Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

EXCITOTOXICIDADE GLUTAMATÉRGICA EM MODELOS EXPERIMENTAIS DE DOENÇAS CEREBRAIS: EFEITOS NEUROPROTETORES DAS PURINAS DERIVADAS DA GUANINA E INOSINA

TESE DE DOUTORADO

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TESE DE DOUTORADO

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(Orientador)

Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas: Bioquímica, como requisito parcial para a obtenção do título de Doutor em Ciências Biológicas - Bioquímica

Porto Alegre, Dezembro de 2010

"A outorga da liberdade é a outorga do amor,

e só o amor pode salvar o mundo"

A. S. Neill

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APRESENTAÇÃO

Os resultados desta tese de doutorado estão apresentados sob a forma de artigos científicos. As seções Materiais e Métodos, Resultados, Discussão e Referências bibliográficas encontram-se nos próprios artigos.

Os itens Introdução, Discussão e Conclusões encontrados nesta tese apresentam interpretações e comentários gerais sobre todos os artigos científicos contidos neste trabalho. As Referências Bibliográficas referem-se somente às citações que aparecem nos itens Introdução e Discussão desta tese.

As informações técnicas mais precisas sobre cada metodologia utilizada poderão ser encontradas nos artigos científicos correspondentes.

Gostaríamos de ressaltar que o Sexto Capítulo foi incluído nesta tese com o principal objetivo de discutir os resultados obtidos até o momento relacionados à identificação do possível sítio de união da guanosina à membrana plasmática cerebral de ratos.

SUMÁRIO

Parte I

RESUMO

A hiper estimulação do sistema glutamatérgico (excitotoxicidade), é um evento tóxico para o sistema nervoso central (SNC) e está envolvida em diversas doenças cerebrais agudas e crônicas. O glutamato, principal neurotransmissor excitatório do SNC, exerce suas funções através da ativação de seus receptores. A modulação da neurotransmissão glutamatérgica envolve a retirada do glutamato da fenda sináptica por transportadores específicos. Diversos estudos têm demonstrado que as purinas derivadas da guanina e a inosina apresentam efeitos neuroprotetores em modelos experimentais de doenças cerebrais relacionadas com a excitotoxicidade. Nesta tese, nós demonstramos que a captação de glutamato em fatias de várias estruturas cerebrais é diferente em ratas que apresentam comportamento tipo depressivo quando comparadas com ratas normais. Ou seja, nas ratas com comportamento tipo-depressivo houve um aumento inicial da captação de glutamato hipocampal e uma diminuição gradual após as 24 h que persiste até 21 dias. Nas ratas normais não houve alterações na captação de glutamato. O hipocampo foi a estrutura cerebral mais sensível ao comportamento de depressão. Alèm disso, evidenciamos o potencial neuroprotetor do GMP, da guanosina e da inosina em diferentes modelos experimentais de doenças cerebrais. A administração sistêmica de GMP induziu um comportamento ansiolítico em protocolos clássicos para avaliar drogas com potencial ansiolítico/ansiogênico em ratos. A administração oral crônica de GMP em camundongos aumentou a resistência do córtex cerebral a um insulto isquêmico *ex vivo* por privação de oxigênio e glicose. Esse efeito neuroprotetor foi correlacionado com modulações do sistema glutamatérgico. Ou seja, a administração de GMP foi capaz de diminuir o imunoconteúdo de algumas subunidades de receptores e de alguns subtipos de transportadores glutamatérgicos. Isto potencialmente pode estar relacionado ao efeito neuroprotetor ocasionado pela diminuição da sinalização e da fonte de glutamato, respectivamente, durante o insulo isquêmico. Além disso, verificamos que a administração intracerebroventricular de inosina exerceu efeito anticonvulsivante em camundongos frente a convulsões induzidas por ácido quinolínico, um potente agonista glutamatérgico. Este efeito foi independente dos receptores benzodiazepínicos. Avaliamos também o efeito do tratamento oral crônico com guanosina frente ao prejuízo cognitivo e dano hipocampal em ratos submetidos à hipoperfusão cerebral crônica. O tratamento com guanosina não foi capaz de prevenir o prejuízo cognitivo, porém nenhum animal tratado apresentou dano hipocampal. Por fim, investigamos o possível sítio de união da guanosina à membrana plasmática cerebral em ratos. Verificamos que as preparações enriquecidas em membrana plasmáticas, comumente utilizadas em protocolos de união, apresentam níveis significativos de guanosina endógena. Entretanto estes níveis elevados de guanosina endógena podem mascarar os níveis de união da guanosina. Por isso apresentamos uma metodologia capaz de diminuir os níveis de guanosina endógena, detectando assim um maior nível de união da guanosina nas preparações enriquecidas em membrana plasmática. Por fim, apresentamos alguns dados que mostram a necessidade de melhorar a purificação das preparações enriquecidas em membrana plasmática para evitar a contaminação com membrana mitocondrial. . De uma maneira geral, esta tese traz contribuições para um melhor entendimento das alterações do sistema glutamatérgico e o potencial neuroprotetor das purinas derivadas da guanina e da inosina em doenças cerebrais envolvendo excitotoxicidade glutamatérgica.

ABSTRACT

The neurotoxicity caused by overstimulation of glutamatergic system is implicated in several acute and chronic brain diseases. Glutamate mediate the excitatory synaptic transmition in the brain by acting on the ionotropic and metabotropic glutamate receptors The uptake mechanisms performed by specific transporter proteins have an important role in modulate the glutamatergic neurotransmission. Several studies have shown that the guanine based-purines and inosine exhibit neuroprotective effects in experimental models of brain disorders caused by excitotoxicity. In this thesis, we showed that the glutamate uptake by different brain structures of female rats with depression-like behavior is different from the normal female rats. For example, in female rats with depression-like behavior there was an initial increase in the hippocampal glutamate uptake followed by a gradual decrease after 24h that persist up to 21 days compared to animals without depressive-like behavior.The hippocampus was the most sensitive brain structure related to this behavior. We also reported the neuroprotective potential effect of GMP, guanosine, and inosine in different experimental models of brain diseases. We showed that systemic administration of GMP induced an anxiolytic-like behavior in classic behavior protocols developed to evaluate potential anxyolityc/anxiogenic drugs in rats. We also demonstrated that an oral chronically administration of GMP in mice increased the resistance of the brain cortex to an ex vivo ischemic insult, the oxygen and glucose deprivation. This neuroprotective effect was correlated with changes in the brain cortical glutamatergic system. The GMP administration decreased the immunocontent of some glutamate receptors subunits and some subtypes of glutamate transporters. Those effects may be related to the neuroprotective effect by decreasing the signaling pathway and the source of glutamate, respectively, during an ischemia insult. Moreover, we reported that an intracerebroventricular administration of inosine caused an anticonvulsive effect in mice against seizures induced by quinolinic acid, a potent glutamate agonist. This effect was independent of benzodiazepines receptors. We also investigated the effect of an oral chronically treatment with guanosine against the cognitive impairment and the hippocampal damage observed in animal model of chronic cerebral hypoperfusion. The guanosine treatment did not prevent the cognitive impairment, however none of the animals treated with guanosine presented hippocampal damage. Finally, we investigated the guanosine binding site at the rat brain plasma membrane. We reported that the plasma membrane enriched preparations, commonly used in binding protocols, shows significant levels of endogenous guanosine. However, the increased levels of endogenous guanosine could mask the guanosine binding. For this reason we proposed a new methodology to decrease the endogenous guanosine in the plasma membrane enrich preparations to be able to detect the increase level of guanosine binding. Moreover, we presented some data that corroborate with the need of a better purify plasma membrane enrich preparations to avoid the contamination with mitochondrial membranes. Therefore, the work developed during this thesis contributes to a better understanding of alterations in the glutamatergic system and the neuroprotective potential of the guanine based purines and inosine in brain disorders related to glutamatergic excitotoxicity.

LISTA DE ABREVIATURAS

SNC – sistema nervoso central

NMDA – N-metil-D-aspartato

AMPA - α-amino-3-hidroxi-5-metil-4-isoxazol-propionato

KA – ácido caínico

EAAT – transportador de aminoácidos excitatórios

HPA – hipófise-pituitária-adrenal

CRF – fator de liberação de corticotropina

mGLUR – receptores metabotrópicos glutamatérgicos

iGLUR – receptores ionotrópicos glutamatérgicos

AVC – acidente vascular cerebral

ATP – adenosina trifosfato

ADP – adenosina difosfato

AMP – adenosina monofosfato

GTP – guanosina trifosfato

GDP – guanosina difosfato

GMP – guanosina monofosfato

Na+ - K+ - ATPase – Sódio-Potássio adenosina trifosfatase

DNA – ácido desoxiribonucléico

RNA – ácido ribonucléico

AMPc – Adenosina monofosfato cíclico

GMPc – Guanosina monofosfato cíclico

MPP+ – 1-metil-4-fenilpiridinium

MAPK – proteína cinase ativadora de mitose

PI3K – fofoinositol-3 cinase

AKT/PKB – proteína cinase B

LCR – líquido cefalorraquidiano

POG – privação de oxigênio e glicose

NR2A/B – subunidade 2 A/B do receptor NMDA glutamatérgico

GluR1 – subunidade R1 do receptor AMPA glutamatérgico

PSD-95 – proteína de densidade pós-sináptica 95

PTZ – pentilenotetrazol

GFAP – proteína glial fibrilar ácida

BDNF – fator neurotrófico derivado do encéfalo

1. INTRODUÇÃO

1.1. Sistema Glutamatérgico e Excitotoxicidade

O sistema glutamatérgico, principal sistema de neurotransmissão excitatório no SNC de mamíferos, é essencial para o desenvolvimento e envelhecimento cerebral, interação entre estruturas cerebrais, comunicação celular e processos de aprendizado e memória (Izquierdo et al., 2006; Segovia et al., 2001; Stevens, 2008). Entretanto, a hiper estimulação do sistema glutamatérgico, decorrente de níveis de glutamato excessivamente aumentados na fenda sináptica, é extremamente prejudicial ao cérebro (Danbolt, 1994; Dong et al., 2009; Rothstein, 1996; Tzingounis and Wadiche, 2007). Esta situação, chamada de excitotoxicidade glutamatérgica, está implicada em diversos distúrbios agudos (como AVC, convulsão, trauma) e crônicos (demência vascular, doenças neurodegenerativas, doenças psiquiátricas, entre outros) que cursam com o comprometimento do SNC (Chao et al., 2010; Danbolt, 2001; Dong et al., 2009; Eulenburg e Gomeza, 2010; Maragakis e Rothstein, 2004; Sheldon e Robinson, 2007; Swanson et al., 2004; Tzingounis e Wadiche, 2007).

O neurotransmissor glutamato exerce suas ações através da ativação de seus receptores ionotrópicos (NMDA, AMPA e KA) e/ou metabotrópicos (acoplados a proteínas G) (Kew e Kemp, 2005). Em condições fisiológicas, o glutamato é liberado na fenda sináptica, ligando-se aos seus receptores. A ativação dos receptores resulta na propagação de um potencial de ação dos neurônios glutamatérgicos. Em condições normais no SNC de mamíferos, a concentração citoplasmática neuronal de glutamato é de 10 mM, 0,6 μM no

espaço extracelular, 20 nM na fenda sináptica, e 100 mM nas vesículas do terminal sináptico (Albrecht et al., 2000). A regulação da neurotransmissão glutamatérgica envolve a remoção do glutamato da fenda sináptica por transportadores específicos, modulando o equilíbrio do tônus fisiológico/tóxico da sinapse glutamatérgica (Danbolt, 2001; Robinson, 2006; Tzingounis e Wadiche, 2007).

Muitos estudos avaliaram o potencial uso de antagonistas de receptores glutamatérgicos no tratamento de doenças cerebrais relacionadas com a excitotoxicidade glutamatérgica; no entanto, a maioria dos estudos em fase clínica demonstrou resultados insatisfatórios, com alto índice de efeitos colaterais, particularmente devido ao bloqueio das ações fisiológicas do sistema glutamatérgico (Chen e Lipton, 2006; Ikonomidou e Turski, 2002; Muir, 2006). Consequentemente, outros alvos terapêuticos associados ao sistema glutamatérgico também podem ser promissores e devem ser investigados como estratégias para a neuroproteção contra a excitotoxicidade glutamatérgica (Hazell, 2007).

Neste contexto, há crescentes evidências de que os astrócitos, e outras células gliais, são capazes de modular o balanço entre os papéis fisiológico/patológico do sistema glutamatérgico, favorecendo o tônus fisiológico e atenuando a excitotoxicidade (Chao et al., 2010; Danbolt, 2001; Eulenburg e Gomeza, 2010; Swanson et al., 2004) 27,36). A captação astrocitária de glutamato é um dos mecanismos endógenos mais importantes para proteger neurônios da excitotoxicidade glutamatérgica.

Os transportadores de glutamato são proteínas com considerável homologia (60% ao nível de aminoácidos) (Beart e O'Shea, 2007). Cinco

subtipos de transportadores foram identificados e estruturalmente caracterizados em cérebro de mamíferos: EAAT1-5 (Arriza et al., 1997; Fairman et al., 1995; Kanai e Hediger, 1992; Pines et al., 1992; Storck et al., 1992). EAAT1 (GLAST) é basicamente astrocitário e o principal transportador durante as fases iniciais do desenvolvimento do SNC (Furuta et al., 1997b). EAAT2 (GLT-1) é o transportador responsável por cerca de 90% de toda a captação de glutamato no cérebro adulto (Danbolt, 1994; Furness et al., 2008; Mallolas et al., 2006; Rothstein, 1996). EAAT3 (EAAC1) é um transportador neuronal presente na membrana pós-sináptica, principalmente em hipocampo, cerebelo e gânglio basal (Furuta et al., 1997a). EAAT4 é um transportador localizado basicamente em células de Purkinje do cerebelo (Furuta et al., 1997a; Furuta et al., 1997b) e EAAT5 é localizado basicamente em fotorreceptores e células bipolares da retina (Pow e Barnett, 2000). A distribuição dos transportadores no SNC é dependente de idade e estrutura e suas atividades variam com o desenvolvimento e em situações de injúria cerebral (Danbolt, 1994; Furuta et al., 1997b; Tzingounis e Wadiche, 2007).

A atividade destes transportadores é dependente de sódio (Bridges et al., 1999; Danbolt, 2001), eles estão localizados em sinapses glutamatérgicas (Danbolt, 1994; Furness et al., 2008; Gegelashvili e Schousboe, 1998) e são sujeitos à regulação e plasticidade (Danbolt, 2001; Eulenburg e Gomeza, 2010; Tzingounis e Wadiche, 2007). Os transportadores de glutamato parecem ser modulados em praticamente todos os níveis possíveis, ou seja, durante a transcrição do DNA, processamento do mRNA, síntese de proteínas ou pós-traducional (Danbolt, 2001; Eulenburg e Gomeza, 2010; Tzingounis e Wadiche, 2007). Esta última pode envolver modulações alostéricas, por exemplo, pelo

ácido araquidônico ou pelo Zn+ (Vandenberg et al., 2004), ou pela translocação dos transportadores entre a membrana plasmática e compartimentos intracelulares (Davis et al., 1998; Duan et al., 1999; Gegelashvili et al., 2000; Lortet et al., 1999; O'Shea, 2002; Robinson, 2002). Já foi demonstrado que a translocação pode envolver diferentes vias intracelulares, incluindo ativação de proteínas cinases e fosfatases e que a ativação dessas vias pode regular diferentemente os transportadores (Davis et al., 1998; Duan et al., 1999; Gegelashvili et al., 2000; Lortet et al., 1999; O'Shea, 2002; Robinson, 2002).

Atualmente, vários estudos têm considerado que a disfunção de transportadores de glutamato pode ser o evento inicial ou parte de uma cascata implicada na patologia de doenças cerebrais agudas e crônicas (Maragakis e Rothstein, 2004; Moussa et al., 2007; Robinson, 2006; Sheldon e Robinson, 2007; Stevens, 2008; Tzingounis e Wadiche, 2007).

1.1.2. Doenças cerebrais envolvendo excitotoxicidade glutamatérgica

1.1.2.1. Depressão

A depressão é a doença psiquiátrica mais comum atualmente e afeta cerca de 20% dos indivíduos no curso de suas vidas (Kessler et al., 1994; Teesson e Vogl, 2006). Ela é duas vezes mais freqüente em mulheres do que em homens e pode começar em qualquer idade, com idade média de início aos 20 anos (Blazer et al., 1994; Teesson e Vogl, 2006).

Nas últimas quatro décadas, os estudos neuroquímicos sobre os transtornos psiquiátricos foram centrados nos sistemas monoaminérgicos, contribuindo de forma significante para o entendimento da fisiopatologia do transtorno da depressão. Além de monoaminas, outros sistemas de

neurotransmissores parecem ter importantes contribuições (Kendell et al., 2005). Recentemente, várias evidências apontam que o sistema glutamatérgico exerce um importante papel na fisiopatologia de depressão (Altamura et al., 1993; Drevets, 2000; Neumeister et al., 2004; Sanacora et al., 2004).

O principal achado que aponta a participação do sistema glutamatérgico na fisiopatologia do transtorno da depressão é que os antagonistas do receptor NMDA mimetizam os efeitos dos antidepressivos clínicos em modelos animais de depressão (Papp et al., 1996; Trullas e Skolnick, 1990). Além disso, existem alguns estudos que mostram uma elevação nos níveis de glutamato na região do córtex pré frontal e límbico em pacientes deprimidos (Drevets, 2000; Sanacora et al., 2004).

O estresse causado pelo ambiente exerce um importante papel na etiologia do transtorno depressivo, porém diferentes indivíduos apresentam respostas diferentes ao mesmo estresse. Alterações morfológicas (Bremner et al., 2000; Sheline, 1996) e histológicas (Ongur et al., 1998; Rajkowska et al., 1999) no hipocampo e no córtex pré-frontal observado em pacientes deprimidos estão fortemente associados a hiper estimulação do sistema glutamatérgico relacionada com o estresse causado pelo ambiente (Sapolsky, 2000a; Sapolsky, 2000b). Vários estudos sugerem que a atividade glutamatérgica está intimamente ligada à resposta do eixo HPA-paralímbico em resposta ao estresse, uma vez que o glutamato é capaz de estimular a liberação do CRF (Cratty e Birkle, 1999; Feldman e Weidenfeld, 1997; Ziegler e Herman, 2000) e que os glicocorticóides (os esteróides adrenais secretados durante o estresse) podem modular a expressão de transportadores de glutamato em cultura de astrócitos (Chou et al., 1994).

1.1.2.2. Ansiedade

A ansiedade é caracterizada por sintomas autonômicos, como taquicardia, sudorese, dispnéia, associado à sensação de medo, desconforto ou estresse psicológico, causando grande sofrimento para os pacientes (Hoffman e Mathew, 2008).

Os mecanismos fisiopatológicos envolvidos na ansiedade permanecem obscuros, no entanto diferentes neurotransmissores parecem estar envolvidos. O sistema glutamatérgico tem recebido atenção nos últimos anos, como potencial alvo para o desenvolvimento de fármacos ansiolíticos (Bergink et al., 2004; Chojnacka-Wojcik et al., 2001; Cryan et al., 2003; Kapus et al., 2008; Palucha e Pilc, 2007). Antagonistas de receptores NMDA não competitivos e competitivos, bem como os antagonistas dos receptores AMPA não-competitivo bloqueiam comportamentos de ansiedade em roedores (Bergink et al, 2004;. Chojnacka-Wojcik et al, 2001;. Kapus et al, 2008;. Kehne et al, 1991;. Plaznik et al, 1994). Além disso, antagonista seletivo de alguns subtipos de receptores mGluR também apresentam efeitos ansiolíticos em roedores (Chojnacka-Wojcik et al, 2001;. Palucha e Pilc, 2007; Spooren e Gasparini, 2004;. Tatarczynska et al, 2001a, b). Estes estudos mostram que a hiperestimulação glutamatérgica está associada ao comportamento ansioso e que o bloqueio desta estimulação é capaz de reduzir o comportamento ansioso.

1.1.2.3. Convulsão

Já este bem estabelecido que a amplificação anormal e a sincronização dos disparos neuronais que levam a episódios de crises convulsivas envolvem

a interação da inibição gabaérgica e de mecanismos excitatórios do glutamato (Bradford, 1995; Meldrum, 1994; Meldrum, 2000; Naylor, 2010; Treiman, 2001). Desta forma, o excesso de estimulação glutamatérgica, o bloqueio de transportadores de glutamato ou as alterações que ativem os receptores de glutamato podem precipitar crises convulsivas. Estas crises podem ser únicas ou crônicas e recorrentes (crises epilépticas) (Meldrum, 1994; Meldrum, 2000).

1.1.2.4. Isquemia

A isquemia cerebral é uma das maiores causas de morte e morbidade em humanos atualmente (Castillo et al., 2003). No Brasil, as doenças cerebrovasculares merecem uma atenção especial, pois são as primeiras causas de morte, apresentam uma alta taxa de morbidade e causam elevado custo econômico e social para o país. A isquemia pode ser focal quando ocorre o bloqueio de artérias (AVC isquêmico) ou de forma global (parada cardíaca) (Castillo et al., 2003).

Inicialmente, o dano isquêmico leva a perda de energia devido à diminuição de oxigênio e glicose para todas as células, o que retarda ou impede a síntese de ATP através da glicólise e da fosforilação oxidativa. Um evento crucial na isquemia cerebral severa ou completa é a inibição da Na +-K +-ATPase que ocorre pela diminuição do ATP intracelular (os níveis de ATP caem para 0-25% comparado com uma perfusão normal) (Lipton, 1999; Lowry et al., 1964). A inibição da Na^{+}-K^{+}-ATPase durante uma isquemia severa, causa uma grande perda dos gradientes iônicos promovendo assim a despolarização da membrana dos neurônios (Benveniste et al., 1988; Rossi,

2006; Silver et al., 1997) e o consequente aumento da concentração extracelular de glutamato (Phillis et al., 1996).

O glutamato é reconhecido por desempenhar um papel importante na patogênese da lesão isquemia cerebral (Choi e Rothman, 1990; Fei et al., 2007). Ele é liberado em altas concentrações na região central da isquemia ou "core" e também na área de "penumbra" que é a região que envolve a área central. A isquemia causa uma hiperativaçao dos receptores NMDA e AMPA, levando a um aumento do influxo de $Ca2^{+}$ e Na^{+} e ativação de genes e vias de sinalização associadas à morte celular (Romera et al., 2004).

1.1.2.5. Demência vascular

A demência vascular é a segunda causa mais comum de demência associada à doença de Alzheimer, sendo assim, responsável por 10-50% de todas as demências (Rockwood et al., 2000). A demência vascular é um grupo de doenças com condições patológicas e mecanismos fisiopatológicos heterogêneos (Desmond, 2004).

A hipoperfusão cerebral crônica é considerada um fator que contribui para a disfunção da memória em doenças neurológicas como a demência vascular (Farkas e Luiten, 2001; Ohnishi et al., 2001). Além disso, o grau de hipoperfusão cerebral tem sido sugerido como um marcador preditivo da transição gradual do comprometimento cognitivo leve à doença de Alzheimer (Borroni et al., 2006).

