões CA1-CA3 e *girus dentatus* é essencial para o desempenho nas tarefas de plasticidade (IZQUIERDO et al. 2006, WALKER et al. 2013, SAJJA et al. 2016).

As consenquências das cascatas inicias após o trauma acabam contribuindo para a diminuição na expressão de proteínas essenciais em processos de plasticidade (BDNF e CREB). Características que estão intimamente ligadas a morte de células neuronais e processos de astrogliose, os quais são capazes de produzir mediadores inflamatórios e citotóxicos que contribuem para o recrutamento e ativação de células da micróglia (Smith et al., 1997, Loane e Kumar, 2016). Este movimento contribui massivamente para excitotoxicidade neuronal, o que influencia ainda mais no processo de neurodegeneração (WERNER et al. 2007, MAAS et al. 2008, KABADI et al. 2014a).

A limitação tanto de eventos agudos (**artigo 1**) quanto crônicos (**manuscrito 1**), torna-se uma ferramenta essencial neste tipo de multipatologia. Neste caso em relação a alterações de médio prazo, a GUO demonstrou um efeito neuroprotetor contra distúrbios comportamentais, moleculares e morfológicos após o TCE. Diversos estudos propõem um papel central para a GUO em diversas condições neurológicas, através da modulação do sistema adenosinérgico e consequentemente atuando como uma molécula neuroprotetora (SCHMIDT et al. 2007,

BETTIO et al. 2016a, LANZNASTER et al. 2016a). Esta hipótese foi confirmada no **manuscrito 1**, visto que a administração de 8-ciclopentil-1,3-dipropilxantina (DPCPX, antagonista seletivo do receptor de adenosina A_1) bloqueou a ação farmacológica da GUO. Acredita-se que a GUO exerceu sua neuroproteção através da modulação da atividade do receptor A1, promovendo a restauração do gradiente iônico e a regulação da transmissão glutamatérgica (BOISON et al. 2010, DAL-CIM et al. 2011, DAL-CIM et al. 2013). Entretanto, não podemos descartar outros alvos moleculares que podem estar intimamente correlacionados com estes efeitos. Desta maneira, a modulação de canais BK (DAL-CIM et al. 2013), a interação oligomérica entre A_1 e A_{2A} (DAL-CIM et al. 2013) e até mesmo uma regulação de outros receptores do tipo P2 não podem ser descartadas (BALLERINI et al. 2006).

Após as evidências encontradas nos dois trabalhos anteriores, algumas dúvidas em relação ao mecanismo farmacológico da GUO ainda permanecem. Neste sentido foram realizados no **manuscrito 2** registros eletrofisiológicos em fatias de hipocampo submetidas a um modelo de excitotoxicidade (NMDA). Evidências apontam para a importância do papel das purinas durante eventos excitotóxicos, uma vez que os níveis de nucleotídeos e nucleosídeos de guanina aumentam durante esse tipo de distúrbio, liberados principalmente pelas células da glia (CICCARELLI et al. 1999, THAUERER et al. 2012, JACKSON et al. 2013, JACKSON et al. 2014). Desta forma confirmou-se o efeito neuroprotetor clássico da GUO, que mostrou ser dependente da atividade astrocitária. Dados da literatura ratificam esta informação, visto que a inibição da função astrocitária em relação ao equilíbrio do sistema glutamatérgico é essencial pra ação da GUO (DAL-CIM et al. 2016, DOBRACHINSKI et al. 2017).

Processos de excitotoxicidade são bem caracterizados em diversas patologias. Nestes casos, a ativação anormal dos receptores NMDA altamente permeáveis ao cálcio resultam em sobrecarga de intracelular deste íon, induzindo cascatas neurotóxicas (WALKER et al. 2013). A regulação destas modificações tornam-se interessantes, afim de evitar processos excitotóxicos. Postula-se que a ação da GUO é vinculada dependentemente da grande condutância de canais de K^+ ativados por Ca^{2+} (canais BK) (DAL-CIM et al. 2012, DAL-CIM et al. 2013). Neste caso, o aumento da captação de glutamato induzido pela GUO depende da ativação dos canais BK, o que resulta numa diminuição dos níveis de Ca^{2+} intracelular evitando assim a perda do potencial de membrana (DAL-CIM et al. 2013). Este fato torna-se um evento importante para a manutenção da atividade dos transportadores glutamatérgicos (DAL-CIM et al. 2013, DAL-CIM et al. 2016, DOBRACHINSKI et al. 2017). Observou-se que os efeitos da GUO são dependentes da homeostase do Ca^{2+} pois a administração do ácido ciclopiazonico (CPA, inibidor da SERCA do reticulo endoplasmático) bloqueou seu efeito.