A cadeia de eventos que eventualmente leva à morte celular neuronal na hipoperfusão cerebral crônica inicia na falha energética neuronal, devido à redução do fluxo sanguíneo, levando a uma deficiência de oxigênio e de

glicose (Briede e Duburs, 2007; Farkas et al., 2007; Otori et al., 2003; Tsuchiya et al., 1993). Este distúrbio energético ocasiona um acúmulo de glutamato extracelular (Chao et al., 2010; Dirnagl et al., 1999; Segovia et al., 2001; Szydlowska e Tymianski, 2010), levando a excitotoxicidade. Desta forma, a excitotoxicidade glutamatérgica tem sido proposta como um dos mecanismos responsáveis pela lesão neuronal observada na hipoperfusão cerebral (Dong et al., 2009; Marosi et al., 2009).

1.2. Sistema Purinérgico

As bases purínicas, como adenina e guanina, e seus respectivos nucleotídeos e nucleosídeos são moléculas amplamente distribuídas dentro e fora das células de organismos vivos. Dentre suas diversas funções biológicas, podemos enfatizar seu papel na construção do DNA e RNA (adenina e guanina), nas vias bioquímicas envolvidas no metabolismo energético celular (ATP) ou nos mecanismos intracelulares de transdução de sinal como mensageiros secundários (AMPc e GMPc) (Barnstable et al., 2004; Bourne et al., 1990). Entretanto, nos últimos 20 anos, diversos trabalhos demonstraram o papel fundamental destas moléculas no espaço extracelular sobre a homeostase (Burnstock, 2007).

As purinas podem ser classificadas em derivados da adenina (ATP, ADP, AMP, adenosina, adenina) e derivadas da guanina (GTP, GDP, GMP, guanosina e guanina). Ainda compõem as purinas os metabólitos diretos dos derivados da adenina e da guanina: inosina, xantina, hipoxantina e ácido úrico.

1.2.1. Efeitos das purinas derivadas da guanina sobre o sistema glutamatérgico

1.2.1.1. GMP

Em relação ao nucleotídeo GMP, diversos estudos demonstraram que este é capaz de inibir a ligação do glutamato e de seus análogos às preparações de membranas cerebrais (Baron et al., 1989; Butcher et al., 1986; Hood et al., 1990; Mendieta et al., 2005; Paas et al., 1996), de prevenir respostas celulares ao glutamato (Aleu et al., 1999; Burgos et al., 2000; Burgos et al., 2003; Regner et al., 1998; Tasca et al., 1995; Tasca et al., 1998) de estimular a captação de glutamato por astrócitos (Frizzo et al., 2003), e de apresentar efeitos neuroprotetores em várias preparações cerebrais submetidas à condições excitotóxicas (Molz et al., 2005; Oleskovicz et al., 2008; Oliveira et al., 2002). In vivo, a administração de GMP apresenta efeitos amnésicos, ansiolíticos e antinociceptivos em roedores (Almeida et al., 2010; Saute et al., 2006; Schmidt et al., 2008).

Ademais, o GMP previne convulsões e dano a neurônios estriatais promovidos pelo ácido quinolínico, um hiper-estimulador do sistema glutamatérgico via receptor NMDA (Malcon et al., 1997; Schmidt et al., 2000; Schmidt et al., 2005; Schmidt e Souza, 2010; Soares et al., 2004). É importante ressaltar que a maior parte dos efeitos do GMP, incluindo o estímulo à captação de glutamato pelos astrócitos e os efeitos amnésicos, antinociceptivos e anticonvulsivantes dependem de sua conversão a guanosina (Frizzo et al., 2003; Saute et al., 2006; Schmidt et al., 2008; Soares et al., 2004).

1.2.1.2. Guanosina

Nos últimos anos, o nucleosídeo guanosina tem ganhado atenção dos pesquisadores devido ao seu potencial efeito neuroprotetor nos modelos experimentais de doenças cerebrais associadas à excitotoxicidade glutamatérgica (Rathbone et al., 2008; Schmidt et al., 2007; Schmidt e Souza, 2010). Nosso grupo demonstrou que a administração sistêmica de guanosina protege roedores de convulsões induzidas por ácido quinolínico (Lara et al., 2001; Schmidt et al., 2000; Schmidt et al., 2005; Schmidt e Souza, 2010; Soares et al., 2004), além de prevenir a queda da captação de glutamato hipocampal em ratos jovens submetidos à hipóxia e isquemia (Moretto et al., 2005; Moretto et al., 2009). A administração sistêmica de guanosina também foi capaz de aumentar a sobrevida e diminuir o déficit cognitivo em modelo animal de isquemia cerebral in vivo (Chang et al., 2008), proteger contra a morte de células neurais e estimular a proliferação celular em modelo animal da doença de Parkinson (Su et al., 2009). Em protocolos in vitro, a administração de guanosina protegeu células SH-SY5Y (linhagem de neuroblastoma humano) da morte apoptótica induzida por β-amilóide, um componente das placas senis que circundam os neurônios na Doença de Alzheimer (Pettifer et al., 2004) e da deprivação de glicose e oxigênio (modelo in vitro de isquemia cerebral) (Chang et al., 2008; Oleskovicz et al., 2008). A guanosina também foi capaz de evitar a fragmentação do DNA provocada pelo MPP+ (um modelo in vivo da doença de Parkinson) (Pettifer et al., 2007).

Os mecanismos moleculares e celulares envolvidos na neuroproteção pela guanosina não são bem conhecidos. O nosso grupo de pesquisa

demonstrou que a guanosina é capaz de estimular a captação de glutamato em cultura de astrócitos e fatias de córtex cerebral (Frizzo et al., 2001; Frizzo et al., 2002; Frizzo et al., 2003; Frizzo et al., 2005; Gottfried et al., 2002). Em condições basais ou fisiológicas, os efeitos da guanosina sobre a captação de glutamato em fatias cerebrais parecem ser idade e estrutura dependentes, ocorrendo mais no córtex e em ratos jovens (Thomazi et al., 2004). Por outro lado, em condições excitotóxicas, guanosina parece ser mais amplamente envolvida na modulação da captação de glutamato (Frizzo et al., 2001; Frizzo et al., 2002; Thomazi et al., 2008). Dependendo da preparação biológica, a concentração mínima de guanosina com efeito sobre a captação foi de 100 nM a 1 µM. A estimulação máxima da captação foi de 60-80% sobre os valores controles (Frizzo et al., 2001; Frizzo et al., 2002).

Como a captação astrocitária de glutamato é o mecanismo mais importante para modular sua ação sináptica e inibir a hiperestimulação dos receptores glutamatérgicos, a estimulação da captação de glutamato pela guanosina pode ser um dos potenciais mecanismos responsáveis pelos seus efeitos de neuroproteção. Entretanto, o mecanismo pelo qual a guanosina estimula a captação de glutamato, e a relação deste com os efeitos neuroprotetores ainda não estão totalmente esclarecidos.

Resultados preliminares sugerem que o efeito estimulador sobre a captação de glutamato astrocitária pela guanosina é promovido extracelularmente visto que a estimulação da captação é incrementada pela administração de um inibidor do transportador de nucleosídeo (dipiridamol) (Frizzo et al., 2001).

Recentemente, Traversa e colaboradores identificaram um sítio de união para a guanosina em preparação de membranas cerebrais de ratos (Traversa et al., 2002; Traversa et al., 2003). Essa identificação foi feita com uma preparação de membranas com grande contaminação de fração mitocondrial. Considerando que a mitocôndria também apresenta metabolismo purinérgico, o sítio de união para guanosina nessa preparação de membrana não pode ser considerado exclusivamente e/ou predominantemente sináptico. Neste sentido, novos estudos sobre a união da guanosina são essenciais, incluindo uma purificação adequada da membrana celular. É aceitável que haja um sítio específico para guanosina no lado extracelular da membrana plasmática, uma vez que alguns efeitos neuroprotetores da guanosina são mediados através de uma rota de sinalização dependente de proteínas G envolvendo nucleotídeos cíclicos e as rotas da p38 MAPK e da PI3K/Akt/PKB, e seus efeitos não são bloqueados por administração de inibidores do transportador de nucleosídeo (Di Iorio et al., 2004; Pettifer et al., 2004; Rathbone et al., 2008; Schmidt et al., 2007).

Desta maneira, alguns estudos sugerem que a guanosina endógena extracelular poderia fazer parte de um mecanismo cerebral envolvido na prevenção da excitotoxicidade (Ciccarelli et al., 1999; Ciccarelli et al., 2000; Ciccarelli et al., 2001; Hagberg et al., 1987; Rathbone et al., 2008; Schmidt et al., 2007; Uemura et al., 1991). Estes estudos demonstraram que o nível extracelular de guanosina aumenta consideravelmente e continua elevado por longos períodos de tempo em modelos in vitro (Ciccarelli et al., 1999; Ciccarelli et al., 2001) e in vivo (Hagberg et al., 1987; Uemura et al., 1991) de isquemia cerebral. Neste sentido, além da ação antiglutamatérgica, a guanosina também

desempenharia efeitos tróficos, pois já foi observado que extracelularmente ela induz a proliferação de células gliais (Christjanson et al., 1993; Kim et al., 1991; Rathbone et al., 2008; Rathbone et al., 1992a; Rathbone et al., 1992b), o crescimento de neuritos (Gysbers e Rathbone, 1996a; Gysbers e Rathbone, 1996b) e a síntese e liberação de outras purinas (Ciccarelli et al., 1999) e de outros fatores tróficos (Gysbers e Rathbone, 1996a; Gysbers e Rathbone, 1996b; Middlemiss et al., 1995).

1.2.2.. Efeitos da inosina sobre o sistema glutamatérgico

Ao contrário da guanosina, poucos trabalhos na literatura científica demonstraram o potencial efeito neuroprotetor da inosina frente a modelos experimentais envolvendo excitotoxicidade glutamatérgica. Dentre os estudos realizados, já foi reportado que inosina reduziu a toxicidade causada pela deprivação de oxigênio e glicose em culturas de astrócitos de ratos (Haun et al., 1996), e preservou a viabilidade celular durante hipóxia química induzida por rotenona em cultura de células de medula (Litsky et al., 1999). O efeito protetor da inosina também já foi demonstrado in vivo. A oclusão da artéria média cerebral levou a liberação de inosina e seu metabólito hipoxantina do córtex isquêmico em animais com derrame (Matsumoto et al., 1993). A inosina administrada estimulou o crescimento axonal e melhorou o perfil comportamental em animais que sofreram isquemia (Chen et al., 2002). Também, a inosina ou o seu análogo inosina 5` trifosfato reduziram respostas mediadas por receptores de glutamato em sinapses na região CA1 hipocampal (Macek et al., 1998) ou a neurotoxicidade mediada por `NMDA em neurônios hipocampais em cultura (Ortinau et al., 2003).

2. Objetivos

2.1. Objetivo geral

Estudar parâmetros bioquímicos e comportamentais em modelos experimentais de doenças cerebrais que envolvem excitotoxicidade glutamatérgica, assim como avaliar o efeito neuroprotetor dos derivados da guanina e/ou inosina em tais modelos e investigar o poissivel sítio de união da guanosina na membrana plasmática cerebral de ratos.

2.2. Objetivos específicos

2.2.1. Estudar a captação de glutamato em fatias de diferentes regiões cerebrais de ratas submetidas a um modelo experimental de depressão.

2.2.2. Estudar os efeitos da administração aguda de GMP sobre comportamentos ansiosos de ratos adultos.

2.2.3. Estudar o potencial neuroprotetor da administração crônica de GMP em camundongos em um modelo de isquemia *in vitro* e avaliar parâmetros do sistema glutamatérgico no córtex cerebral.

2.2.4. Estudar o potencial anticonvulsivante da administração intracerebroventricular de inosina contra convulsões induzidas por ácido quinolínico em camundongos e o envolvimento dos receptores benzodiazepinicos.

2.2.5. Estudar o efeito do tratamento crônico com guanosina sobre o prejuízo cognitivo e o dano hipocampal em um modelo de demência vascular, promovidos pela oclusão das carótidas comum em ratos.

2.2.6. Estudar o possível sítio de união da guanosina à membrana plasmática cerebral de ratos.

Parte II

Capítulo I

Effects of Depressive-Like Behavior of Rats on Brain Glutamate Uptake

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ORIGINAL PAPER

Effects of Depressive-Like Behavior of Rats on Brain Glutamate Uptake

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Abstract Learned helplessness paradigm is a widely accepted animal model of depressive-like behavior based on stress. Glutamatergic system is closely involved with the stress-neurotoxicity in the brain and recently it is pointed to have a relevant role in the pathophysiology of depression disorder. Glutamate uptake is the main mechanism to terminate the glutamatergic physiological activity and to neuroprotection against excitotoxicity. We investigated the profile of glutamate uptake in female rats submitted to the learned helplessness paradigm and to different classes of stress related to the paradigm, in slices of brain cortex, striatum and hippocampus. Glutamate uptake in slices of hippocampus differ between learned helplessness (LH) and non-learned helplessness (NLH) animals immediately persisting up to 21 days after the paradigm. In addition, there were a decrease of glutamate uptake in the three brain structures analyzed at 21 days after the paradigm for LH animals. These results may contribute to better understand the role of the glutamatergic system on the depressive-like behavior.

Keywords Stress behavior · Depression · Learned helplessness paradigm · Glutamate uptake · Hippocampus

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Introduction

Major depression is the most common psychiatric disorder and affects over 17% of individuals in the course of their lifetimes [1]. It is twice more frequent in women than in men and can begin at any age, with average age of onset in the mid-20s [2, 3].

Although environmental stressors play a strong role in the etiology of depressive disorder, there is an individual susceptibility to similar environmental stressors. This might indicate that vulnerability to the depression-inducing effects of environmental stress may be, at least in part, genetically determined [4, 5].

In animals, inescapable stress leads to the phenomenon known as learned helplessness (LH), which is a widely accepted animal model of depressive-like behavior [6]. LH rats fail to exhibit escape behavior to escapable shock 24 h after exposure to inescapable shock, whereas non—learned helplessness (NLH) rats will continue to attempt escape regardless of prior inescapable shock exposure [7, 8]. A number of studies have previously demonstrated that LH is a syndrome of changes in brain activity involving multiple neurotransmitter systems and brain regions, such as cortex, hippocampus and striatum [9].

Over the past four decades, the studies concerning psychiatric disorders have focused on brain monoaminergic systems, which have contributed significantly to our understanding of the pathophysiology of depression disorder [8], although, in addition to monoamines, others neurotransmitter systems seem to have important contributions [10]. Recently, numerous evidences pointed to a relevant role for the glutamatergic system in the pathophysiology of depression disorder [11–14].

Glutamate is the major excitatory neurotransmitter in mammalian brain being involved in most of central

nervous system (CNS) synapses [15–17]. Glutamate exerts its excitatory action via ionotropic (AMPA, NMDA and kainate) or metabotropic glutamate receptors [18]. The high affinity uptake through Na^+-dependent excitatory amino acid transporters (EAAT1-5) is responsible for the clearance of glutamate from the synaptic cleft, which is the main mechanism to terminate its physiological activity and avoid the glutamatergic excitotoxicity [16]. To date, five different mammalian glutamate transporters have been cloned: GLAST [19] and GLT-1 [20], responsible for most of the glutamate uptake activity are predominantly localized in astrocytes [16, 21], while EAAC1 [22], EAAT4 [23] and EAAT5 [24] are mostly localized in neurons [25]. Overstimulation of the glutamatergic system may lead to excitotoxicity, a process that involves intracellular increase of free Ca^{2+}, ATP levels exhaustion and the neurotoxic process thereafter [26]. This phenomenon is involved in the pathophysiology of various acute and chronic disorders of CNS [27].

The most convincing finding supporting the involvement of the glutamatergic system in the pathophysiology of depression disorder is that functional NMDA receptor antagonists mimic the effects of clinically effective antidepressants in animal models commonly used to evaluate potential antidepressant activities [28, 29]. Additionally, there are some reports showing an abnormal elevation of glutamate neurotransmission and glutamate levels in cortico/limbic brain areas of depressed patients [11, 13].

In addition, acute injection of glutamate into frontal neocortex of naive rats produced a deficit in escapable shock performance similar to that produced by exposure to uncontrollable shock [30]. Regarding the LH behavior, there are studies showing that blocking NMDA receptors during inescapable stress (IS) prevented the usual impact of IS on escape responding [31] and intraperitoneal administration of MGS0039 (a selective antagonist for group II metabotropic glutamate) to rats for 7 days elicited a significant reduction in escape failures in the LH paradigm [32].

Morphological [26, 33] and histological [34, 35] alterations in hippocampus and prefrontal cortex observed in depressive patients are tightly associated to overstimulation of glutamatergic system by a stress-responsive neurotoxicity [36, 37]. Both acute and repeated stress increase extracellular glutamate concentrations [38–41] and alter NMDA and AMPA receptor binding profiles in several brain regions [42–44]. Several studies suggest that glutamatergic activity is closely tied to the stress-responsiveness of the paralimbic-HPA axis, since glutamate is capable to stimulate CRF (corticotrophin releasing factor) release into the portal circulation [45–47] and that glucocorticoids (the adrenal steroids secreted during stress) can modulate the glutamate transporters expression in astrocyte culture [48].

Thus, considering the relevant modulating role of glutamate uptake in the glutamatergic synapse efficacy and the involvement of the glutamatergic system with the depression disorder and stress neurotoxicity, in this study we investigated the glutamate uptake by slices from different brain regions of animals submitted to the LH paradigm and after different kind of a single stress session related to the paradigm protocol.

Experimental Procedure

Materials

L-[3H]glutamate (specific activity 30 Ci/mmol) was purchased from Amersham International, UK. N-methyl-d-glucamine was purchased from Sigma Chemical CO (St. Louis, MO, USA). Other chemicals were of analytical grade.

Animals

Female adult Wistar rats (200–300 g) were kept on a 12 h light/dark cycle (light on at 7:00 am) at a constant temperature of 22 ± 1°C, with experiments occurring during the light phase of the cycle. They were housed in plastic cages (5 animals/cage) with commercial food and tap water ad libitum and were acclimated in the animal research facilities for at least 2 weeks prior the experiments. Our institutional experimental protocols, designed to minimize suffering and limit the number of animals sacrificed, were performed according regulations of local animal house authorities.

LH Paradigm

As described by Vollmayr and Henn [49] with some minor adaptation. The protocol consists of an induction LH behavior session 24 h prior a LH behavior test session.

Induction LH Behavior Session (Inescapable Footshock Session)

Animals were individually placed into a conditioning chamber consisting in a 50 × 25 × 25 cm acrylic box without lever or signal light, with a stainless-steel grid floor (parallel 1.0 mm-caliber stainless steel bars spaced 1.0 cm). A pulsate-current shocking device was used to deliver inescapable shocks (0.7 mA), randomly ranging from 5 to 15 s, with inter-shock times randomly ranging from 5 to 15 s, in a 40 min time session. The sum of shock duration and inter-shock time is 20 min for each one.

LH Behavior Test Session (Escapable Footshock Session)

24 h after inescapable shock session, animals were placed individually into the conditioning chamber with lever and a turn off lamp at this time, and allowed to habituate the environment for 5 min. Immediately thereafter, 15 trials shocks (0.7 mA), accompanied by a light clue to facilitate the distinction from the inescapable shock session were delivered lasting up to 60 s each with an inter-trial time of 24 s. Animals could stop the shock by pressing the lever (that turn off the light), been the rest of the 60 s considered in the total time in each trial without receiving footshock. The length in each shock trial was recorded. The score for the test session was provided by counting the number of trials the rat pressed the lever.

Groups

Naive: animals only manipulated (weighted and transported to the behavioral session room);

Habituated: animals only individualized for 40 min in the conditioning chamber, without receiving shock;

Inescapable stressed: animals only stressed in the inescapable shock session;

Escapable stressed: animals individualized for 40 min in the conditioning chamber, without receiving shock, and submitted 24 h later to the escapable learned helplessness test session.

Learned Helplessness: animals stressed in inescapable shock session and submitted 24 h later to the escapable test session. The LH animals pressed the lever only 5 times or less (from 15 trials) in the test session;

Non—Learned Helplessness: animals stressed in the inescapable shock session and tested 24 h later in the escapable test session. The NLH animals pressed the lever 10 or more times (from 15 trials) in the test session;

Glutamate Uptake Assay

Glutamate uptake was performed according to Thomazi et al. [50]. Striatal, hippocampal and brain cortical (parietal area) slices (0.4 mm) were obtained through a McIlwain chopper and pre-incubated for 15 min at 37°C in a Hank's balanced salt solution (HBSS) containing (in mM): 137·NaCl; 0.63·Na_2HPO_4; 4.17·$NaHCO_3$; 5.36·KCl; 0.44·KH_2PO_4; 1.26·$CaCl_2$; 0.41; $MgSO_4$; 0.49·$MgCl_2$ and 1.11·glucose, in pH 7.2. Then, 0.66 and 0.33 μCi.ml-1 L-[^{3}H]glutamate for striatum/hippocampus and cortex, respectively, in a 100 μM final concentration glutamate was added. Incubation was stopped after 3, 5 or 7 min for striatum, hippocampus and cortex, respectively, with two ice-cold washes of 1 ml HBSS, immediately followed by the addition of 0.5 N-NaOH, which was then kept overnight. The times were specifically adapted to each structure, in order to evaluate the initial rate of uptake and the glutamate concentration used results in assessment of Vmax for transport, as we previously determined by Thomazi et al. [50]. Unspecific uptake was measured using the same protocol described above, with differences in the temperature (4°C) and medium composition (N-methyl-D-glucamine instead of sodium chloride). Na^+-dependent uptake was considered as the difference between the total uptake and the unspecific uptake. Both uptakes were performed in triplicate. Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409).

Statistical Analysis

Data were expressed as means ± SEM of the mean, and were analyzed using two-way ANOVA for the different kind of a single stressed animals (factors were different kind of a single stress session and time after the session), and for the LH behavior (factors were development of depressive-like behavior and time after the test session) followed by the Bonferroni post test, when indicated. Correlations between total time of shock exposition during test session, test score and glutamate uptake were analyzed with Pearson's tests. Significance level was accepted as different when the P value was equal or less than 0.05.

Results

Glutamate Uptake in Brain Slices of Rats Submitted to LH Paradigm

We submitted 57 animals to the LH paradigm. 17 animals (as already described criteria) were considered as LH, 34 animals as NLH and the remaining animals were discarded. All animals that had not been previously stressed, but were tested in the escapable shock session (escapable stress group) learned the task (data not shown).

The Two-Way ANOVA analysis of variance was performed using the development of depressive-like behavior X time after the test session as factors and the glutamate uptake as the variable. This analysis showed an interaction between the factors in hippocampal [$F1.45 = 9.48$, $P = 0.0004$, Fig. 1c] and striatal [$F1.45 = 5.67$, $P = 0.006$, Fig. 1b] but not in cortical slices [$F1.45 = 0.58$, $P = 0.56$, Fig. 1a].

In LH group, the glutamate uptake by cortical, striatal and hippocampal slices was lower at 21 days after the test session, compared to the uptake immediately after ($P < 0.05$). In hippocampal slices, this decrease was observed yet 24 h after. In NLH group, the glutamate uptake by hippocampal slices 24 h after was higher than immediately and 21 days after.

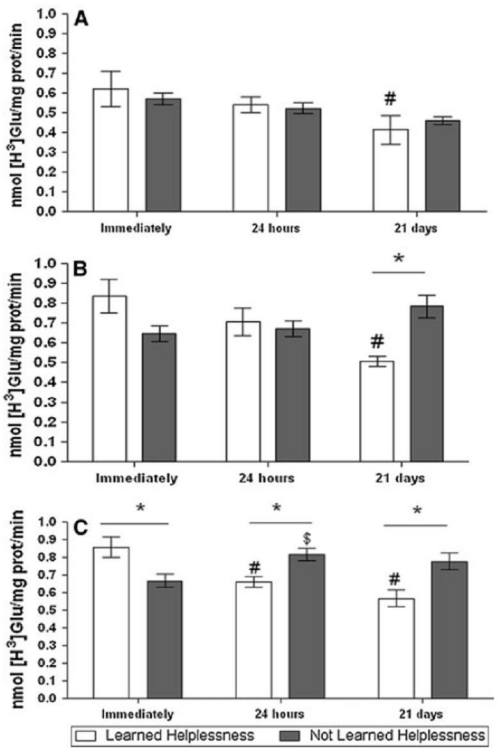

Fig. 1 Glutamate uptake by slices of cortex (**a**), striatum (**b**) and hippocampus (**c**) of rats submitted to learned helplessness paradigm at different times following the test session. The paradigm is described in the material and methods session. Bars represent mean ± S.E.M from learned helplessness (LH) and not learned helplessness animals (NLH). (*) Indicates statistically significant differences between the LH and NLH groups bellow the dash. (#) Indicates a statistically significant difference compared to LH immediately evaluated glutamate uptake. ($) Indicates statistically significant difference from NLH immediately and 21 days after evaluated glutamate uptake. Differences among groups were determined by two-way analysis of variance (ANOVA), followed by Bonferroni post test when applicable. $P < 0.05$ was considered of statistical significance. (n = 4–10 animals for each group)

By comparing LH and NLH groups, the uptake was lower by striatal (21 d) and hippocampal (24 h and 21 d) slices in LH than in NLH; and was higher in hippocampal slices immediately after.

Glutamate Uptake by Brain Slices of Rats Submitted to a Single Stress Session

No statistically significant interaction was observed among groups after the different kind of stress sessions (naive, habituation, inescapable and escapable groups), on Two-Way ANOVA, in slices of cortex [$F1.11 = 0.36$, $P = 0.70$, Fig. 2a, striatum [$F1.11 = 1.20$, $P = 0.31$, Fig. 2b, or hippocampus [$F1.11 = 0.32$, $P = 0.81$, Fig. 2c, a]. In addition, no differences between the groups were observed ($P > 0.05$).

Correlation Between Total Time of Shock Exposition or Learning and Memory Events During Test Session and Glutamate Uptake in Brain Slices

In order to investigate if the learning ability of animals in LH paradigm or if the total time of shock exposition during test session trials could be the responsible for the differences observed in glutamate uptake, we carried out some statistical correlations between test score and glutamate uptake, and between total time shock exposition and glutamate uptake, immediately and 24 h after test session and in all structures investigated, in LH and NLH groups in separate (since both parameters have an intra-group variation), and with both groups together. No statistically significant correlations were found in such analysis ($P > 0.05$, data not shown).

Discussion

The discovery that stress and depression, as well as other psychiatric illnesses, are characterized by brain structural alterations, which results from atrophy and loss of neurons and glia in specific limbic regions and circuits, has contributed to a fundamental transformation in our understanding of these illnesses. These structural changes are accompanied by dysregulation of neuroprotective and neurotrophic signaling mechanisms that are required for the maturation, growth, and survival of neurons and glia cells [51].

Most studies in the literature on behavioral models of depressive-like behavior, such as learned helplessness, are performed with male animals, even though that women are twice as men expected to suffer from major depressive disorder [52]. Certainly, this choice is due to differences in the neurobiology of brain and behavior when comparing female and male animals. Today it is known that there are many factors that contribute to the neurobiological and behavioral differences between the genders such as hormonal influences. However, it is hard to attribute all genders differences only to one factor [53]. For example, the few studies that investigated females subjected to the LH paradigm, that took into account the different phases of estrous cycle, reported controversial results, requiring additional investigation [54, 55].

In the present study, the main aim was to evaluate the glutamate uptake in different brain regions of female rats

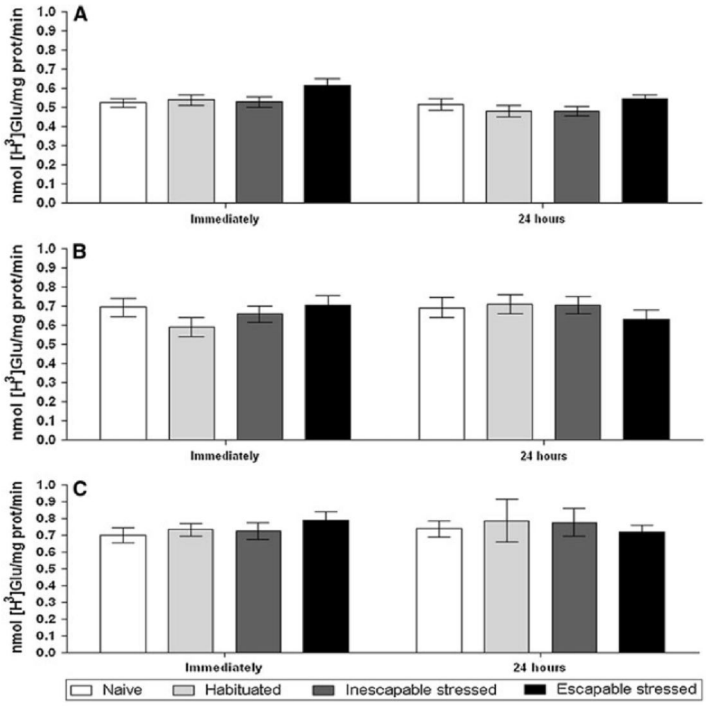

Fig. 2 Glutamate uptake by slices of cortex (**a**), striatum (**b**) and hippocampus (**c**) of rats submitted to a single different kind of stress session in different times following the stress. The stress sessions are described in material and methods session. The statistical analyses among groups were determined by two-way ANOVA. (n = 8–12 animals or each group). No statistical differences were determined among the groups

showing a depressive and not depressive-like behavior. We submitted 57 animals to the LH paradigm, which were randomly separated into several groups, which tends to reduce the variability due to the influence of specific sex parameters. However, the fact that we did not verify the estrous cycle phase is a methodological bias of this work.

We used a LH paradigm protocol well established in neurobiological research on molecular pathogenesis and therapy of depression and encompasses good construct and face validity [56–58]. The protocol enable to identify individual animals with LH for subsequent neurobiological studies and compare them with selected animals displaying most pronounced not helpless behavior.

Recently, the glutamate uptake have been reported as a strong mechanism involved in the fear conditioning, which is a tightly behavior associated to the LH behavior [59]. Tsvetkov et al. [60], found that the input specificity of LTP (long term potentiation) in fear conditioning pathways in auditory fear conditioning is determined by the glutamate uptake and it is compromised when glutamate is allowed to diffuse from stimulated synapses to heterosynaptic sites. The importance of uptake activity, specifically in fear learning is supported by a recent finding that both LTP induction and contextual fear conditioning, a form of fear conditioning which requires both the amygdala and the hippocampus, lead to an increase in glutamate uptake in area CA1 of the hippocampus [61].

Taking into account we could hypothesize that the higher glutamate uptake by hippocampal slices in LH compared to NLH immediately after the test session, observed in the present work, could indicate that this increase during the contextual fear can be achieve right after the LH paradigm. Also, this effect seems to be unspecific promoted by shock exposure or learning events, since no modulation was observed neither in animals submitted only to inescapable shock nor to escapable shock session. In addition, our results show that the hippocampus is the brain structure more sensitive to this protocol compared to cortex and striatum, concerning the differences on glutamate uptake between LH and NLH rats. There are several studies in the literature that investigated the hippocampal consequences after the development of depressive-like behavior in rats. Profound structural changes have been observed in hippocampal neurons of animals exposed to depression paradigms—like atrophy of apical dendrites of pyramidal neurons in CA3 hippocampal region [62–64], atrophy in granule and CA1 pyramidal cells and synaptic loss [65] as well as a significant diminution of light

neurofilaments of the cytoskeleton in hippocampal neurons [66].

In our study we also investigated the glutamate uptake in LH animals 21 days after the protocol. This time was choose because Ferrero et al. [67] in one of the few studies about the involvement of the glutamatergic system after the development of LH behavior in rats, reported that the potassium-evoked glutamate release in hippocampal slices of LH rats 21 days after the test session are lower than the NLH rats. Our data show that the LH hippocampal glutamate uptake also decreased in this time, as well as in striatum and parietal cortex.

It is also important to highlight that in the present study it was not addressed the type(s) of glutamate transporter(s) involved in the observed modulation of glutamate uptake. Considering that the neurobiology of depression is influenced by the glutamatergic signal transduction, Zink et al. [68] published a study analyzing the genotype- and phenotype-associated alterations (trait and state markers, respectively) of glutamate transporters expression on established genetic strains with congenital likelihood to helpless (cLH) or not helpless (cNLH) behaviours. Their results showed that LH male animals presented a significant reduced expression and immunocontent levels of some glutamate transporters and pointed that there are both trait (genetic factors predisposing to helplessness—reduced EAAT4 in occipital cortex and retrosplenial granular cortex) and states (which can be induced in vulnerable animals by stress—reduced GLT-1 in hippocampal subregions CA1, CA3 and dentate gyrus, plus occipital and retrosplenial granular cortex) markers of depressive-like behavior. As glutamate uptake observed in hippocampal slices is mainly attributed to GLT-1 [69], and the present study investigated the time-course of glutamate uptake after the onset or not of depressive-like behaviour (phenotype alterations), the decrease expression and immunocontent of GLT-1 observed by Zink group could point to the molecular basis of our finding, concerning the glutamate uptake. All these glutamatergic alterations suggest the involvement of the glutamatergic system in the depressive-like behavior, in both male and female rats.

Summarizing, our results showed that the glutamate uptake is different between LH and NLH animals immediately after the test session and as long as 21 days later, mainly in hippocampus. These results indicate that a persistent modulation of the glutamate uptake mechanism is occurring after the development of LH behavior. However, few studies have investigated others parameters of the glutamatergic system after the onset of LH behaviors; therefore more studies are necessary to better clarify the contribution of mechanisms involved in the modulation of glutamate transporters in this depressive-like behavior model. Finally, a better understanding of the glutamatergic system involvement in this model may lead to new insights into the pathophysiology of the depression disorder and conceivably to new pharmacological antidepressants.

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Capítulo II

Systemic administration of GMP induces anxiolytic-like behavior in rats

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Systemic administration of GMP induces anxiolytic-like behavior in rats

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ABSTRACT

The glutamatergic system has received considerable attention over the last few years as potential target to develop anxiolytic drugs. Guanine based purines (GBPs) play an important neurmodulatory effect in the glutamatergic system. Several studies have shown the ability of the GBPs to reduce glutamatergic activity. In the present study, we investigated the anxiolytic effect of GBPs — by Guanosina Monophosphate (GMP) administration — in rodents. Adult male Wistar rats were pretreated with GMP (10, 25, 50, 100 and 150 mg/kg: i.p.); or saline (NaCl 0.9%; i.p.) (control); or, diazepam (2 mg/kg: i.p.) (positive control). One hour after the injection, the anxiety-related behaviors for each animal was evaluated in the light/dark, elevated plus-maze, and open field tasks. Additionally, purines concentration in the cerebrospinal fluid (CSF) was verified. The administration of 25 and 50 mg/kg GMP was able to promote anxiolytic-like behavior, in the light/dark and elevated plus-maze task, similar to diazepam effect. However, no changes in the open field task, or CSF purines concentration were found for either GMP or diazepam treated animals, when compared with saline group. Thus, this study suggests that acute administration of GMP was able to decrease the levels of anxiety in classical behavioral tasks.

1. Introduction

Anxiety disorders are common psychiatric diseases in medical practice. Anxiety is characterized by autonomic symptoms, such as, tachycardia, sweating, dyspnea, associated with feeling of fear, discomfort or psychological stress, causing great pain to the patients (Hoffman and Mathew, 2008).

The pathophysiological mechanisms underlying anxiety remain relatively obscure. However, different neurotransmitter seems to be involved. It is widely accepted that the GABAergic system plays an important role in this disorder (Nutt and Malizia, 2001). Benzodiazepines, which act by increasing the inhibitory GABAergic neurotransmission, have been established as a standard treatment for anxiety. However, their sedative-hypnotic effects, muscle relaxant properties, and memory impairing effects may limit their therapeutic application (Hoffman and Mathew, 2008). Therefore, the search for new effective pharmacological targets to treat anxiety with less adverse side effects has gained attention.

The glutamatergic system has received considerable attention over the last years as potential target to develop anxiolytic drugs (Bergink et al., 2004; Chojnacka-Wojcik et al., 2001; Cryan et al., 2003; Kapus et al., 2008; Palucha and Pilc, 2007). Glutamate, the main excitatory neurotransmitter in mammalian central nervous system (CNS), is essential for brain activity, modulates brain plasticity (such as, learning and memory), pain, and several brain responses to external stimuli (Izquierdo et al., 2006; Ozawa et al., 1998; Schmidt et al., 2008; Segovia et al., 2001). However, overstimulation of the glutamatergic system, caused by excess of extracellular glutamate levels (excitotoxicity), is implicated in various acute and chronic brain diseases, including neurodegenerative disorders, traumatic brain injury, cerebral ischemia, and seizures (Lipton and Rosenberg, 1994; Maragakis and Rothstein, 2006; Meldrum, 1994; Sheldon and Robinson, 2007). In addition, it is also widely accepted that the glutamatergic system is involved in the pathophysiology of some psychiatric disorders, such as anxiety and depression (Bergink et al., 2004; Cryan et al., 2003).

The glutamate acts on the ionotropic (ion channels — NMDA, AMPA and KA) and metabotropic (mGlu — coupled to G proteins) receptors (Maragakis and Rothstein, 2006; Ozawa et al., 1998; Sheldon and Robinson, 2007). NMDA receptors uncompetitive and competitive antagonists, as well as AMPA receptor non-competitive

Abbreviations: ADO, adenosine; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; BBB, blood brain barrier; CNS, central nervous system; CSF, cerebrospinal fluid; Dz, diazepam; GABA, γ-aminobutyric- acid; GBPs, guanine based purines; GDP, guanosine diphosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; GUO, guanosine; HIPOX, hypoxanthine; HPLC, high-performance liquid chromatography; iGluR, ionotropic glutamate receptor; IMP, inosine monophosphate; INO, inosine; mGluR, glutamate metabotropic receptor; MK-801, dizocilpine; NaCl, sodium chloride; NMDA, N-methyl-D-aspartate; RA, risk assessment behavior; AU, uric acid; XANT, xanthine.

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antagonists can profoundly block anxiety-like behavior in rodents (Bergink et al., 2004; Chojnacka-Wojcik et al., 2001; Kapus et al., 2008; Kehne et al., 1991; Plaznik et al., 1994). In addition, selective antagonist of some subtypes of mGluR receptors can also display anxiolytic-like effects in rodents (Chojnacka-Wojcik et al., 2001; Palucha and Pilc, 2007; Spooren and Gasparini, 2004; Tatarczynska et al., 2001a,b). These studies indicate that anxiolytic-like effect can be achieved by blocking glutamatergic neurotransmission and that hyper stimulation of the glutamatergic transmission can be associated with anxiety related behavior. Thus, new drugs able to antagonize the glutamatergic neurotransmission could be effective to treat anxiety (Bergink et al., 2004; Palucha and Pilc, 2007; Spooren and Gasparini, 2004; Tatarczynska et al., 2001a,b).

Over the past 10 years, our group have shown that guanine-based purines (GBPs), such as, the nucleotides GTP, GDP, GMP and the nucleoside guanosine (GUO) exhibit important neuromodulatory function, including the extracellular antagonism of glutamatergic system (Schmidt et al., 2007). In vitro studies demonstrated that GBPs prevent cellular responses evoked by glutamate (Paz et al., 1994; Regner et al., 1998; Tasca et al., 1995), and stimulate glutamate uptake (Frizzo et al., 2002, 2003). In vivo studies had shown that GUO, administered either intraperitoneally (i.p.) or orally (p.o.), prevented seizures induced by the glutamatergic agents (quinolinic acid (QA), kainate and α-dendrotoxin) in rodents. These data suggest the neuroprotective role of GBPs in events caused by the glutamatergic excitotoxicity (Lara et al., 2001; Schmidt et al., 2000, 2007; Soares et al., 2004; Vinadé et al., 2003, 2005).

Behavioral experiments performed by our group demonstrated that GUO exerted an amnesic effect on inhibitory avoidance task in rats (Roesler et al., 2000; Saute et al., 2006; Vinadé et al., 2003, 2005). On the other hand, no obvious motor disturbance or sedative effects were observed after acute or chronic administration of GBPs, as evidenced with other glutamate antagonists such as MK-801 (Lara et al., 2001; Tort et al., 2004; Vinadé et al., 2003). In addition, GUO exhibited an anxiolytic effect in mice tested by the hole board task when administered ad libitum in the water for two weeks (Vinadé et al., 2003).

Considering the effects of GBPs on the glutamatergic system and the involvement of the glutamate in the pathophysiology of anxiety, the main objective of this study was to evaluate the effects of GMP on behavioral models of anxiety in rats.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (60–90 days old, weighing 250–350 g) were kept under a 12-hour light/dark cycle (light on at 7:00 AM) at constant temperature of 22 ± 1 °C. They were housed in plastic cages (5 per cage) with water and commercial food ad libidum. All behavioral procedures were conducted between 9:00 AM and 5:00 PM. All experimental procedures were performed in accordance with the Brazilian Society for Neuroscience and Behavior's recommendations for animal care.

The animals were handled in the same room during the 6 days preceding the experiment. At the day of behavioral task, the animals were acclimatized in the testing room for 1 h before the task.

Each animal was used once, because our previous observation that, 2 sequential different experiments with the same animal, promoted behavioral disturbances like anxiety-related behaviors (data not shown).

2.1.1. Chemicals

Guanosine monophosphate (GMP) was obtained from Sigma Chemicals (St. Louis, MO, USA). Diazepam was purchased from União Química Nacional S/A (Pouso Alegre, MG, Brazil). GMP was dissolved in saline 0.9% and diazepam was dissolved in saline solution containing 0.5% Tween 80. The anesthetic sodium thiopental was obtained from Cristália (Itapira, SP, Brazil).

2.1.2. Drugs administration

The animals were divided into the following groups: Saline (NaCl 0.9%) (control group); GMP 10, 25, 50, 100 and 150 mg/kg (GMP group); and diazepam 2 mg/kg (positive control group). The diazepam dose was chosen based on dose response curve performed prior the study (data not shown). The standard anxiolytic compound diazepam was employed as a positive anxiolytic control in all experiments. All groups received a 1 mL/kg intraperitoneally (i.p.) administration of the drugs 1 h before each behavioral task. To minimize the interference of the natural variability of the animals on the tasks, all plastic cages contained 1 animal that received saline, other that received diazepam and 3 animals that received different doses of GMP.

2.2. Behavioral tasks

2.2.1. Light/dark task

The light/dark task was performed as previously described (Crawley and Goodwin, 1980) with some modifications. The light/dark apparatus consisted of an acrylic rectangular box with two separated chambers. One chamber had black walls and floor, with size of 210 × 350 × 410 mm (height × length × width) and was not illuminated. The other side had white walls and floor, with size of 210 × 450 × 410 mm (height × length × width) and illuminated by a 100 W white lamp overhead. The two compartments were separated by a wall, which had a small opening (80 × 50 mm, height × length) at floor level. For each experiment, the animal was placed in the white chamber and allowed to explore the two-chamber area for 5 min. The following parameters were recorded by a trained and blinded-to-treatment observer: number of transitions between the two chambers, time spent in the light chamber, latency time to enter for the first time in dark chamber and the risk assessment behavior (RA, i.e., the number of times the animal in the dark compartment explored the light compartment). After each experiment, the apparatus was cleaned with alcohol 70° and dried before the next animal.

2.2.2. Elevated plus-maze task

The elevated plus-maze was performed as previously described (Pellow et al., 1986). The elevated plus-maze apparatus, entirely made of wood, consisted of two open arms (50 × 10 cm, length × width) and two enclosed arms (50 × 10 × 40 cm; length × width × height) separated by a central platform (5 × 5 cm; length × width) arranged so that the two identical arms of each type were opposite to each other. The height of the maze was 70 cm, and the experiments were conducted under dim red light in a quiet room. The animals were placed individually on the central platform of the plus-maze facing an open arm, and observed individually for 5 min by a trained and blinded-to-treatment observer. The number of transitions between the open and enclosed arms and of the total arm entries, and the time spent into open arms were recorded. After each session the apparatus was cleaned with alcohol 70° and dried before the next animal.

2.2.3. Open field task

Exploratory activity, locomotor activity and anxiety-like behavior of rats were evaluated during an open field session of 8 min (the first 3 min was considered as a measure of novelty exploratory activity and the last 5 min as locomotor activity). The test was performed by placing each individual animal without previously habituation in the center of a square arena (50 × 50 × 50 cm, length × width × height) with a black floor and walls. All sessions were recorded by a video-camera (positioned above and at ca. 90° to the square arena) connected to a monitor. Videotapes were blinded scored by a trained

observer using dedicated software (ANY-maze®). The videos were subsequently placed in randomized order in a separate ANY-maze protocol for a trained observer to score using a keyboard-based behavioral tracking system, blinded to the treatment group. The time spent in the inner area of the apparatus was considered as a anxiolytic-like behavior. After each session the apparatus was cleaned with alcohol 70° and dried before the next animal.

2.3. Cerebrospinal fluid (CSF) purines analysis

2.3.1. Cerebrospinal fluid (CSF) sampling

To evaluate whether changes in CSF purines profile could be correlated with the observed anxiolytic-like effect, we measured the CSF purines concentration on the GMP or diazepam treated animals. One hour after receiving GMP (50 and 150 mg/kg; i.p), saline (NaCl 0.9%; i.p.) or diazepam (2 mg/kg; i.p), the rats were anesthetized with sodium thiopental (40 mg/kg, 1 mL/kg, i.p.), and placed in a stereotaxic apparatus. The CSF was collected (40 to 80 mL) by direct puncture of the cisterna magna with an insulin syringe (27 gauge× 1/2-inch length). Individual samples with visible blood contamination were discarded. All samples were centrifuged at 10.000 g at 4 °C in an Eppendorf centrifuge for 10 min to obtain cell-free supernatants and then stored in single tubes at −70 °C.

2.3.2. High-performance liquid chromatography (HPLC) procedure

HPLC was performed with aliquots obtained from the CSF cell-free supernatants to measure purines levels. The measurement was done as described previously (Schmidt et al., 2009). The levels of the following purines were determined: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (ADO), guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine (GUO), inosine monophosphate (IMP), inosine (INO), hypoxanthine (HIPOX), xanthine (XANT) and uric acid (AU). Analyses were performed with the Shimadzu Class-VP chromatography system, consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto injector valve with 50 mL loop and a UV detector (Shimadzu, Kyoto, Japan). Separations were achieved on a Supelco 250 mm×4.6 mm, 5 μm particle size column (Supelco, St Louis, MO, USA). The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24 °C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 10 μl were injected into the injection valve loop. Absorbance was read at 254 nm. CSF concentrations of purines are expressed as mean ± SEM in micromoles.

2.4. Statistical analysis

Statistical analysis among groups were performed by one way ANOVA, plus the Tukey post hoc test if the variances of the data were homogenous, or Kruskal–Wallis analysis of variance followed by a Mann–Whitney *U* test when the variances of the data were not homogenous. All differences with $P<0.05$ were considered significant.