Corroborando com estes fatos, pode-se obervar que a presença de um bloqueador dos canais de K+ [Trimetilamonio (TEA)] e mais especificamente um bloqueador dos canais de K^+ sensível a Ca^{2+} [Charybdotoxina (CHA)], bloquearam o efeito da GUO. Este resultado está de acordo com os dados demonstrados na literatura em modelos de OGD (DAL-CIM et al. 2013), caracterizando o envolvimento direto ou indireto destes canais afim de estabelecer um equilibrio iônico e seu mecanismo neuroprotetor. Entretanto, na presença de um bloqueador de canais de Ca^{2+} do tipo-L [Nifedipina (NIFE)], o efeito da GUO não foi observado. Estas informações reforçam que o efeito deste nucleosideo purinérgico está intimamente relacionado ao metabolismo do Ca^{2+} e não diretamente aos canais BK, como afirma a literatura (DAL-CIM et al. 2013). Pois quando bloqueados, os canais BK, os canais de Ca^{2+} do tipo-L e a bomba de Ca^{2+} ATP-ase do retículo endoplasmático, observa-se uma inibição do efeito neuroprotetor da GUO em modelo de excitotoxicidade causado por NMDA.

Outra hipótese está relacionada a modulação de receptores de adenosina (A_1 e A_{2A}) (THAUERER et al. 2012, DAL-CIM et al. 2013). No **manuscrito 1,** destacam-se fortes indícios de que os receptores adenosinérgicos, mais especificicamente A_1, participem de alguma maneira da neuroptoteção causada pela GUO. No entanto, GUO e nucleotídeos purinérgicos em geral apresentam uma afinidade muito baixa de ligação a receptores de adenosina (RATHBONE et al. 1991, MULLER et al. 1993, CICCARELLI et al. 2000), mostrando que sua ação não está provavelmente vinculada a ligação direta com estes receptores. Teoria esta confirmada, pois observou-se que a GUO não deslocou a curva inibitória da transmissão sináptica basal causada pela 2-cloroadenosina, nem a ação inibitória do DPCPX sobre o receptor A_1. Contudo, na presença de adenosina deaminase (ADA), a GUO mostrou influência sobre o aumento da transmissão sináptica basal causada pela ADA. Evidências sugerem que a GUO pode contribuir para a sinalização celular, através de um mecanismo indireto envolvendo o sistema adenosinérgico (JACKSON et al. 2013, JACKSON et al. 2014), conduzindo ao aumento dos níveis de adenosina extracelular (JACKSON et al. 2013, BETTIO et al. 2016b). Isso leva-nos a acreditar que a GUO pode afetar a liberação de adenosina para o meio extracelular muitas vezes proveniente do ATP, ou mesmo interferir diretamente na atividade da ADA.

O possível aumento dos níveis de adenosina causados pela GUO em condições patológicas pode ser correlacionado com um aumento da liberação de ATP / ADP para as células da glia (JACKSON et al. 2013, JACKSON et al. 2014, KOVACS et al. 2015). Desta forma, a modulação específica dos receptores P2 (P2X e P2Y) pela GUO não pode ser excluída (BALLERINI et al. 2006). A estimulação de receptores P2 localizados nos astrócitos pelo

aumento extracelular de ATP/ADP representa um componente importante das respostas fisiológicas e patológicas da glia, incluindo neuromodulação, neurotransmissão e neuroreparação (JAMES et al. 2002, THAUERER et al. 2012). Após estas evidências, a relação de GUO com outros receptores purinérgicos (P2) não pode ser excluída, assim estudos específicos para este sistema aparecem como possíveis alvos da ação da GUO.