3. Results

3.1. Light/dark task

Animals treated with diazepam (2 mg/kg; i.p.) or GMP (25 and 50 mg/kg; i.p) exhibited a significant increase in the total exploration time on the light compartment of the apparatus (Fig. 1A, $P<0.05$). The number of transitions between the light and dark compartments was also increased in the diazepam treated animals and in the GMP group that received 50 mg/kg (Fig. 1B, $P<0.05$), when compared with saline group. No changes in the number of transitions were observed for animals receiving GMP 25 mg/kg. However, no changes in the number of RA, or the latency to enter in the dark chamber of the apparatus, was observed for all groups (data not shown).

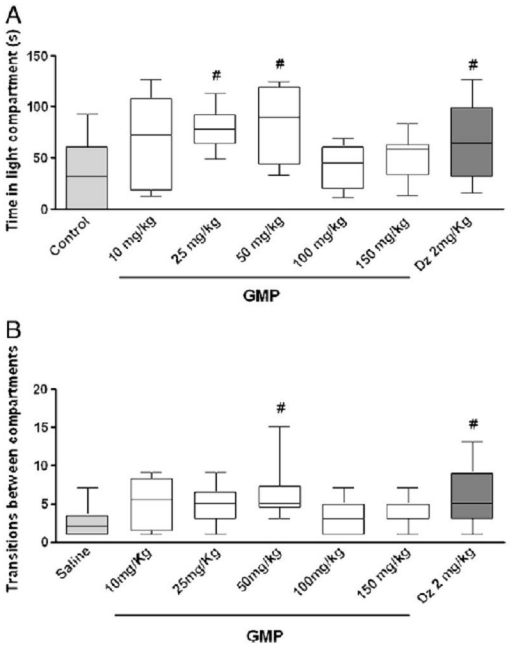

Fig. 1. Light/dark task. Effect of Dz or GMP on the total time spent in the light side (A) and on the total number of transitions (B). Data are reported as medians (interquartile ranges) analyzed by one-way ANOVA, $n = 10$ per group, # $P<0.05$ compared to saline. Dz: Diazepam; GMP: Guanosine Monophospate.

Animals treated with the lowest (10 mg/kg), and the highest (100 and 150 mg/kg) doses of GMP did not exhibit alterations of the analyzed parameters when compared with controls (data not shown).

3.2. Elevated plus-maze task

Animals treated with diazepam (2 mg/kg; i.p.) or GMP (25 or 50 mg/kg; i.p) presented a significant increase in the time spent in open arms, compared with the saline group (Fig. 2, $P<0.05$).

On the other hand, the administration of diazepam or GMP was not able to change the number of entries in open, closed, and total arms, when compared with the saline group (data not shown).These data suggest that diazepam and GMP had no effect on the locomotor activity.

No alterations were observed in animals that received GMP 10 mg/kg, 100 and 150 mg/kg (data not shown).

3.3. Open Field task

I.p. administration of diazepam (2 mg/kg) or GMP (50 and 150 mg/kg) did not modify the total distance traveled by the animals in the 8 min of the open field session (Fig. 3A). There were no

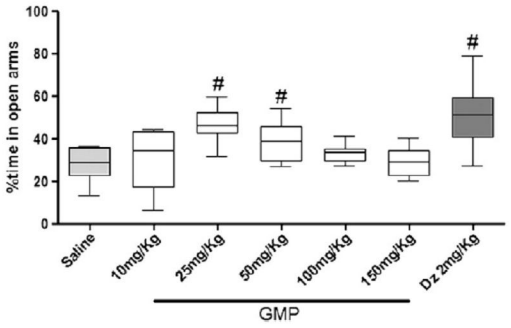

Fig. 2. Elevated plus-maze task. Effect of Dz or GMP on the time spent in open arms. Data reported as medians (interquartile ranges) analyzed by Kruskal–Wallis, followed by Mann–Whitney test, $n = 10$ per group, # $P<0.05$ compared to saline. Dz: Diazepam; GMP: Guanosine Monophospate.

alterations on the exploratory activity in the first 3 min (Fig. 3B) and on the locomotor activity in the last 5 min among groups (Fig. 3C).

No difference was observed in the time spent in the inner area of the open field for all groups (data not shown).

3.4. Cerebrospinal fluid (CSF) analysis

The analysis of the CSF 1 h after the i.p. administration of diazepam (2 mg/kg) or GMP (50 and 150 mg/kg) shows no alteration on the purines concentrations when compared with control group (data not shown).

4. Discussion

In the present study, we investigated the anxiety-like behavior in rats by the light/dark and elevated plus-maze tasks which are the two widely used protocols to predict anxiolytic-like or anxiogenic-like activity in rodents. The light/dark task is based on the innate aversion of rodents to brightly illuminated areas and on their spontaneous exploratory behavior in response to mild stressors, as novel environment and light. The elevated plus-maze is based on the natural aversion of rodents for open and elevated areas, as well as on their natural spontaneous exploratory activity in novel environments (Bourin and Hascoët, 2003; Prut and Belzung, 2003). We also evaluated the anxiety-like behavior in the open field task by measuring the time that the animal remain in the center of the open field arena which could indicate an anxiolytic-behavior effect, since it is well known that rodents prefer to walk close to the walls, a behavior called thigmotaxis.

The observation of an anxiolytic-like behavior in rodents is complex. The extent to which an anxiolytic compound can facilitate exploratory activity depends on the baseline behavior of the control group. Differences between the type and severity of external stressors might account for the variable results reported by different works. In the present study, we used a positive control group (classical anxiolytic drug — diazepam) in all behavioral tasks to establish which parameter(s) could indicate an anxiolytic effect compared with control group. We choose diazepam as a positive control because benzodiazepines are used to treat a broad spectrum of anxiety disorders. For the implementation of our experimental protocol, we changed the time of administration and the anxiolytic doses of diazepam described previously (Bourin and Hascoët, 2003; Prut and Belzung, 2003). We observed that the administration of lower doses of diazepam did not produce any effect on the behavior for all animals, while higher doses were sedative. These emphasize the difficulties to implement a reliable positive control of anxiolytic-like effects. According the dose response curve performed prior the study; the best suitable dose of diazepam to achieve an anxiolytic-like effect was 2 mg/kg, i.p., 60 min before the behavioral task.

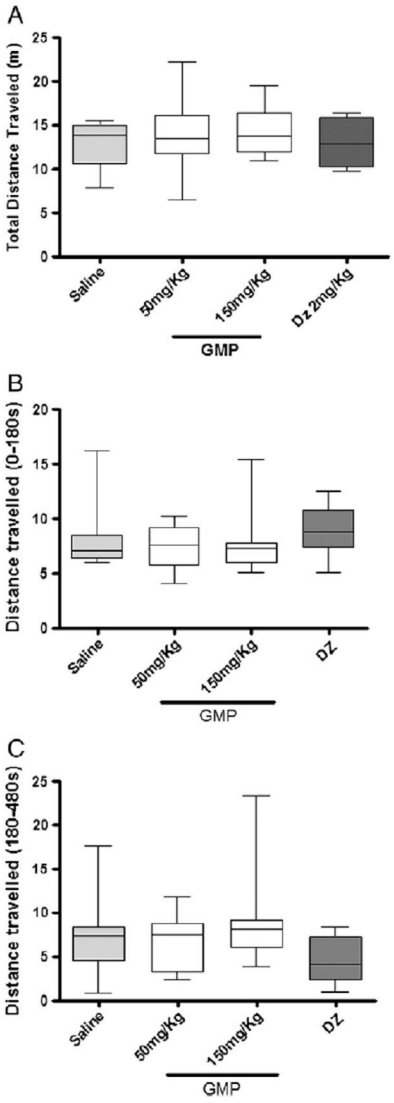

Fig. 3. Open field task. Effect of Dz or GMP on the total distance traveled in 8 min (A), in the exploratory activity in the first 3 min (B), and the locomotor activity in the last 5 min (C). Data reported as medians (interquartile ranges), analyzed by one-way ANOVA, $n = 7–9$ animals per group. No significant differences were observed among the groups. Dz: Diazepam, GMP: Guanosine Monophospate.

The main finding of the present work was that GMP at the dose of 50 mg/kg was able to consistently reproduce the anxiolytic effects of diazepam. On the other hand, in the open field task, neither diazepam nor GMP produced anxiolytic-like behavior. Therefore, predictive

value of this task appears to have limitations to test the anxiolytic effect of these drugs. Additionally, no locomotor activity alteration was observed after GMP or diazepam administration.

Interesting, GMP at intermediate doses (25 and 50 mg/kg) was able to decrease the anxiety levels in the classical behavioral tasks. Since, to our knowledge, no studies about the pharmacokinetics of i.p. injection of GMP were performed, it is difficult to understand why the GMP induces anxiolytic-like behaviors in a bell-shape dose response. Accordingly, more studies focusing the pharmacokinetics of GMP administration may help to clarify the dose-response effects observed in the present work.

The original reason that lead us to investigate whether GMP could induce anxiolytic-like behaviors was based on the fact that GMP can directly, but weakly, antagonizes iGluR (Baron et al., 1989; Mendieta et al., 2005; Paas et al., 1996; Porciúncula et al., 2002; Souza and Ramirez, 1991), and, that the administration of iGluR antagonists can induce anxiolytic-like behaviors in rodents (Bergink et al., 2004; Chojnacka-Wojcik et al., 2001; Kapus et al., 2008; Kehne et al., 1991; Plaznik et al., 1994). Considering that the behavioral changes observed 60 min after GMP administration were not accompanied by the increase in CSF GMP levels, we cannot confirm that the mechanism by which GMP induced anxiolytic-like behavior involved iGluR antagonism.

In addition, as changes in CSF GMP levels were not found, we cannot confirm that GMP crosses the blood brain barrier (BBB). However, organic anion transporter proteins, responsible to transport nucleotides analogs across BBB, were already described (Takeda et al., 2002; Strazielle and Ghersi-Egea, 2005). Thus these proteins could also transport GMP across the BBB. Moreover GMP seems to have a neuromodulatory role on CNS. This was attributed to the neuroprotective and behavioral effects after the intracerebroventricular, intrahippocampal and intrastriatal administration of GMP in rodents (Malcon et al., 1997; Rubin et al., 1996; Saute et al., 2006; Schmidt et al., 2005; Soares et al., 2004).

A previous study from our group reported that GMP (7.5 mg/kg; i.p.) increases CSF GUO levels 30 min after the administration, without altering GMP or adenosine CSF levels (Soares et al., 2004). Therefore, we cannot rule out the possibility that the CSF GBPs level varied before 60 min after GMP administration, causing the modulation of the glutamatergic system, which might be involved with the anxiolytic-related behavior observed.

GMP is metabolized both, systemically and in the CNS (Saute et al., 2006), suggesting that the metabolites of GMP may also be responsible for the anxiolytic-like effects observed. In agreement with this hypothesis, some effects of i.p. injection of GMP, such as, the anticonvulsant effect against QA-induced seizure (Soares et al., 2004) and the amnesic effect (Saute et al., 2006), are dependent on its conversion to GUO. In addition, GMP, through its conversion to GUO, increases glutamate uptake activity in astrocytes (Frizzo et al., 2003) which is the main mechanism to terminate glutamate physiological activity and avoid the glutamatergic excitotoxicity (Danbolt, 2001). As anxiety per se might ultimately arise from a shift towards neuronal hyper excitability (Bergink et al., 2004; Chojnacka-Wojcik et al., 2001; Cryan et al., 2003; Kapus et al., 2008; Palucha and Pilc, 2007), higher glutamate uptake ability might promote anxiolytic-like effect.

Additionally, there is a lack of studies focusing on the interaction between GBPs with other neurotransmitter systems besides glutamate. So, since the mechanisms underlying anxiety involves other neurotransmitters, the exactly mechanism by which GMP exert the anxiolytic effects needs more investigation.

To our knowledge, this is the first study showing anxiolytic-like effects after systemic administration of GMP in classical behavioral tasks. As GMP is an endogenous compound, apparently well tolerated with minor toxicity, it could eventually be developed as a new drug for anxiety treatment.

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Capítulo III

Chronic GMP oral administration increases the resistance of the mouse brain cortex to oxygen and glucose deprivation: correlation with glutamatergic system modulation

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Keywords: GMP, guanine based purines, glutamatergic system, excitotoxicity, oxygen and glucose deprivation, neuroprotection

Abstract:

Ischemic stroke is a major cause of death and illness in the Western world. In cerebral ischemia, the extracellular levels of glutamate rapidly increase due to disruption of brain aerobic metabolism and ATP production, leading to neural damage. Several in vitro and in vivo studies reported that the guanine based purines (GBPs), namely the nucleotides guanosine tri- di and -monophosphate (GTP, GDP and GMP) and the nucleoside guanosine may exert antiglutamatergic effects, with putative neuroprotective roles against excitotoxicity. In the present work, we aimed to investigate the neuroprotective and neuromodulatory potential of in vivo GMP treatment. Thus, mice were orally administered for 3 weeks with GMP 1.5 mg/kg or saline. GMP administration protected cortical slices against oxygen and glucose deprivation (OGD) insult and promoted functional and structural changes in basal glutamatergic system. GMP administration promoted a decrease in the cortical glutamate binding and uptake, as well as a decrease in the levels of ionotropic receptors subunits for NMDA and AMPA (NR2A/B and GluR1, respectively), the post synaptic protein PSD-95, and the glutamate transporters EAAC1 and GLT-1. GMP administration increased the CSF level of GMP and its metabolites and the serum GMP hydrolysis. Taken together, in vivo GMP administration increased the resistance of the mouse brain cortex to OGD and modulated the cortical glutamatergic system. The putative correlations among the neuroprotective effect and the neurochemical glutamatergic changes caused by GMP are discussed.

1. Introduction

Ischemic stroke is a major cause of death and illness in the Western world (Goldstein et al., 2006; Murray and Lopez, 1997). An interruption of blood supply to the brain in an ischemic stroke results in oxygen and glucose deprivation and thus, in a reduction in energy source available to neural cells (Chao et al., 2010; Dirnagl et al., 1999). Particularly neurons become unable to maintain the ionic gradients necessary for cellular function and homeostasis, which result in excessive neuronal depolarization and release of excitatory neurotransmitters, as well as in reduced neurotransmitter re-uptake from the extracellular space. This process ultimately leads to excessive cellular accumulations of Ca^{2+} ions which, in turn, lead to the overactivation of several deleterious enzymes and signaling processes that impair neural cells function (Dirnagl et al., 1999; Szydlowska and Tymianski, 2010). Glutamate is known to play a predominant role in the pathogenesis of ischemia brain injury (Chao et al., 2010).

Glutamate is the main excitatory neurotransmitter in the mammalian Central Nervous System (CNS) and it is essential for the normal brain functions. Glutamate acts via ionotropic (ligand gated ion channel: NMDA, KA and AMPA receptors) and metabotropic (coupled to G proteins) receptors, modulating several plastic brain processes, such as learning and memory, development and aging, and adaptation to the environment (Beart and O'Shea, 2007; Danbolt, 2001; Izquierdo et al., 2006; Segovia et al., 2001).

However, overstimulation of the glutamatergic system when extracellular glutamate levels increase over the physiological range (excitotoxicity), is involved in various acute (such as ischemia and seizure) and chronic brain

(such as epilepsy and Alzheimer diseases) diseases (Dong et al., 2009; Lau and Tymianski, 2010; Maragakis and Rothstein, 2004; Rossi et al., 2000; Tzingounis and Wadiche, 2007). The maintenance of extracellular glutamate concentrations below neurotoxic levels is achieved through the high affinity sodium-dependent glutamate transporters activity (Anderson and Swanson, 2000; Beart and O'Shea, 2007). To date, five different mammalian glutamate transporters have been cloned: GLAST (Storck et al., 1992) and GLT-1 (Pines et al., 1992), quantitatively the main glutamate transporters and responsible for most of the glutamate uptake activity (Anderson and Swanson, 2000; Danbolt, 2001; Maragakis and Rothstein, 2006), are predominantly localized in astrocytes (Lehre and Danbolt, 1998; Rothstein et al., 1994); EAAC1 (Kanai and Hediger, 1992), EAAT4 (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997) are localized in neurons (Gegelashvili and Schousboe, 1998). Notably, EAAT4 and EAAT5 are specifically located in cerebellar Purkinje cells and in retina, respectively.

Current evidences indicate that the guanine-based purines (GBPs), namely the nucleotides guanosine tri-, di- and mono-phosphate (GTP, GDP and GMP, respectively) and the nucleoside guanosine (GUO) modulate the glutamatergic activity, thus they are able to be neuroprotective against excitotoxicity (for review see Schmidt et al., 2007; Schmidt and Souza, 2010).

Concerning the nucleotide GMP, several studies reported that this nucleotide is able to inhibit the binding of glutamate and its analogs to brain membrane preparations (Baron et al., 1989; Butcher et al., 1986; Hood et al., 1990; Mendieta et al., 2005; Monahan et al., 1988; Paas et al., 1996; Sharif and Roberts, 1981), to prevent cell responses to glutamate (Aleu et al., 1999;

Burgos et al., 1998; Burgos et al., 2000; Burgos et al., 2003; Paz et al., 1994; Regner et al., 1998; Tasca et al., 1995; Tasca et al., 1998; Tasca et al., 1999), to stimulate glutamate uptake by astrocytes (Frizzo et al., 2003), and to present neuroprotective effects in several brain preparations submitted to excitotoxic conditions (Molz et al., 2005; Oleskovicz et al., 2008; Oliveira et al., 2002). *In vivo*, GMP administration presents amnesic, anxiolytic and antinociceptive effects in rodents (Almeida et al., 2010; Saute et al., 2006; Schmidt et al., 2008). Additionally, GMP prevents seizures and striatal neuronal damage promoted by quinolinic acid, a hyper stimulator of the glutamatergic system (Malcon et al., 1997; Schmidt et al., 2000; Schmidt et al., 2005; Schmidt and Souza, 2010; Soares et al., 2004). Noteworthy, most of the GMP effects depend on its conversion to GUO, including the stimulation of the astrocytic glutamate uptake, and the amnesic, antinociceptive and anticonvulsant effects (Frizzo et al., 2003; Saute et al., 2006; Schmidt et al., 2008; Soares et al., 2004).

Several works have reported that GMP and GUO promote neuroprotective effects on different experimental models of cerebral ischemia. Hippocampal slices submitted to oxygen glucose deprivation (OGD) followed by reperfusion have their viability restored when GMP or GUO is added to the incubation medium (Frizzo et al., 2002; Molz et al., 2005; Oleskovicz et al., 2008; Oliveira et al., 2002; Thomazi et al., 2008). Moreover, GUO systemic administrated prevent the decrease in brain glutamate uptake observed after *in vivo* ischemia in neonatal rats (Moretto et al., 2005; Moretto et al., 2009).

In view of the reported *in vitro* neuroprotective effects of GMP against OGD; this study was designed to further investigate the neuroprotective effect of a chronic oral administration of GMP to mice against an *ex vivo* OGD model.

Attempts have been made to investigate some of the possible involvement of the glutamatergic system that could underlie the neuroprotective effect of GMP.

2.Materials and methods

2.1. Reagents

GMP, N-methyl-D-glucamine, bovine serum albumin, protease and phosphatase inhibitor cocktail and antibodies against glial fibrillary acid protein (GFAP), Snap 25 and Actin were from Sigma Chemicals. Antibody against NR2 A/B (NMDA receptor subunit) was from Chemicon; antibodies against GluR1 (AMPA receptor subunit) was from UpState; antibody against PSD-95 was from Affinity BioReagents; antibody against EAAC1 was from Alpha Diagnostic; antibodies against GLAST (batch Ab#314) and GLT-1 (batch Ab#360) where from the same preparations as previously published (Holmseth, et al., 2009). The horseradish peroxidase-conjugated secondary antibody against rabbit and mouse, and the ECL were from Amersham Pharmacia Biotech. X ray films were from Kodak X-Omat, Rochester, NY, USA. L-[^{3}H] glutamate (specific activity 30 Ci/mmol) was from Amersham International, UK. The anesthetic sodium thiopental was from Cristália (Itapira, SP, Brazil). All other chemicals were of analytical grade or higher.

2.2. Animals

Male adult Swiss albino mice (3–4 months of age, 30–40 g) were kept on a 12 h light/dark cycle (light on at 7:00 am) at temperature of 22 ± 1 °C, housed in plastic cages (4 per cage) with commercial food *ad libitum*. All procedures were carried out according to the Brazilian Society for Neuroscience and

Behavior's recommendations for animal care and the US National Institutes of Health guide for the care and use of laboratory animals, designed to minimize the suffering and the number of animals used. All behavioral procedures were conducted between 3:00 and 6:00 PM.

2.3. Treatment

The mice received water (control group) or GMP solution (1.5 mg/ml) for 1 or 3 weeks *ad libitum* from the bottle water. The GMP dose used was based on a previous pilot study, which indicated that GMP administration for 3 weeks with a lower (0.5 mg/mL) or a higher (3 mg/mL) dose had none or the same effect of the dose here used, respectively, on the viability of cortical slices submitted to OGD (data not shown). The water consumption was monitored every 2 days.

2.4. Behavioral parameters.

The animals were weighed every 2 days during the period of the treatment. The motor coordination and the rectal temperature of the animals were analyzed after 1 or 3 weeks by the rotarod test and with a rectal probe, respectively, as previously described (Vinade et al., 2003).

2.5. *Ex vivo* and *in vitro* experiments

The GMP effects were evaluated on the brain cortical preparations, based on previous studies indicating that this brain structure is responsive to guanine-based purines (GBPs) (Frizzo et al., 2002; Thomazi et al., 2004).

Briefly, the animals were decapitated, their brains immediately removed and the cerebral cortices were dissected onto Petri dishes. Cortices from both brain hemispheres from each animal were used. The right one was micro sliced and the left one was wholly frozen at -20 ^{0}C in 25 mM Hepes buffer (pH 7.4), containing 0.1% SDS and a protease and phosphatase inhibitor cocktail.

The cortical coronal slices from the parietal area (0.4 mm) were obtained using a McIlwain tissue chopper, as described previously (Thomazi et al., 2004). The slices were used for OGD or glutamate uptake assay.

The intact cortices were used up to 1 month after decapitation, to isolate plasma membrane or to analyze the immunocontent of specific proteins.

The animals were randomly assigned to the experiments.

2.5.1. *Ex vivo* ischemic model

2.5.1.1. Oxygen and glucose deprivation (OGD)

Six slices were used from each animal. The cortical slices were separated in 2 equal sets (control and OGD group), placed into separated 24-well culture plates, and pre-incubated for 30 min at 37°C in the modified Krebs–Henseleit solution containing (in mM): 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$; 25 HEPES and 5.5 glucose (pH 7.2). After preincubation, the medium in the control plate was replaced with a new modified Krebs–Henseleit solution and the plate was maintained at 37°C for 60 minutes. For ischemic conditions, the slices were exposed to a model of OGD, as previously described (by Strasser and Fischer, 1995), with some modifications (Moreira et al., 2009). Briefly, after pre-incubation, OGD slices were washed twice with modified Krebs-Helseleit

glucose free solution and incubated for 60 min (OGD period) at 37°C in a chamber saturated with nitrogen.

After the 60 min period, the media from both control and OGD slices were replaced by incubation solution (with glucose) and cellular viability was immediately verified. The period of 60 minutes of was chosen after a time curve of OGD, evaluating cell viability by the MTT assay (data not shown).

2.5.1.2. Analysis of cellular viability (MTT assay)

Cellular viability assay was performed by the colorimetric [3(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) method. After the 60 min period, both control and OGD slices were incubated with 0.5 mg/mL of MTT at 37 °C for 20 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide (DMSO) and measured at 560 and 630 nm. Only viable cells are able to reduce MTT.

2.5.2. Glutamate uptake

Glutamate uptake was assessed as fully detailed elsewhere (Thomazi et al., 2004). For each animal, 6 slices were used. The cortical slices were separated in 2 equal sets, placed into separated 24-well culture plates, one plate was maintained at 37°C and the other on ice, for evaluation of total and Na^+-independent glutamate uptake, respectively, with HBSS solution containing (in mM): 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 5.5 glucose (pH 7.2). For the total glutamate uptake, the slices were washed once with 1 mL of 37°C HBSS, and then pre-incubated at 37°C for 15 min, followed by the addition of 0.33 Ci/mL L-

[^{3}H]glutamate and 100 uM (final concentration) glutamate. Incubation was stopped after 7 min with 2 ice-cold washes of 1mL HBSS, immediately followed by the addition of 0.5N NaOH, which were then kept overnight. Na^{+}-independent uptake was measured using the same protocol described above, with differences in the temperature (4°C) and medium composition (N-methyl-d-glucamine instead of sodium chloride). Results (Na^{+}-dependent uptake) were considered as the difference between both uptakes. The incorporated radioactivity was measured in a Wallac 1409 liquid scintillation counter.

2.5.3. [^{3}H] Glutamate binding to brain plasma membranes

2.5.3.1 Brain plasma membrane preparations

Plasma membrane preparations were carried out as previously described (Martini et al., 2007). The frozen isolated parietal cortices were thawed at room temperature and all subsequent isolation procedures were performed at 4°C. The cortices were homogenized in 0.32 M sucrose (10% w/v) and centrifuged twice at 1000 x g, both supernatants were joined and centrifuged at 11,000 × g for 20 min. The pellet was osmotically shocked by resuspension in 5 mM Tris/HCl pH 7.4 for 30 min. Following, the resuspended pellet was centrifuged at 60,000 x g in a sucrose density gradient (49 %, 28.5 % and 10 %). The plasma membranes were collected from the interface between 49 % and 28.5 % sucrose layer, which were then washed twice in Tris/HCl buffer at 18,000 x g for 15 min.

2.5.3.2 [^{3}H] Glutamate binding protocol

The binding assay of [^{3}H] glutamate was performed at 35°C in small polycarbonate tubes (total incubation volume 500 uL) containing 10 mM Tris/HCl pH 7.4 and 40 nM [^{3}H] glutamate. Incubation was started by the addition of plasma membrane preparation (50 µg protein). After 30 min, tubes were centrifuged at 16,000 x g for 10 min at 4°C. The supernatant was discarded, and the walls of the tubes and the pellets surface were quickly and carefully rinsed with cold distilled water. The pellets were solubilized with 0.3 mL of NaOH 0.1 M overnight. Bound radioactivity was measured by using a Wallac scintillation counter. Unspecific binding (10–20% of the total binding) was determined by adding 200 µM non radioactive glutamate to the medium in a parallel assay. Specific binding was considered as the difference between total binding and unspecific binding. Experiments were performed in triplicate.

2.5.4. Western blotting proteins analysis

For Western blotting analysis, the frozen isolated parietal cortices were thawed at room temperature and then homogenized in 25 mM Hepes buffer (pH 7.4) containing 0.1% SDS and a protease and phosphatase inhibitors cocktail and normalized with a sample buffer (4% sodium dodecylsulfate, 2.1 mM EDTA, 50 mM Tris and 5 % β-mercaptoethanol). For each protein evaluated, a concentration curve (5-50 ug) was made in the same gel to determine the adequate amount of protein to be used in the experiments; accordingly, samples (30 µg protein/well), with the exception for glutamate transporters (20 µg protein/well), were subjected to electrophoresis and transferred to a nitrocellulose membrane. Membranes were processed as follows: (1) blocking with 5% bovine serum albumin for 2 h; (2) incubation with primary antibody

overnight; (3) incubation with horseradish peroxidase conjugated secondary antibody for 2 h; (4) chemioluminescence (ECL) was detected using X-ray films. Protein loading was controlled by additionally staining blots with β-actin antibodies. The films were scanned and bands intensity were analyzed using ImageJ (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

2.6. Cerebrospinal fluid (CSF) purine levels measurement

2.6.1. Cerebrospinal fluid (CSF) sampling

For CSF purines concentration measurement, we used another animals not used in the other experiments. Mice were anesthetized with sodium thiopental (60 mg/kg, 10 ml/kg, i.p.) and placed in a stereotaxic apparatus, where the CSF was drawn (10–20 µl per mouse), with the help of a magnifying glass, by direct puncture of the cisterna magna with an insulin syringe (27 gauge × 1/2 in length). All samples were centrifuged at 10.000 x g in an Eppendorf centrifuge for 10 min at 4°C and the supernatant was frozen at -70°C until analysis.

2.6.2. High-performance liquid chromatography (HPLC) procedure

HPLC was performed with CSF cell-free supernatants aliquots for determination of purines concentration (Schmidt et al., 2009). CSF concentrations of the following compounds were determined: GTP, GDP, GMP, GUO, xanthine (XAN), and uric acid (UA). Analyses were performed with Shimadzu Class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu

SIL-10AF auto injector valve with 50 µl loop, and an UV detector, 254 nm. Separations were achieved on a Supelco C18 250 mm × 4.6 mm, 5 µm particle size column. The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24°C. Buffers composition remained unchanged (A: 150 mmol/l phosphate buffer, pH 6.0, containing 150 mmol/L KCl; B: buffer A with 15% acetonitrile). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 10 µl were injected into the injection valve loop.

2.7. Measurement of serum GMP hydrolysis

Serum GMP hydrolysis was determined evaluating the inorganic phosphate (Pi) released (Oses et al., 2004). The reaction mixture containing GMP (3.0 mM) as a substrate, in 10 mM Tris–HCl, pH 7.5, was incubated for 40 min with 1.0–1.5 mg serum protein per tube at 37°C in a final volume of 0.2 ml. The reaction was stopped by the addition of 0.2 ml 10% trichloroacetic acid (TCA). The Pi released was determined as previously described (Chan et al., 1986). Controls to correct for non-enzymatic hydrolysis were performed by adding the serum after stopping the reaction with TCA. All samples were assayed in triplicate. Enzyme activities were expressed as nmol Pi released per min per mg of protein.

2.8. Protein determination

Protein concentration was determined by the method of Lowry (Lowry et al., 1951) for glutamate uptake and by the Coomassie Blue method (Bradford, 1976) for the others parameters. Bovine serum albumin was used as standard.

2.9. Statistical analysis

The data obtained are expressed as means ± S.E.M. of mean. As the variances of the data were homogenous (analyzed by the Kolmogorov-Smirnov test), we used two-way ANOVA for analyzing the data from OGD experiment (factors were submission or not to OGD and duration of GMP administration) followed by the Bonferroni post test, and unpaired Student's t-test for analyzes of the others experiments. A $P<0.05$ was considered to represent a significant difference. The number of animals used in each experiment is described in the figure legends.

3. Results

3.1. Effects of chronic GMP oral treatment on behavioral parameters:

Ad libitum 1.5 mg/mL GMP oral treatment had no effects on water and food consumption or body weight during 3 weeks (data not shown). No effects were observed on rotarod task performance, as well as on rectal temperature up to 3 weeks of GMP treatment (data not shown).

3.2. Effects of chronic GMP oral treatment on cell viability (MTT) on cortical slices submitted to OGD:

The Two-Way ANOVA analysis of variance was performed using the submission or not to OGD X duration of GMP administration as factors and the

cell viability (by MTT assay) as the variable. This analysis showed an interaction between the factors [F1.40 = 9.16, P = 0.0005]. As observed in Figure 1, the basal viability of cortical slices was not affected by GMP administration. However, cellular injury caused by OGD was prevented by GMP orally administrated for 3 but not 1 week of GMP administration ($P < 0.05$). Based upon these results, in the further experiments the study was focused on GMP administration for 3 weeks.

3.3. Effects of 3 weeks GMP oral treatment on functional and structural brain cortical glutamatergic system parameters:

To investigate GMP effects on glutamatergic system, firstly it was evaluated the glutamate binding to cortical brain plasma membranes and the glutamate uptake by slices from parietal cortex. GMP administration by 3 weeks caused a decrease in [^{3}H] glutamate binding (Fig. 2A) and in [^{3}H] glutamate uptake (Fig. 2B), compared to control animals ($P < 0.05$).

Since these parameters were altered, it was investigated the immunocontent of several proteins related to the glutamatergic neurotransmission. In parietal cortex samples, oral GMP administration for 3 weeks reduced the immunocontent of the NMDA receptor subunit NR2A/B (to 63 ± 5 %), the AMPA subunit GluR1 (to 69 ± 9 %), and PSD95 (to 73 ± 10 %), compared to control animals (Fig. 3 A, B and C; $P<0.05$). The immunocontent levels of the glutamate transporter proteins EAAC1 and GLT1 were also reduced to 69 ± 7 % and to 86 ± 2 %, respectively, compared to control (Fig. 4 A and B, $P<0.05$). The content of Snap 25 (Fig. 3 D), the transporter GLAST and GFAP (Fig. 4 C and D) were not affected.

3.4. Effects of 3 weeks GMP oral treatment on purinergic parameters:

GMP, GUO, XAN and UA levels increased with GMP treatment (Table 1); the GTP levels were not affected. The CSF GDP content was not reliably detected (data not shown). Furthermore, there was an increase in serum GMP hydrolysis ($P<0.05$, Figure 5).

4. Discussion

In the present study, 3 weeks *ad libidum* GMP 1.5 mg/ml to mice increased the resistance of brain cortex to OGD insult. This neuroprotective effect was accompanied by functional and plastic changes in some parameters of the cortical glutamatergic system.

Glutamatergic excitotoxicity plays a pivotal role in the cellular damage during OGD. Several works have reported that a blockade of glutamate ionotropic receptors or a decreasing in their signaling pathway is neuroprotective against excitotoxic events ((Aarts et al., 2002; Arias et al., 1999; Moskowitz et al., 2010; Sun et al., 2008). Our results show that GMP administration reduced some parameters of glutamate signaling pathway at the same time that it was neuroprotective against OGD. GMP reduced the binding of glutamate and the amount of ionotropic glutamate receptors subunits, NR2A/B and GluR1 - NMDA and AMPA receptors, respectively - and of a protein implicated in connecting NMDA receptors to specific signal transduction pathways, PSD-95; Fig 3 A, B and C). Therefore, it could be speculated that the decrease in the glutamate signaling pathway parameters is involved with the neuroprotection promoted by GMP administration.

In a condition of energy failure, as in an ischemic episode, high levels of glutamate accumulate in the synaptic cleft, mainly due to neuronal EAAC1 transporter reverse activity (Hamann et al., 2002; Rossi et al., 2000) and to increased pre synaptic release (Hazell, 2007). In the present work, GMP treatment reduced the EAAC1 levels (Fig 3A), which could point to an adaptative/neuroprotective response, putatively contributing to a decrease in the extracellular glutamate accumulation during ischemia. However, GMP administration did not affect the levels of Snap25 (Fig 3D), a protein essential in promoting vesicular fusion (Bragina et al., 2007) and involved in glutamate release.

Although EAAC1 plays a pivotal role during ischemia, it has a little contribution to the physiological total glutamate uptake (Danbolt, 2001; Haugeto et al., 1996). In contrast, GLT1 does not contribute significantly to glutamate release or glutamate removal from the extracellular space during ischemia (Hamann et al., 2002; Rossi et al., 2000), while it is the principal transporter responsible for physiological glutamate uptake (Danbolt, 2001; Furness et al., 2008). Noteworthy, GMP reduced the glutamate uptake activity by cortical slices (Fig. 2B) and the levels of GLT1 (Fig 3B). Of note, it has been recently identified that the GLT-1 located at glutamatergic nerve terminals are the main responsible for physiological glutamate uptake despite that 80 % of GLT1 is located in astroglia (Furness et al., 2008). This raises the question if the reduced uptake was due to a selective effect on neuronal GLT1 rather than total (neuronal plus astrocytic) GLT1. In fact, two astrocytes protein markers, the glutamate transporter - GLAST and the cytoskeleton protein - GFAP, were neither altered by GMP treatment (Fig 3 C and D).

Concerning GBPs effects on glutamatergic uptake, our group has several works demonstrating that acute *in vitro* administration of GBPs stimulates glutamate uptake by cultured astrocytes and cortical slices from rats (Frizzo et al., 2002; Thomazi et al., 2004), and *in vivo* administration prevents the decrease in the glutamate uptake after excitotoxic stimuli (de Oliveira et al., 2004; Moretto et al., 2005; Moretto et al., 2009; Vinade et al., 2005). These data point that the reduced glutamate uptake observed in the present work could be looked at as inconsistent, mainly due to the fact that this protocol was neuroprotective against OGD insult. However, this is the first study that investigated the effects of a chronic *in vivo* administration of GMP on glutamate uptake by cortical slices of adult mice (which also affected other glutamatergic parameters), thus pointing that the GBPs effects on glutamate uptake may depends of the experimental models, as the GBP used, the time and administration route used, the general parameters affected, and the protocol for excitotoxic stimuli investigated.

Our results show that the CSF GMP as well as its metabolites levels (Fig 4) increased after 3 weeks of GMP administration. As there is no study in the literature, to our knowledge, about GMP CSF transport across the blood brain barrier, our group is carrying studies aiming to elucidate this topic. In a previous study, we reported that acute GMP administration increases GUO but not GMP CSF level (Soares et al., 2004). Since GMP is derived from GUO (Schmidt et al., 2007), it is possible that the increase in CSF GMP levels here observed came from its conversion to GUO. This hypothesis is reinforced by the fact that serum GMP hydrolysis is increased by the GMP administration (Fig.5), which in turn could increases serum GUO availability to CSF. Furthermore, since several

GMP effects are dependent of its conversion to GUO, we can not rule out the hypothesis that both GMP and GUO are involved with the observed effects caused by GMP administration.

Despite all these effects reported, no obvious side effect was observed, as GMP administration did affect neither the motor coordination, nor the ability to consume food or water, nor the body weight or temperature.

In summary, this study points that GMP administration is able to increase the resistance of the mouse brain to oxygen and glucose deprivation and is also able to modulate some parameters of the cortical glutamatergic system in adult mice, pointing that they are possibly related to each other. As GMP is an endogenous compound apparently well tolerated, it could be developed as a useful drug for brain diseases related to excitotoxicity. This study provides additional evidence on the role of extracellular GMP in the CNS, although its mechanism of action remains unclear. We are continuing to investigate the neuroprotective effects of GMP and the mechanisms underlying these effects.

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6. Legends

Figure 1 – Effects of GMP oral administration or 1 or 3 weeks on cellular viability (MTT assay) of cortical slices submitted to *ex vivo* oxygen glucose deprivation - OGD (B). Data are expressed as means ± S.E.M., n = 12 animals per group. Statistical comparison among groups was performed with TWO-way ANOVA followed by the Bonferroni post test. *Indicates a difference from all others groups at $P<0.05$.

Figure 2 – Effects of GMP oral administration for 3 weeks on glutamate binding to brain cortical plasma membranes (A) and on glutamate uptake by cortical slices (B). Data are expressed as mean ± S.E.M., n = 12 per group for both experiments. Statistical comparison among groups was performed with unpaired student-t test. *Indicates a difference from control group at $P < 0.05$. 100% represents 16.3 ± 1.8 pmol/mg (A) and 0.40 ± 0.02 nmol/mg/min (B).

Figure 3 – Effects of GMP oral administration for 3 weeks on the cortical levels (evaluated by Western blotting) of glutamate ionotropic receptors subunits: A - NMDA (NR2a/b); B – AMPA (GluR1)and of glutamatergic synaptic proteins: C - PSD-95; D – Snap 25. Densitometric analysis of the bands corresponding to each proteins and normalization to actin allowed comparison between the groups. Data are expressed as means ± S.E.M. from 3 independent experiments, n = 6 per group. Statistical comparison between groups was performed with unpaired student-t test. *Indicates a difference from control group at $P<0.05$. Representative Western blot images are shown under

the figures. Protein loading of the gels was controlled by additionally staining blots with β-actin antibodies.

Figure 4 – Effects of GMP oral administration for 3 weeks on the cortical levels (evaluated by Western blotting) of glutamate transporters subunits: A - EAAC1; B – GLT-1; C – GLAST and of astrocytic protein - H – GFAP. Densitometric analysis of the bands corresponding to each proteins and normalization to actin allowed comparison between the groups. Data are expressed as means ± S.E.M. from three independent experiments, n = 6 per group. Statistical comparison between groups was performed with unpaired student-t test. *Indicates a difference from control group at $P<0.05$. Representative Western blot images are shown under the figures. Protein loading of the gels was controlled by additionally staining blots with β-actin antibodies.

Figure 5 - Effects of GMP oral administration for 3 weeks on serum GMP hydrolysis. Data are expressed as means ± S.E.M., n = 12 per group. Statistical comparison between the groups was performed with unpaired student-t test. *Indicates a difference from control group ($P < 0.05$). 100% represents 0.73 ± 0.61 nmolPi/mg/min.

Table 1 – Effects of GMP oral administration for 3 weeks on cerebrospinal fluid (CSF) concentration of purines. Data are expressed as means ± S.E.M., n = 6 per group. Statistical comparison between the groups

was performed with unpaired student-t test. *Indicates a difference from control group ($P < 0.05$).

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Table 1:

Treatment	Control	GMP
CSF purine Concentration (µM)		
GTP	1.41±0.05	1.61±0.06
GMP	0.84±0.02	1.01±0.03*
GUO	0.39±0.02	0.47±0.02*
XAN	2.20±0.04	3.30±0.18*
UA	5.25±0.32	6.43±0.22*

Figure 1

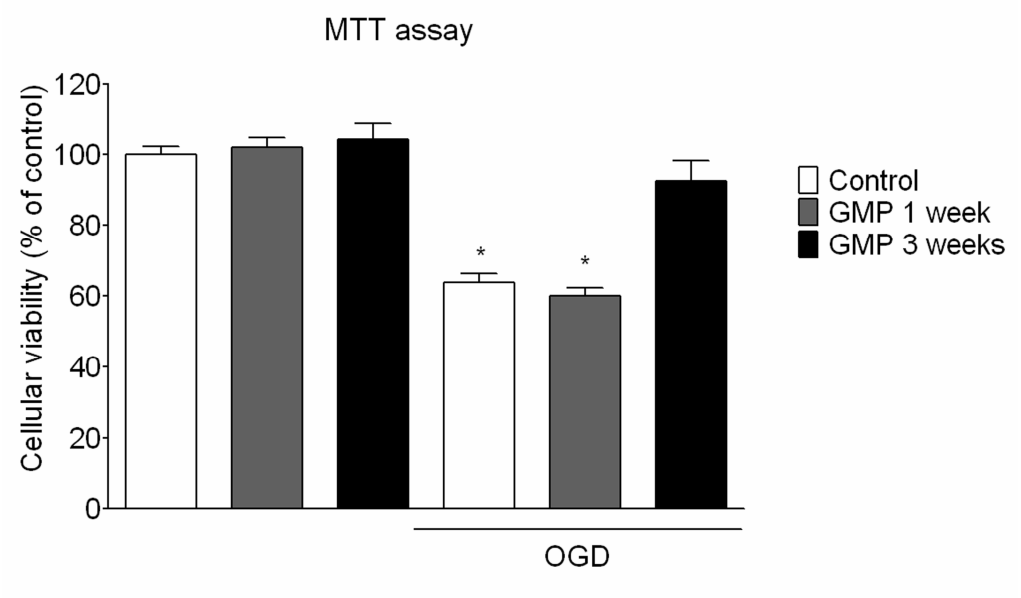

MTT assay
Cellular viability (% of control)
120
100
80
60
40
20
0
*
*
OGD
Control
GMP 1 week
GMP 3 weeks

Figure 2

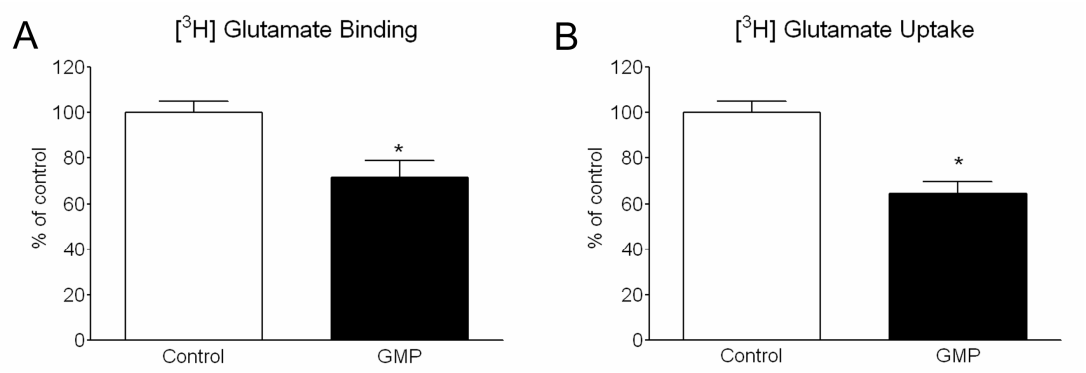

A
[3H] Glutamate Binding
% of control
120
100
80
60
40
20
0
*
Control
GMP
B
[3H] Glutamate Uptake
% of control
120
100
80
60
40
20
0
*
Control
GMP

Figure 3

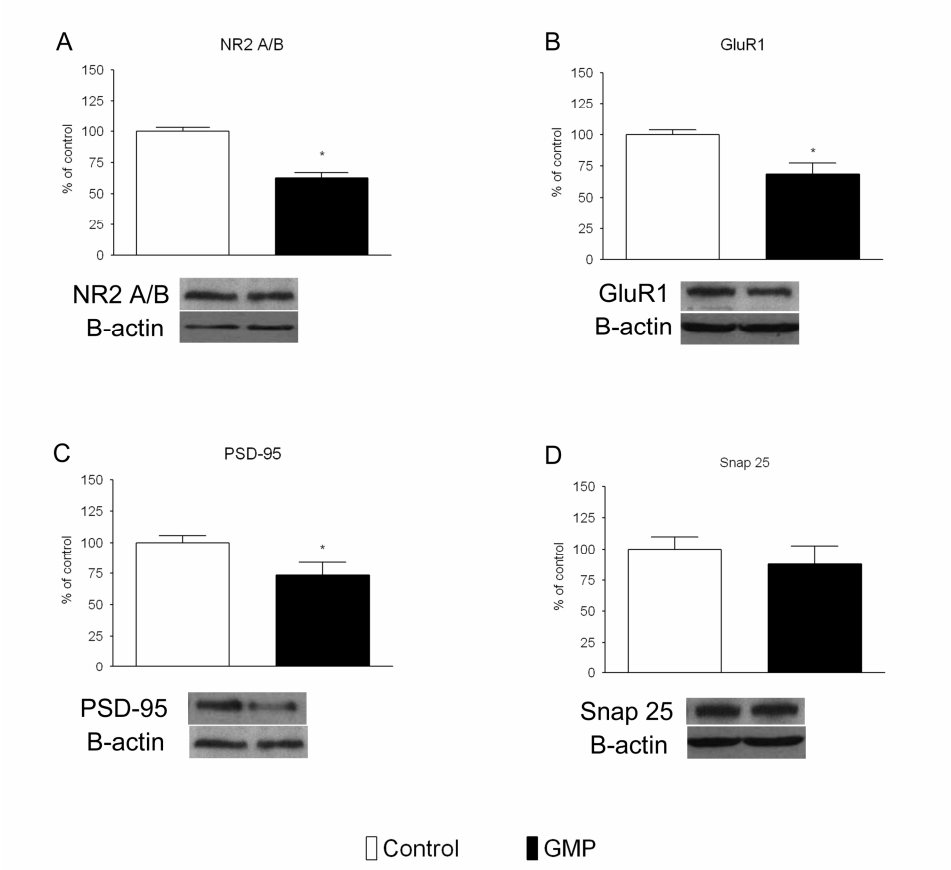

A
NR2 A/B
% of control
150
125
100
75
50
25
0
*
NR2 A/B
B-actin
B
GluR1
% of control
150
125
100
75
50
25
0
*
GluR1
B-actin
C
PSD-95
% of control
150
125
100
75
50
25
0
*
PSD-95
B-actin
D
Snap 25
% of control
150
125
100
75
50
25
0
Snap 25
B-actin
Control
GMP