Outra hipótese que torna-se interessante é a interação/modulação da GUO com receptor purinérgico $P2Y_1$ ($P2Y_1R$). Este receptor tem demonstrado uma importante função nos processos de plasticidade e excitotoxicidade (RODRIGUES et al. 2015, PEDATA et al. 2016). A modulação destes subtipos de receptores conduz a um aumento da ativação das ondas de Ca^{2+} intracelulares, por meio de sinalização via PI3K/Akt, MAPK e consequentemente a fosforilação da ERK1/2, contribuindo para o papel neuroprotetor em modelo isquêmica em astrócitos (IWABUCHI et al. 2009). Além disso, os $P2Y_1$ localizados nas sinapses centrais tem ação associada ao controle da liberação de glutamato (MENDOZA-FERNANDEZ et al. 2000, RODRIGUES et al. 2005), na atividade de receptores NMDA (LUTHARDT et al. 2003) e controle da condutância de canais de Ca^{2+} e K^+ (GEREVICH et al. 2004, COPPI et al. 2012). Informações que vão de encontro com os mecanismos moleculares até então associados ao efeito da GUO em modelos de DGO (MOLZ et al. 2011, DAL-CIM et al. 2013). Isso confirmou-se, onde agonista do receptor $P2Y_1$ (MRS 2365) causou um efeito neuroprotetor contra a excitotoxicidade causada pelo modelo de NMDA. Essa hipótese foi farmacologicamente ratificada com o bloqueio do efeito do agonista $P2Y_1$ pelo seu antagonista (MRS 2179) em nosso modelo.

Confrontando estas informações obtidas pelos experimentos e com os dados da literatura, observou-se que o efeito neuroprotetor da GUO em modelo de excitabilidade foi bloqueado pela ação do antagonista do receptor $P2Y_1$ (MRS 2365). Contudo, o agonista não apresentou efeito aditivo junto com a GUO. Isso caracteriza a possibilidade de que o nucleosídeo purinérgico possa estar exercendo sua atividade neuroprotetora através deste receptor.

É importante ressaltar com base em nossos resultados e na literatura, que a GUO pode modular o sistema adenosinérgico através do aumento nos níveis de adenosina e a atividade de receptores P2Y1. Além disso, não podemos descartar a regulação do funcionamento do receptor A1 induzida pela ativação do receptor P2Y1 ou uma interação oligomérica entre estes dois receptores (TONAZZINI et al. 2007, TONAZZINI et al. 2008, DELEKATE et al. 2014).

Considerando os aspectos discutidos, que constituem o **artigo 1 e os manuscritos 1 e 2,** este estudo consolidou em grande parte a ação da GUO contra os danos causados pelo TCE e auxiliou na compreensão dos seus mecanismos de ação.

5. CONCLUSÃO

A partir dos resultados obtidos nesta tese, podemos concluir que a GUO:

- Apresentou ação protetora interelacionando aspectos iniciais da cascata secundária após o TCE.
- Reverteu os danos crônicos causados pelo TCE, sendo este efeito bloqueado pela administração de DPCPX, indicando uma participação do sistema adenosinérgico (mais especificamente o receptor A_1) em seu mecanismo;
- Reforçou aspectos da sinalização adenosinérgica, demonstrando que seu efeito pode estar vinculado ao aumento de adenosina extracelular e que não está ligado a atividade de um único canal iônico.
- Estabeleceu uma nova hipótese de modulação dos receptores do tipo P2 ($P2Y_1$), através de sua ação neuroprotetora em um modelo de hiperexcitabilidade glutamatérgica.

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ANEXO A – Carta de Aprovação da Comissão de Ética no Uso de Animais-UFSM:

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Possiveis efeitos da administração de guanosina em ratos com traumatismo cranioencefálico sobre a suscetibilidade a crises epilépticas."

Número do Parecer: 153/2014

Pesquisador Responsável: Prof. Dr. Felix Alexandre Antunes Soares

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

*** Foram Aprovados 140 animais (Rato heterogênico).**

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DE APROVAÇÃO: 25/03/2015.

Santa Maria, 25 de março de 2015.

Vania Lucia Loro

Prof.ª Dr.ª Vania Lucia Loro
Coordenadora da Comissão de Ética no Uso de Animais- UFSM

Comissão de Ética no Uso de Animais - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362