Figure 4

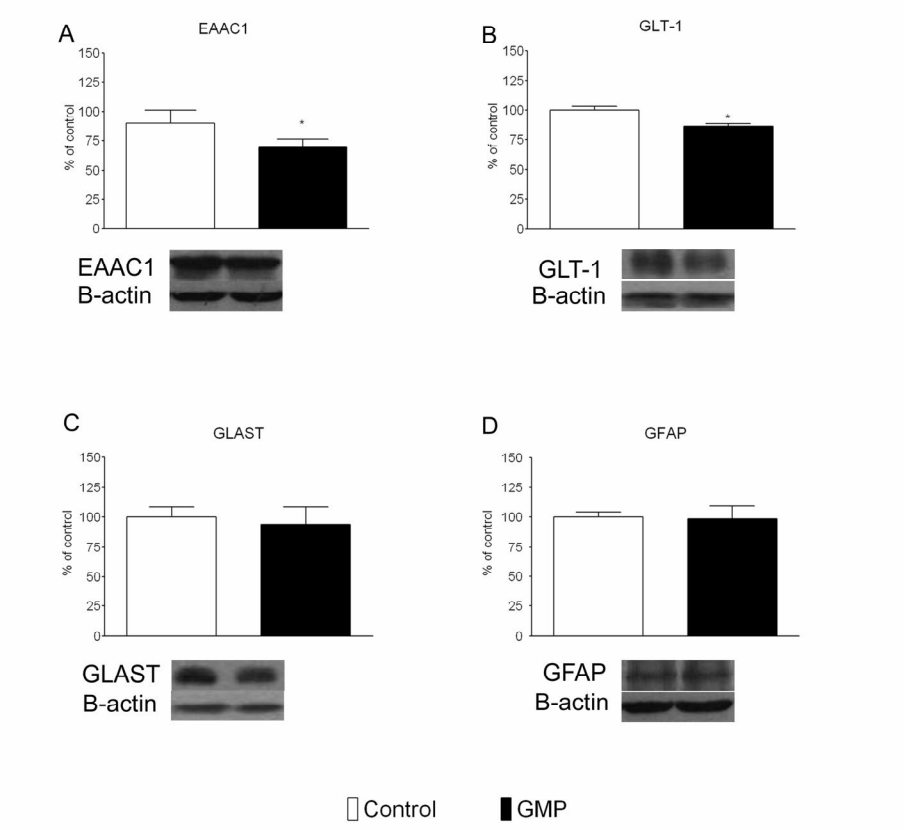
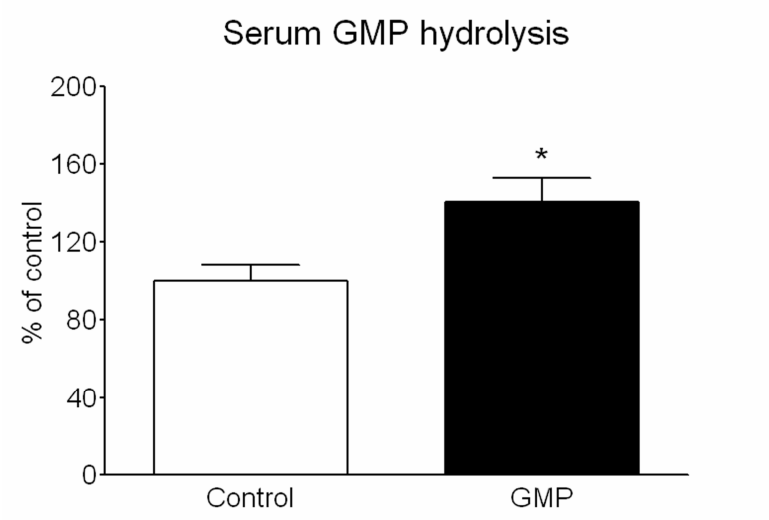

A
EAAC1
% of control
150
125
100
75
50
25
0
*
EAAC1
B-actin
B
GLT-1
% of control
150
125
100
75
50
25
0
*
GLT-1
B-actin
C
GLAST
% of control
150
125
100
75
50
25
0
GLAST
B-actin
D
GFAP
% of control
150
125
100
75
50
25
0
GFAP
B-actin
Control
GMP

Figure 5


Serum GMP hydrolysis
200
160
120
80
40
0
% of control
*
Control
GMP

Capítulo IV

Intracerebroventricular administration of inosine is anticonvulsant against seizures induced by quinolinic acid in mice, an effect independent of benzodiazepine receptors

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Title: Intracerebroventricular administration of inosine is anticonvulsant against seizures induced by quinolinic acid in mice, an effect independent of benzodiazepine receptors.

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Keywords: Inosine, Seizure, Quinolinic acid, Glutamatergic hyperactivity, Benzodiazepine, Diazepam

Abstract

CSF level of inosine (INO), a purine nucleoside, significantly increases after a single seizure episode. When administratered both centrally or systemically, INO is able to promote anticonvulsant effects against seizures induced by antagonists of the GABAergic system. Several works have postulated that INO exerts its anticonvulsant effects by acting on benzodiazepines (BZ) receptors, since INO competitively inhibited the binding of [^{3}H] diazepam to brain membrane. Despite GABAergic system inhibition, glutamatergic system hyperactivity has also been implicated in the neurobiology of seizures in the mammalian CNS. In the present study, we investigated the anticonvulsant effect of intracerebroventricular (i.c.v.) INO administration against quinolinic acid (QA)-induced seizures in mice. Furthermore, we investigated whether the BZ receptors were involved in INO effects. Animals were pretreated with an i.c.v. injection of vehicle or INO before an i.c.v. administration of 4 µl QA (36.8 nmol). All animals pretreated with vehicle followed by QA presented seizures. INO time- and dose-dependently protected mice against QA-induced seizures, up to 60% at 400 nmol, 5 min before QA injection. Moreover, i.c.v. Diazepam (DZ) administration was anticonvulsant against QA induced seizures. Additionally, i.p. administration of flumazenil, a BZ antagonist did not change the anticonvulsant potency of i.c.v. INO injection but completely abolish the DZ anticonvulsant effect. Thus, this study suggests that INO exert anticonvulsant activity against hyperactivity of glutamatergic system independently of BZ receptors activation.

1.Introduction

The purinergic system including adenine, guanine and hypoxanthine, as well as their respectively nucleosides and nucleotides, has important roles in the modulation of central nervous system (CNS) activity (Abbracchio et al., 2009; Hasko et al., 2004; Schmidt et al., 2007; Schmidt and Souza, 2010). They are found both intra- and extracellularly in CNS and their levels are regulated by neural cell release/uptake and by nucleotidases activities (Abbracchio et al., 2009; Hasko et al., 2004; Schmidt et al., 2007; Schmidt and Souza, 2010). It has been shown that purine extracellular levels are modulated by physiological and pathological conditions (Abbracchio et al., 2009; Hasko et al., 2004; Schmidt et al., 2007; Schmidt and Souza, 2010).

Several works have reported that the cerebral spinal fluid (CSF) levels of purine nucleosides (adenosine, guanosine and inosine) are augmented after a single seizure episode (Dunwiddie and Masino, 2001; Latini and Pedata, 2001; Lewin and Bleck, 1981; Oses et al., 2004; Winn et al., 1980). Most of these studies suggest that the purine nucleosides have an important role to attenuate seizures.

In the late of the 70`s, and beginning of the 80`s, the potential anticonvulsive effect of inosine (INO) started to be investigate (Lewin and Bleck, 1985; Marangos et al., 1981a; Marangos et al., 1981c; Skolnick et al., 1979). It was demonstrated that intracerebroventricular (i.c.v) (Skolnick et al., 1979) and subcutaneous (s.c.) (Lewin and Bleck, 1985) INO injection was able to increase the latency of pentylenetetrazol (PTZ) elicited seizure in mice. Additionally, the threshold for seizures induced by both bicuculline and picrotoxin were significantly raised after INO s.c. administration (Lewin and Bleck, 1985). To

note, PTZ, bicuculline and picrotoxin are inhibitors of the GABAergic complex receptor GABA-A, which contains binding sites for GABA, barbiturates, benzodiazepines (BZ), picrotoxin, and neurosteroids (McKernan and Whiting, 1996). GABA is recognized as the principal inhibitory neurotransmitter in the cerebral cortex and is generally accepted that administration of GABA agonists suppresses seizures (Treiman, 2001). BZs are classical anticonvulsant clinically used due to their ability to enhance the binding of GABA to GABAergic receptors, thus increasing GABA-mediated inhibition (Treiman 2001).

Several works have proposed that INO exerts its anticonvulsant action by acting on BZ binding sites in GABAergic receptors (BZ receptors) (Marangos et al., 1979; Marangos et al., 1981c; Skolnick et al., 1979). INO competitively displaces the binding of diazepam (DZ - a classical BZ receptors ligand) to brain membrane binding sites, an effect more potent when DZ binding is stimulated by GABA (Asano and Spector, 1979; MacDonald et al., 1979; Marangos et al., 1981b; Marangos et al., 1981c). Moreover, INO promoted a fast desensitizing excitatory response by activation of BZ receptors in nerve cells (MacDonald et al., 1979).

It is well established that the abnormal amplification and synchronization of neuronal firing that leads to seizure episodes is likely to involve interaction of GABAergic inhibitory and glutamatergic excitatory mechanisms (Bradford, 1995; Meldrum, 1994; Naylor, 2010; Treiman, 2001). Accordingly, most of seizures animal models are based on hiper-activation of the glutamatergic neurotransmission and/or inhibition of the GABAergic neurotransmission by administration of glutamate analogs and/or GABAergic antagonists, respectively (Naylor, 2010). However, it is noteworthy that many different animal models

may reflect a diversity of seizures etiology (Siniatchkin and Koepp, 2009). To our knowledge, the anticonvulsive effect of INO was never investigated on glutamate analogs based models.

Quinolinic acid (QA), an endogenous compound from the metabolism of tryptophan, may be involved in the pathogenesis of various CNS disorders including epilepsy (Heyes et al., 1990; Stone, 2001). QA is a well know NMDA agonist (Stone, 1993) and more recently it is considered an over-stimulator of the glutamatergic system by stimulating synaptosomal glutamate release (Tavares et al., 2005), increasing glutamate uptake by synaptic vesicles (Tavares et al., 2008) and inhibiting glutamate uptake by astrocytes (Tavares et al., 2002). Thus, QA i.c.v. administration is used as a seizure animal model for experimental hyperactivation of the brain glutamatergic system (Schmidt et al., 2007; Schmidt and Souza, 2010).

Concerning BZ agonists effects on seizure models based on hyperactivation of glutamatergic system, DZ intraperitoneally (i.p.) administrated abolished QA-induced seizures (Lapin et al., 1986) and NMDA-induced tonic convulsions (Moreau et al., 1989). The prevention of NMDA induced convulsions by BZ agonist was mediated by the BZ receptor and not by the NMDA receptor, as shown by the specific blockade with flumazenil (FLU - a specific BZ receptor antagonist) (Moreau et al., 1989).

So, in order to investigate if INO could promote anticonvulsant effects against direct glutamatergic system hyperactivity, we tested whether INO could afford anticonvulsive effects against QA-induced seizures. We also investigated if the BZ receptors were involved in the observed INO neuroprotective effect.

2.Material and methods

2.1.Animals

Male adult Swiss albino mice (35–45g) were kept on a 12h light/dark cycle (light on at 7:00 AM) at temperature of 22±1°C, housed in plastic cages with tap water and commercial food *ad libitum*. All procedures were carried out according to the Brazilian Society for Neuroscience and Behavior's recommendations for animal care and the US National Institutes of Health guide for the care and use of laboratory animals, designed to minimize the suffering and the number of animals used. All behavioral procedures were conducted between 3:00 and 6:00 PM. Each animal was used once.

2.2.Chemicals

INO, FLU and DZ were purchased from Sigma (St. Louis, MO, USA). The anesthetic sodium thiopental was obtained from Cristália (Itapira, SP, Brazil). All other chemicals of analytical grade were from standard suppliers. INO was dissolved in phosphate buffer saline (PBS, pH=7.4) containing (in mM): Na_2HPO_4 7.7, NaH_2PO_4 2.7 and NaCl 154. DZ and FLU were solubilized in PBS plus 20% Tween 80 (PBS+Tween). We tried to dissolve INO with PBS+Tween but it did not improve INO solubility.

2.3.Surgical procedure

Surgery and i.c.v. infusion techniques were adapted from Schmidt et al., 2000 (Schmidt et al., 2000). Animals were anesthetized with sodium thiopental (60mg/kg, 10ml/kg, i.p.). In a stereotaxic apparatus the skin of the skull was removed, and an i.c.v. guide cannula for infusion was implanted. Stereotaxic

coordinates were 1.5 mm posterior to bregma, 1 mm right of the midline. The guide cannula was implanted 1.7 mm ventral to the superior surface of the skull and fixed with jeweler's acrylic cement. Experiments were performed 48 h after surgery. I.c.v. treatments were performed with a 30-gauge cannula, which was fitted into the guide cannula and connected by a polyethylene tube to a microsyringe. The tip of the infusion cannula protruded 1 mm beyond the guide cannula, aiming the right lateral brain ventricle.

2.4.QA-induced seizures

An i.c.v. infusion of 4 µl of QA (9.2 mM, the lowest dose causing seizures in all animals – vehicle as control) was performed. Mice were observed for 10 min in Plexiglas chambers for the occurrence of wild running, clonic, tonic or tonic–clonic seizures lasting more than 5 s. Animals not displaying seizures during these 10 min were considered protected. After experiments, methylene blue (4 µl) was injected through the cannula and animals without dye in the lateral brain ventricle were discarded (Schmidt et al., 2000).

2.5.Treatments

We performed i.c.v. administration of INO and DZ and i.p. administration of FLU. DZ was used as agonist and FLU as antagonist of BZ receptors. The dose of DZ (8 nmol) here used was chose because it was the maximal concentration well solubilized in PBS+Tween, and it promoted anticonvulsive effect similar of INO against QA-induced seizures. The FLU dose here used was chose based upon previous studies investigating its antagonistic effect on DZ anticonvulsive activity (Moreau, et al. 1989).

2.5.1.Treatment 1

Mice received 4 μl i.c.v. infusion of a dose curve of INO (100 – 400 nmol – PBS as control) 5min before QA injection. The solubility of INO did not allow for testing higher doses.

2.5.2.Treatment 2

Mice received 4 μl i.c.v. infusion of INO (400 nmol – PBS as control) 1-10min before QA injection.

2.5.3.Treatment 3

Mice received i.p. injection of FLU (10 mg/kg, 10 ml/kg - PBS+Tween as control). After 15 min, 4μl i.c.v. injection of vehicle (PBS+Tween) or INO (400nmol) or DZ (8 nmol) was performed and 5 min later seizures were induced by QA administration.

2.6.Statistical Analysis

Statistical analyses were performed by two-tailed Fisher's exact test and $P \leq 0.05$ was considered significant.

3.Results

I.c.v. QA administration induced seizures in all animals. The latency and duration of the tonic–clonic seizures was 24.2 ± 12 s and 20.9 ± 10 s, respectively. PBS and/or PBS+Tween i.c.v. did not evoke any seizure.

Fig. 1 shows that INO pretreatment protected mice against QA-induced seizures. This anticonvulsant effect was dose-dependent (A) and varied with the time of administration before QA (B). The strongest anticonvulsant effect

(protecting 60% of the animals) was observed with the administration of 400 nmol INO 3 minutes before QA infusion. When INO did not provide protection, it had no effect on latency neither on duration of seizures (data not shown).

Figure 1 should be included here.

Fig. 2 shows that DZ (4 μl, i.c.v., 8 nmol), as INO (4 μl, i.c.v., 400 nmol), partially prevented QA-induced seizures, while FLU (10 mg/kg, i.p.) had no effect. Moreover, FLU pretreatment (10 mg/kg, i.p., 15 min before INO or DZ injection) did not modify the anticonvulsive effect of INO (4 μl, i.c.v., 400 nmol) but abolished the anticonvulsive effect of DZ (4 μl, i.c.v., 8 nmol).

Figure 2 should be included here.

4.Discussion:

The results of the present study clearly demonstrate an anticonvulsant effect of INO against QA-induced seizures in adult mice. Additionally, this effect seems not to be mediated by activation of BZ receptor, since FLU did not block INO effect while abolished the DZ anticonvulsant effect.

INO anticonvulsive potential was previously demonstrated by using seizure models based on inhibition of GABA-A receptor complex. Several studies had proposed that INO effect could be mediated by activation of BZ receptors based upon in vitro evidences that INO is able to displace BZ agonists binding to brain membrane (Marangos et al., 1979; Marangos et al., 1981c; Skolnick et al., 1979). Interesting, it was already demonstrated by an in vitro study that INO is also able to inhibit excitability by increasing membrane conductance through an unknown mechanism independently of BZ receptors (MacDonald et al., 1979). This unknown inhibitory mechanism may be involved

with the anticonvulsant potential of INO, but more studies are necessary to clarify this hypothesis. Until now, there are no specific receptors for INO identified on extracellular brain membrane. To our knowledge, our work was the first that investigated if a BZ antagonist could be able to block INO effect.

Furthermore, INO anticonvulsant activity seems to be more potent against seizures evoked by hyper-excitation than those evoked by stressing inhibitory tonus of the CNS. When comparing INO effects against QA- and PTZ-induced seizure models, the dose and time of INO infusion that promoted the best protective effect were very similar in both models, however, the seizure behavior were completely blocked when seizures were promoted by QA infusion while only the latency to start the seizure were increased in the PTZ model (Skolnick et al., 1979).

The anticonvulsive INO potential reported here is very similar to the effect observed by centrally guanosine (GUO) infusion (Schmidt et al., 2005). In previous studies, we demonstrated that GUO (400 nmol), administrated 5 min before QA, protected mice against seizures induced by QA up to 60% (Schmidt et al., 2005). Interesting, both compounds were unable to protected 100% of the QA injected animals. Using eletrophysiological measures, we observed that dynamic variables within rats (pharmacokinetic and pharmacodynamic factors or to the current internal state of the brain), in contrast to basal electrophysiological parameters, seem to play a major role in the successfulness of GUO to prevent seizures (Torres et al., 2010). Indeed, this hypothesis may also be applied for INO effect.

To our knowledge, this is the first evidence that INO, in an experimental model, may exert anticonvulsant activity against the over activity of the

glutamatergic system. Since INO is an endogenous compound and apparently well tolerated and non-toxic to humans (Hasko et al., 2004), we suggest that our results warrants the investigation on the promissory role of INO in seizure activity and its potential neuroprotective role against excitotoxic insults.

5.Figure legends

Fig.1. Effect of dose (A) and pretreatment time (B) of INO against QA-induced seizures. Fig 1A - Different doses of INO were administered i.c.v. 5 min before QA administration. Fig 1B - INO 400 nmol were administrated i.c.v at different times before QA-induced seizures. After QA administration, mice were observed during 10 minutes for the occurrence of seizures. N=12-15 animals per group. P≤0.05 (Fisher's exact test). * different from control group, # different from 100 nmol group.

Fig.2. FLU effect on INO or DZ anticonvulsant potential against QA-induced seizures. FLU or vehicle (PBS+Tween) was i.p. injected 15 mintutes before INO (400 nmol) or DZ (8 nmol) or vehicle i.c.v. administration. Seizures were induced by i.c.v. QA infusion 5 minutes after INO or DZ or vehicle infusion and mice were observed for the occurrence of seizures; n=12-15 per group.*P ≤ 0.05 (Fisher's exact test), as compared to control, FLU+ Veh and FLU+DZ groups.

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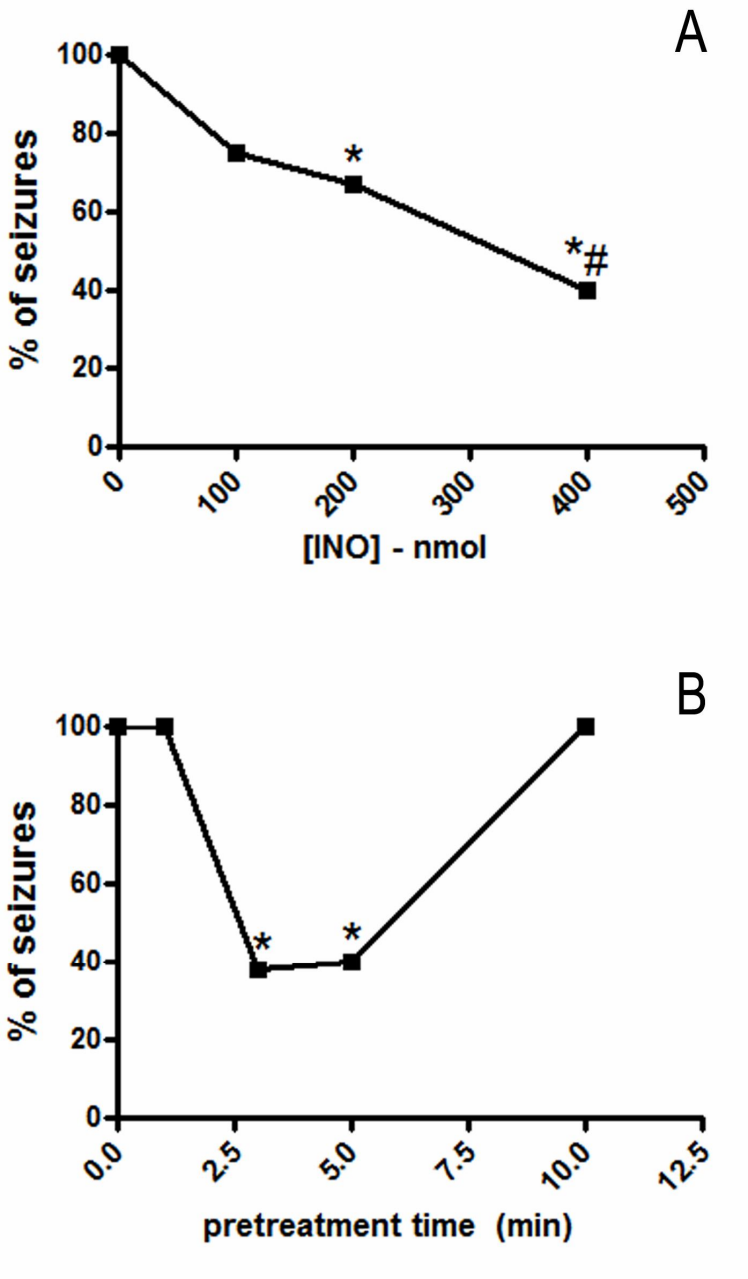
Figure 1


A
100
80
60
40
20
0
% of seizures
*
*#
0
100
200
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400
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[INO] - nmol
B
100
80
60
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20
0
% of seizures
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0.0
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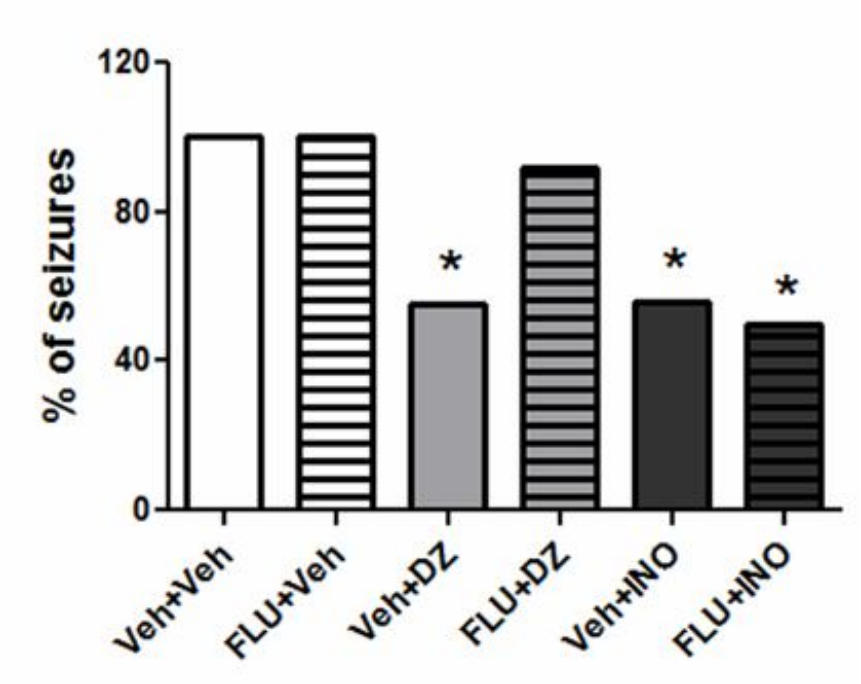
10.0
12.5
pretreatment time (min)

Figure 2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% of seizures
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FLU+DZ
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FLU+INO

Capítulo V

Effects of chronic guanosine treatment on hippocampal damage and cognitive impairment of rats submitted to chronic cerebral hypoperfusion

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Artigo Submetido ao periódico Neroscience Research

Title: **Effects of chronic guanosine treatment on hippocampal damage and cognitive impairment of rats submitted to chronic cerebral hypoperfusion**

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Abstract:

Chronic cerebral hypoperfusion contributes to a cognitive decline related to brain disorders. Its experimental model in rats is a permanent, bilateral common carotid artery occlusion (2VO). Overstimulation of the glutamatergic system due to brain energetic disturbance in 2VO animals seems to play a pivotal role as a mechanism of cerebral damage. The nucleoside guanosine (GUO) exerts extracellular effects including antagonism of glutamatergic activity. Accordingly, our group demonstrated several neuroprotective effects of GUO against glutamatergic excitotoxicity. So, in this study we evaluated a chronic GUO treatment effects in rats submitted to 2VO. We evaluated the performance of animals in the Morris water maze and hippocampal damage by neurons and astrocytes immunohistochemistry. Also, we investigated the cerebrospinal fluid (CSF) brain derived neurotrophic factor (BDNF) and serum S100B levels. GUO treatment did not prevent the cognitive impairment promoted by 2VO. However, none of the 2VO animals treated with GUO showed differences in the hippocampal regions compared to control, while 20% of 2VO not treated with GUO rats presented loss of pyramidal neurons and increased reactive glial labeling cells in CA1 hippocampal region. Additionally, we did not observe differences in CSF BDNF nor serum S100B levels among the groups. In conclusion, GUO treatment did not prevent the cognitive impairment observed in 2VO animals, but our data suggest that GUO could be neuroprotective against hippocampal damage induced by 2VO.

Keywords: guanosine, cerebral hypoperfusion, cognitive impairment, hippocampal damage, neuroprotection

Introduction:

Vascular dementia (VD), which is a group of diseases with heterogeneous pathological conditions and physiopathological mechanisms, is the second most common cause of dementia associated with Alzheimer's disease and accounts for 10–50% of all dementias (Desmond, 2004; Rockwood et al., 2000). Chronic cerebral hypoperfusion is considered a factor that contributes to memory dysfunction in neurological diseases such as VD (Farkas and Luiten, 2001; Ohnishi et al., 2001) . Moreover, the degree of cerebral hypoperfusion has been suggested as a predictive biomarker of the gradual transition from a mild cognitive impairment to the Alzheimer's disease (Borroni et al., 2006).

Permanent bilateral occlusion of the common carotid arteries (also denominated two vessels occlusion - 2VO) in rats is used as a chronic cerebral hypoperfusion model (Farkas et al., 2007). This procedure results in significant reduction of cerebral blood flow (Farkas et al., 2007; Marshall et al., 2001; Ni et al., 1994; Ni et al., 1995) and can cause progressive, long-lasting cognition deficits and neuronal damage resembling the effects observed in VD (Ni et al., 1995; Sarti et al., 2002). 2VO in rats provides an useful model to understand the pathophysiology of chronic cerebral vascular disorders and to screen drugs with potential therapeutic value in VD.

The chain of events that eventually leads to neuronal cell death in chronic cerebral hypoperfusion begins with neuronal energy failure due to the blood flow reduction and the consequent oxygen and glucose deficiency (Briede and Duburs, 2007; Farkas et al., 2007; Otori et al., 2003; Tsuchiya et al., 1993). The disturbance of the energetic metabolism leads accumulation of extracellular

glutamate (Chao et al., 2010; Dirnagl et al., 1999), the main excitatory neurotransmitter in the central nervous system, essential for the brain function (Chao et al., 2010; Lau and Tymianski M., 2010; Segovia et al., 2001). However, overstimulation of the glutamatergic system (excitotoxicity), which increases the calcium influx triggers several intracellular processes such as proteolytic hydrolysis, lipid peroxidation, and generation of reactive oxygen species, causing neuronal death (Lau and Tymianski, 2010; Szydlowska and Tymianski, 2010). Thus, excitotoxicity has been proposed as a mechanism of neuronal damage impairing cellular energetics, as observed in hypoperfusion (Dong et al., 2009; Marosi et al., 2009).

The nucleoside guanosine (GUO) exerts various extracellular signaling effects, such as trophic effects on neural cells (Bau et al., 2005; Ciccarelli et al., 2000; Ciccarelli et al., 2001; Kim et al., 1991; Middlemiss et al., 1995; Rathbone et al., 2008) and *in vitro* and *in vivo* antagonism of the glutamatergic system (Schmidt et al., 2007; Schmidt et al., 2009b; Schmidt and Souza, 2010). Accordingly, our group has demonstrated by several works that GUO is neuroprotective in different *in vitro* and *in vivo* experimental models of glutamatergic excitotoxicity (Lara et al., 2001; Schmidt et al., 2000; Schmidt et al., 2005; Schmidt et al., 2007; Schmidt et al., 2008; Schmidt et al., 2009a; Schmidt et al., 2009b; Schmidt and Souza, 2010; Soares et al., 2004; Vinadé et al., 2003; Vinadé et al., 2005), including ischemic insults (Moretto et al., 2005; Moretto et al., 2009). The mechanism of action of GUO is not fully understood, however, our group demonstrated that GUO stimulates glutamate uptake by cultured astrocytes and brain slices (Frizzo et al., 2001; Frizzo et al., 2002;

Frizzo et al., 2003; Frizzo et al., 2005; Gottfried et al., 2002), a physiological process that prevent the glutamate toxicity.

Therefore, considering the involvement of excitotoxicity in the 2VO model and the neuroprotective potential of GUO, the aim of the present study was to investigate the effects of GUO in rats submitted to the 2VO model. We evaluated the 2VO and GUO effects in the performance in a behavioral task and in hippocampal cells damage. We also investigated the cerebrospinal fluid (CSF) brain derived neurotrophic factor (BDNF) levels and the serum S100B levels as possible markers of brain insult.

Material and Methods:

Animals

Male adult Wistar rats (90-100 days old, weighing 300–350 g) were kept on a 12-hour light/dark cycle (light on at 7:00 AM) at constant temperature of 22 ± 1°C. They were housed in plastic cages (5 per cage) with water and commercial food *ad libitum*. All behavioral tasks were conducted between 9:00 am and 5:00 pm. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and with the Federation of Brazilian Societies for Experimental Biology (FESBE), and were approved by the Research Ethics Committee of Universidade Federal do Rio Grande do Sul.

Surgery procedure

Chronic cerebral hypoperfusion was performed (20 animals) by a modified protocol of permanent bilateral occlusion of the common carotid

arteries (2VO) (Cechetti et al., in press); twenty animals served as sham-operated controls (SHAM). Rats were anesthetized with halothane. The common carotid arteries were exposed via a neck ventral midline incision, separated from their sheaths and vagal nerves. Rats were submitted to the modified 2VO protocol: carotids were permanently occluded with 5-0 silk suture with a 1-week interval between interventions, the right common carotid being the first to be assessed and the left one being occluded 1 week later. Sham-operated controls received the same surgical procedures without carotid artery occlusion.

Treatment and groups

Rats received water (for control group) or GUO solution (0.5 mg/ml) for 6 weeks *ad libitum* in the bottle water immediately from the 2VO surgery. The GUO dose was chosen based on a previously study, which demonstrated that it was neuroprotective against seizures induced by quinolinic acid (Vinadé et al., 2003). The water consumption and body weight were monitored during the period of treatment every 2 days. The animals were randomly assigned to four different groups: sham-operated animals receiving water (SHAM-CT) or GUO (SHAM-GUO) and 2VO operated animals receiving water (2VO-CT) or GUO 2VO-GUO).

Morris water maze

Cognitive function was evaluated 6 months after surgery in the Morris water maze task (Vicente et al., 2009). The maze consisted of a black circular pool (200 cm diameter, 60 cm height) filled to a depth of 40 cm with water (22 ±

1 °C). A circular platform (10 cm diameter) was submerged 2 cm below the surface of the water and hidden from the rat's view. Four points, equally spaced along the circumference of the pool, were arbitrarily assigned as North, South, East and West. The pool, therefore, was divided into four quadrants (Northeast, Southeast, Southwest, and Northwest). These points served as the starting positions where the rats were gently lowered into the water, with its head facing the wall of the water maze. We submitted the rats to a reference memory protocol for spatial memory analyzes.

Reference memory protocol. In this protocol, rats received 5 training sessions (one session/day) and a probe trial in the 6th day. Each session consisted of 4 trials with a 15-min inter-trial interval. The location of the escape platform was fixed throughout training in the middle of the NE quadrant, 30 cm from the wall. A trial began when the rat was placed in the water at one of the 4 starting positions, randomly chosen, facing the wall. The order of the starting position varied in each trial and any given sequence was not repeated. A trial ended when the rat escaped onto the platform, and the escape latency for each trial was recorded. When the animal did not succeed, it was gently guided to the platform and left on it for 10s and the escape latency was recorded as 60 s. Rats were dried and returned to their home cages after each trial. The mean escape latency of daily trials was then calculated.

Probe trial. In this 1-day test, each rat was subjected to a probe trial (60s) without the platform. The latency to reach the original platform position, the number of crossings over that place and the time spent on the target quadrants were measured.

Sessions were recorded with a video acquisition system. Videotapes were used by a trained observer using dedicated software (ANY-maze®). Videos were subsequently placed in randomized order in a separate ANY-maze protocol to be scored by a trained observer blind to the experimental condition using a keyboard-based behavioral tracking system.

Cerebrospinal fluid (CSF) and serum sampling

One day after the completion of the behavioral study, the rats were anesthetized with sodium thiopental (40mg/kg, 1mL/kg, i.p.), and then positioned in a stereotaxic holder for cerebrospinal fluid collection from the cisterna magna. The puncture was performed using an insulin syringe (27 gauge 9 1/200 length). Rats were then removed from the stereotaxic apparatus and placed in a flat place; whole blood was obtained through an intracardiac puncture using a 0.37-mm diameter needle that was inserted into the intercostal space above the sternum. Serum was separated by centrifugation at 3000g for 5 min. CSF and serum samples were frozen (-20^{0}C) until analysis.

Quantification of cerebrospinal fluid (CSF) BDNF

BDNF levels in CSF were measured by anti-BDNF sandwich-ELISA, according to the datasheet from DuoSet kit (R&D Systems, Inc, USA) in a Spectra Max M5 molecular Devices (USA). Microtiter plates (96-well flat-bottom) were coated 2 h with samples and reference curve standards (ranging from 23 to 1500 pg/ml BNDF). The plates were then washed 4 times with PBS+Tween 0.05% and a diluted biotinylated mouse anti-human BDNF monoclonal (1:1000) was added to each well and incubated for 2 h at room

temperature. After this time, the plates were washed 4 times and then a diluted streptavidin-HRP conjugate solution was added to each well and incubated at room temperature for 20 minutes. Wells were then washed 4 times before adding TMB chromogen (Tetramethylbenzidine) and maintained at room temperature for 20 minutes before the addition of stop solution. The amount of BDNF was determined at 450 nm and expressed as ng/mL.

Quantification of serum S100B

S100B concentrations were measured using an enzyme linked immunosorbent assay (Diasorin® S100 ELISA Kit) in a Spectra Max M5 molecular Devices (USA). Calibrators and serum samples (100 µL) were incubated in a plate previously coated with anti-S100B antibody. The S100 ELISA was a two-site, one-step, enzyme linked immunosorbent assay. In the assay calibrators, controls and serum samples react simultaneously with 2 solid phase capture antibodies and a detector antibody conjugated with horseradish peroxidase (HRP) during the incubation in the microtiter wells for 2 hours. After a washing step (with PBS+Tween 0.05%) a TMB chromogen (Tetramethylbenzidine) was added and the reaction was allowed to proceed for 15 minutes. The enzyme reaction was stopped by adding stop solution and the absorbance was measured at 450 nm. S100B concentrations were derived by comparison with the calibration curve based on the total absorbance for each given calibrator provided with the assay. All determinations were carried out within the same experiment. The S100B calibration curve is cubic spline up to 5 µg/L, and the CVs for duplicates across the entire concentration range for the

calibrators and samples were 5%. The detection limit of the assay is 0.03 µg/L. The results are expressed as ng/mL.

Histological analysis

After CSF and serum sampling, the animals were injected with 400 IU of heparin. They were then submitted to transcardiac perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. The brains were removed and post-fixed in the same solution at room temperature for 4 h. For the morphological analysis, brains were cryoprotected with a 30% sucrose solution for 2 days. Coronal sections (50 µm) were obtained using a Vibratome (Leica, Germany).

One section in 6 random series was collected for immunohistochemistry. Immunohistochemical detection was carried out in the sections with all fields of the hippocampus (an area of the hippocampus extending between the Bregma ±2.30 and ± 3.80, Plate 29–34, according to Paxinos & Wattson Atlas). The sections were incubated for 48 h at 4^0C with polyclonal rabbit GFAP antiserum (Dako, UK, 1:500) and NeuN (Chemicon, 1:250) in phosphate buffered (pH 7.4) saline (PBS)-Tx containing 2% bovine serum albumin (BSA). After being washed several times with PBS, the sections were incubated with 594 alexa-conjugated donkey antirabbit and 488 alexa-conjugated donkey antimouse antibodies for 2 h at room temperature. The sections were mounted on slides coated with 2% gelatin with chromium and potassium sulfate, using Vectashield mounting medium (Vector Laboratories, São Paulo, Brazil). All sections were photographed with a confocal microscope (Olympus, Japan).

Statistics analysis

The data obtained are expressed as mean ± S.E.M. of mean. As the variances of the data were homogenous (analyzed by the Kolmogorov-Smirnov test), behavioral performance in reference memory was analyzed by two-way repeated-measures analysis of variance (ANOVA) followed by the Bonferroni post test when indicated. Mean escape latency was the dependent variable, day was the within-subjects variable, and the four groups were the between-subjects variables. Remaining data were analyzed by two-way ANOVA (factors were surgery and treatment). In all statistical comparisons, $P < 0.05$ was used as the criterion for significance.

Results:

Behavioral and treatment parameters

Chronic cerebral hypoperfusion had no effect on liquid and food consumption or body weight during 6 months (data not shown). Additionally, *ad libitum* 0.5 mg/mL GUO oral treatment did not affect the parameters mentioned above.

Morris water maze learning

The escape latency of both sham groups (CT and GUO) decreased from the 1st to the 5th day of the task, while both 2VO groups (CT and GUO) did not present this performance, indicating that 2VO procedure impaired the learning of this task and that GUO did not interfere with this impairment (Fig. 1A). All rats showed similar escape latencies on training day 1, a two-way ANOVA with one between factor (4 groups) and one repeated measure (5 days) on escape latencies indicated a significant difference among groups ($F(3, 144) = 6.36$, $p <$

0.05), and a significant difference over training days (F (4, 133) = 7.64, p < 0.01). 2VO-CT rats showed significantly longer escape latencies than SHAM-CT rats from training day 3 to 5 (two-way ANOVA followed by Bonferroni post-test).

In the probe test, both sham groups (CT and GUO) spent more time in the former platform quadrant (Fig. 1B) than both 2VO groups (CT and GUO) [by two-way ANOVA, F (1,35) = 5.20, p < 0.01]. Again, GUO had no effect on this performance.

We observed that SHAM-CT rats spent more time in the training quadrant where the platform was previously located compared to the 2VO-CT animals by two-way ANOVA, F (1,35) = 5.20 (Fig. 1B, p < 0.01). Additionally, it was not observed any motor deficit: the mean swimming speed was 0.15 m/s for control group and 0.17 m/s for 2VO group.

Brain insult markers

As shown in Fig. 2, CSF BDNF (A) and serum S100B (B) levels were not affected by 2VO procedure and/or by GUO administration, by two way ANOVA (p > 0.05).

Hippocampal Damage

Immunohistochemistry was carried out using NeuN and GFAP for labeling neurons and astrocytes, respectively. It was evaluated the hippocampal damage 6 months after 2VO-surgery. Evident loss of CA1 pyramidal neurons and increased reactive glial labeling cells was observed in 2 of 10 animals in the 2VO-CT group (Fig 3). It was not detected any difference in the NeuN or GFAP

labeling in CA3 or DG regions. Images of hippocampal CA1, CA3 and DG (Dentate Gyrus) from rats of the 4 groups are shown in Fig 3, 4 and 5, respectively. With the exception of 2 animals (showed in Fig. 3), none animal presented any cell damage in CA3 and DG hippocampal regions. (Figs. 3, 4 and 5).

Discussion

The results of the present study demonstrate that chronic cerebral hypoperfusion produced by 2VO, impaired spatial learning and memory and caused severe hippocampal damage only in 20% of the animals. The behavioral effect was not affected by GUO treatment. However no hippocampal damage was observed in the 2VO animals treated with GUO. Additionally, it was not observed any alteration neither in the CSF BDNF levels nor in the serum S100B concentration caused by 2VO surgery and/or by GUO treatment.

Studies involving the use of the 2VO model have demonstrated that an impaired spatial learning function is associated with hippocampal damage (Farkas et al., 2007; Otori et al., 2003; Sarti et al., 2002). In our study we did not observe this correlation: animals presenting behavioral alteration did not present cell hippocampal damage, as measured in this study. We can not exclude that other cell damage not investigated may be present in the 2VO group. In fact, the relationship between chronic cerebral hypoperfusion produced by 2VO and impaired cognitive function has not been completely elucidated. Other studies also found almost no correlation between the loss of CA1 neurons and the Morris maze performance (Institoris et al., 2007; Jaspers et al., 1990; Lyeth et al., 1990). The pyramidal and granular cells in the

hippocampus are not the exclusive cells involved in spatial memory. Other regions of the hippocampal formation such as the entorhinal cortex, the parahippocampal gyrus, and the rhinal and cingular gyri are also involved in spatial memory (Meunier et al., 1996; Wiig and Bilkey, 1994). Moreover, the memory deficit correlates with the white matter damage in 2VO rats (Farkas et al., 2007; Lee et al., 2006; Wakita et al., 1994; Watanabe et al., 2006). Thus, a direct link cannot be established between 2VO-induced memory failure and a specific appearance of neuronal damage in the hippocampus.

The hippocampal damage here observed in 2 animals was evidenced by a massive neuronal loss in the CA1 region that was accompanied by an intense increase in GFAP labeling, indicating an astrogliosis process. No alteration in the CA3 or DG areas was observed. This is consistent with the selective vulnerability of CA1 neurons that has been observed in others studies using the 2VO model and with various acute ischemia models (Bowler, 2004; Farkas et al., 2007; Ni et al., 1994). GFAP is commonly used as a marker for changes in astroglial cells during brain development and injury (Eng et al., 2000). In fact, CNS injury, as consequence of brain diseases as trauma, ischemia, genetic disorders, neurodegenerative disorders or chemical insult causes reactive astrogliosis to become reactive, a condition accompanied by an increase in GFAP levels (O'Callaghan and Sriram, 2005). However, some studies indicate no glial changes, as evaluated by immunohistochemistry for GFAP, in 2VO rats evaluated in the first week after the reduction of cerebral blood flow (Farkas et al., 2007; Schmidt-Kastner et al., 2005).

Thus, it is noteworthy that variable hippocampal damage has been reported by others (Bennett et al., 1998; Farkas et al., 2007; Pappas et al.,

1996; Plaschke et al., 2001; Schmidt-Kastner et al., 2005). As in most vascular models, individual anatomical differences in the cerebrovascular anatomy at the circle of Willis of rodents can influence experimental variability (Schmidt-Kastner and Eysel, 1994; Schmidt-Kastner and Ingvar, 1994). Differences in the focal lesions outcome after 2VO in different laboratories may be explained possibly by differences in rat strain, animal age at the time of occlusion or anesthesia.

In our study, 2VO procedure did not affect the CSF BDNF level and the serum S100B concentration in rats. BDNF and S100B are 2 proteins commonly used as biomarkers for brain diseases, including vascular diseases and Alzheimer's diseases (Chaves et al., 2010; Grillo et al., 2007; Haas et al., 2010; Kessler et al., 2007; Lee et al., 2005; Machado-Vieira et al., 2007; Schaf et al., 2005; Zhang et al., 2008). BDNF in the hippocampus is critical for the acquisition and/or consolidation of spatial memory (Alonso et al., 2002a; Alonso et al., 2002b; Bekinschtein et al., 2007). Previous studies showed that chronic cerebral hypoperfusion induced down-regulation of hippocampal BDNF (Zheng et al., 2008). Additionally, 2VO rats presenting an increase in hippocampal BDNF levels also presented better performed in the water maze task (Zheng et al., 2008). S100B is a calcium-binding protein found in brain tissue, predominantly in astrocytes. This protein has putative intra and extracellular functions. Intracellular roles include regulation of protein phosphorylation, cytoskeleton components and transcriptional factors (Donato, 2001; Gonçalves et al., 2008); extracellularly, S100B plays trophic roles on neuronal and glial cells, but elevated levels of this protein could induce apoptosis in neural cells (Van Eldik and Wainwright, 2003). In a previous work, we demonstrated that the hippocampal S100B levels increased, while CSF S100B levels decreased, after

the 2VO surgery (Vicente et al., 2009). To note, previous works evaluated the BDNF and S100B proteins 8 and 10 weeks after the 2VO surgery, respectively (Vicente et al., 2009; Zheng et al., 2008). In the present work, we investigated the 2VO effects 6 months after the surgery. Therefore, we can not rule out that alteration in the CSF BDNF level or serum S100B concentration may be altered in a other periods after 2VO surgery.

Glutamatergic excitotoxicity is suggested to play a pivotal role in the behavioral alterations and neuronal damage observed in ischemic insult, including hypoperfusion (Dong et al., 2009; Marosi et al., 2009; Vicente et al., 2009). In a previous work, we demonstrated that 2VO animals presented a decrease of hippocampal glutamate uptake, indicating a higher susceptibility of these animals to excitotoxicity (Vicente et al., 2009). A correlation between hippocampal glutamate uptake and Morris water maze performance was also evidenced (Vicente et al., 2009). Our group has demonstrated that acute *in vitro* administration of GUO stimulates glutamate uptake (Frizzo et al., 2001; Frizzo et al., 2003; Gottfried et al., 2002), and *in vivo* administration prevents the decrease in the glutamate uptake after excitotoxic stimuli (de Oliveira et al., 2004; Moretto et al., 2005; Moretto et al., 2009; Vinade et al., 2005). Therefore, we expected that GUO treatment would be able to prevent the behavioral alterations observed in 2VO rats, which did not occur in this study. Noteworthy, none animal submitted to the 2VO surgery and received GUO treatment showed neuronal injury.

GUO was given orally for 6 weeks immediately from the 2VO surgery. We chose to treat the animals with chronic orally treatment because we previously demonstrated that GUO chronic administration was neuroprotective

against excitotoxicity insults (Vinade et al., 2003; Vinade et al., 2005). We chose this time after the 2VO surgery because the reduction in the cerebral blood flow values has been reported during this period (Ohta et al., 1997; Otori et al., 2003). However, several studies have demonstrated significant time-dependent changes in the cellular markers in the hippocampus, including cellular alterations starting after 6 weeks postoperative (Farkas et al., 2007; Schmidt-Kastner et al., 2005). Other protocols using GUO treatment with different administration schedule should be design to investigate the GUO effects in 2VO animals.

In summary, we did not observed correlation between the cognitive impairment and hippocampal cell damage in the 2VO animals. Additionally, CSF BDNF and serum S100B levels were not brain injury markers, at least 6 months after the 2VO surgery. Although the GUO neuroprotective potential in experimental models involving excitotoxicity events has been well demonstrated, in the present work GUO chronic orally treatment did not prevent the behavioral alterations observed in 2VO animals. However, our data suggest that GUO could be neuroprotective against hippocampal damage induced by 2VO. More studies are necessary to better characterize the GUO effects on the behavioral and histological alterations observed by our model of chronic cerebral hypoperfusion.

Legends:

Fig 1- Performance of rats in the water maze task submitted to chronic cerebral hypoperfusion evaluated. (A) Curves in the Morris water maze performance in the reference memory protocol. 2VO groups showed significantly longer escape latencies than SHAM groups, respectively (*, $P < 0.05$; #, $P < 0.05$). (B) Memory time spent in the target quadrant showing memory impairment of 2VO-CT and 2VO-GUO compared to SHAM-CT and SHAM-GUO groups (*, $P < 0.05$).

Fig. 2 – CSF BDNF and serum S100B levels. No differences were observed in CSF BDNF concentration (A) and in serum S100B ($P > 0.05$).

Figure 3 - Pyramidal neurons and GFAP immunoreactivity in the CA1 hippocampal region of 2VO-CT rats six month after chronic cerebral hypoperfusion. Immunofluorescence showing NeuN (red) and GFAP (green) positive cells in SHAM-CT (A - C), SHAM-GUO (D - F), 2VO-CT (G - I) and 2VO-GUO (J - L) groups. Magnification 20x, scale bar = 100µm.

Figure 4 - Pyramidal neurons and GFAP immunoreactivity in the CA3 hippocampal region of rats. Immunofluorescence showing NeuN (red) and GFAP (green) positive cells in SHAM-CT (A - C), SHAM-GUO (D - F), 2VO-CT (G - I) and 2VO-GUO (J - L) groups. Magnification 20x, scale bar = 100µm.

Figure 5 - Pyramidal neurons and GFAP immunoreactivity in the DG hippocampal region. Immunofluorescence showing NeuN (red) and GFAP

(green) positive cells in SHAM-CT (A - C), SHAM-GUO (D - F), 2VO-CT (G - I) and 2VO-GUO (J - L) groups. Magnification 20x, scale bar = 100µm.

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Figure 1

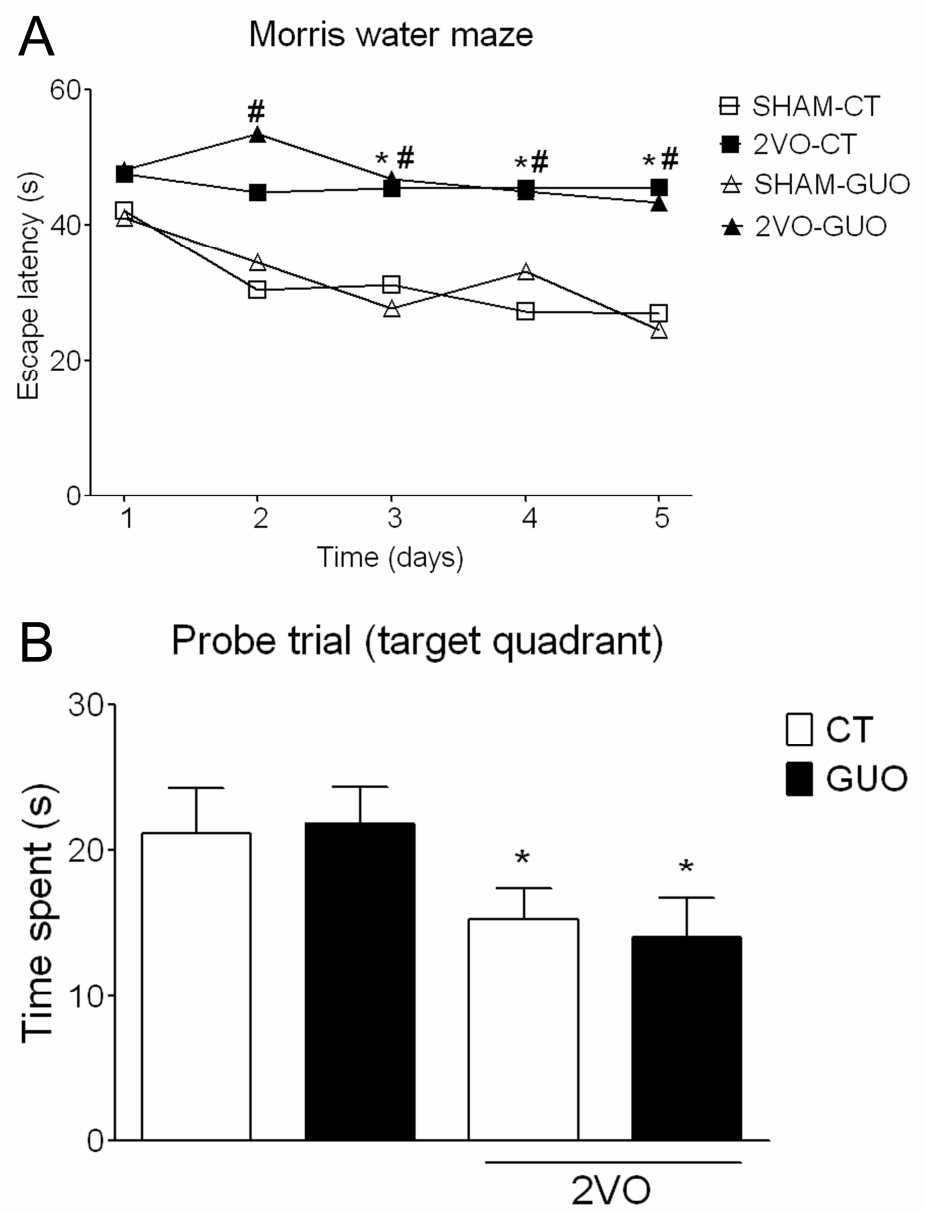

A
Morris water maze
Escape latency (s)
60
40
20
0
1
2
3
4
5
Time (days)
#
*#
*#
*#
SHAM-CT
2VO-CT
SHAM-GUO
2VO-GUO
B
Probe trial (target quadrant)
Time spent (s)
30
20
10
0
*
*
CT
GUO
2VO

Figure 2

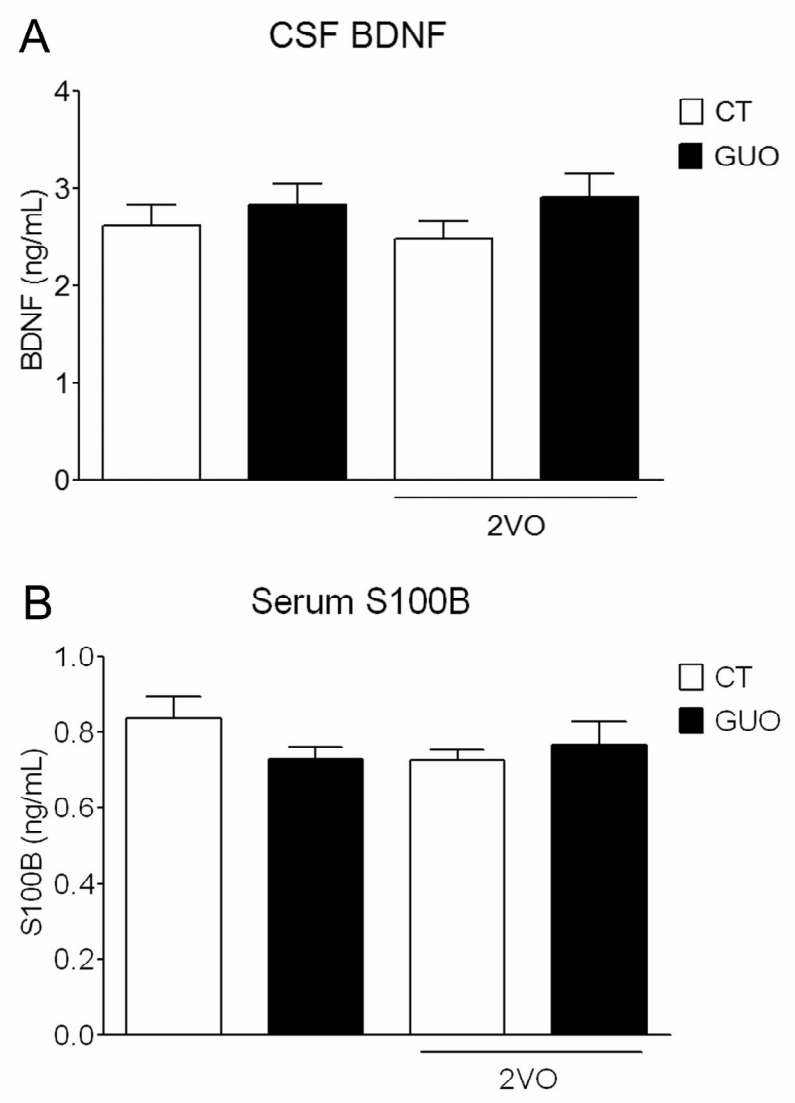

A
CSF BDNF
BDNF (ng/mL)
0
1
2
3
4
CT
GUO
2VO
B
Serum S100B
S100B (ng/mL)
0.0
0.2
0.4
0.6
0.8
1.0
CT
GUO
2VO

Figure 3

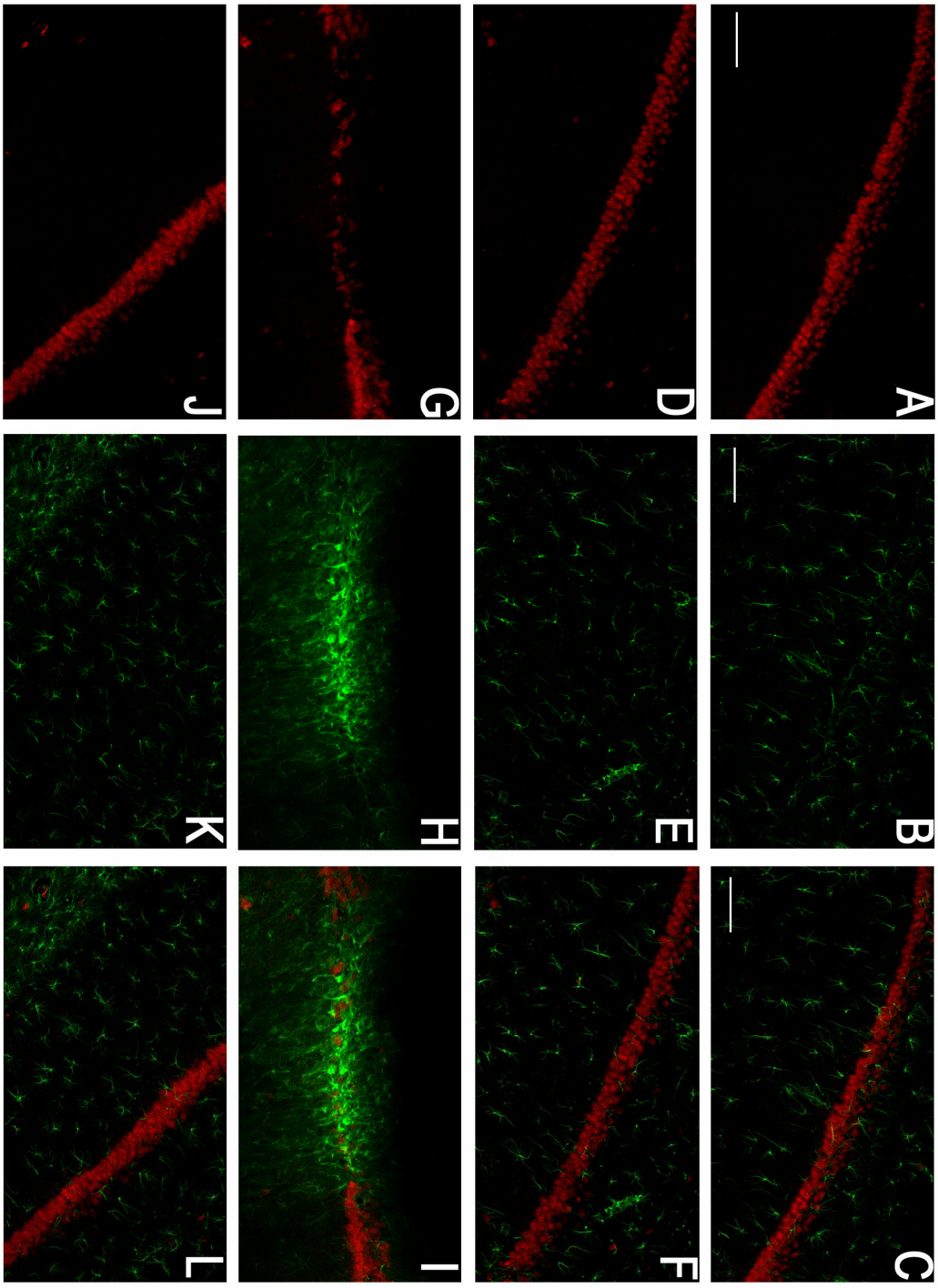


Figure 4

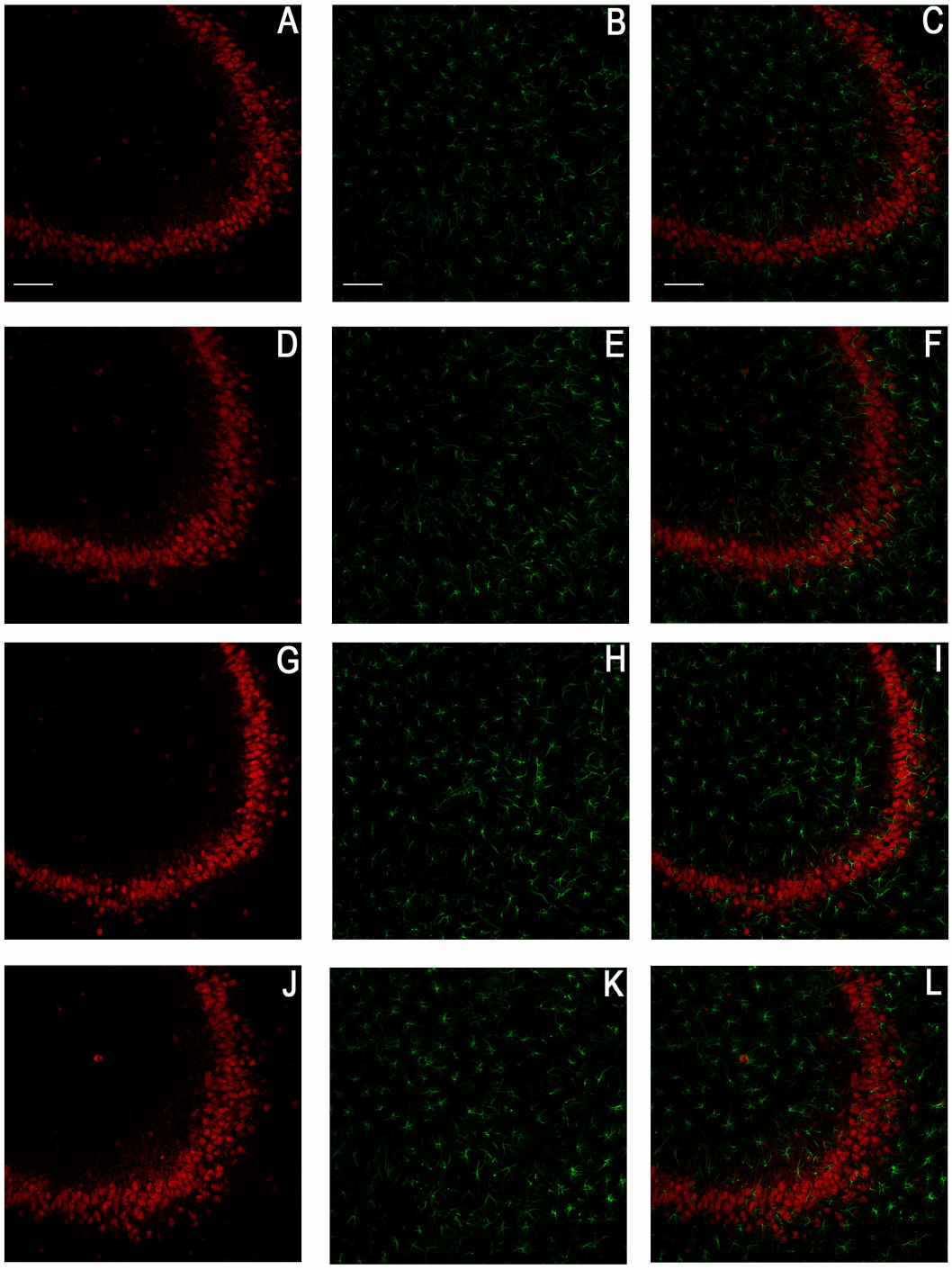
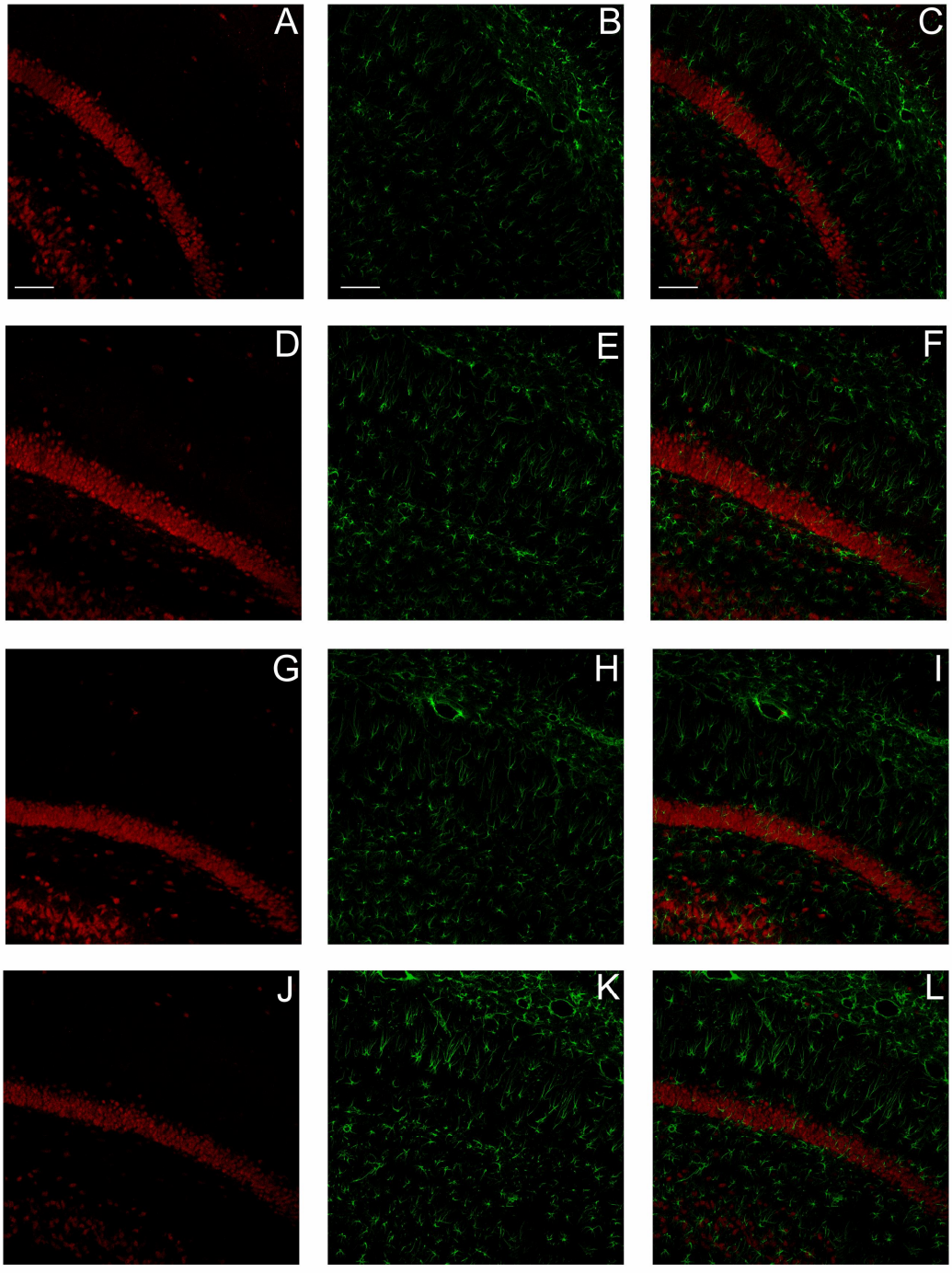


Figure 5



Capítulo VI

Estudo sobre o possível sítio de união da guanosina na membrana plasmática cerebral de ratos

Marcelo Ganzella, Roberto Farina Almeida, Denise Barbosa Ramos, Ana Elisa Böhmer, Gisele Hansel, Fernanda Lopes, Moema Queiroz, Bruno Siqueira Mietto, Gustavo da Costa Ferreira, Franciele Stefanello, Maria Cristina Faccioni-Heuser, Ana Maria Blanco Martinez, Ângela Teresinha Wyse, Moacir Wajner, Fabio Klamt, Diogo Onofre Souza

Artigo para discussão

Estudo sobre o possível sítio de união da guanosina a membrana plasmática cerebral de ratos

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1. Introdução

Já está bem estabelecido que a excitotoxicidade glutamatérgica está envolvida em vários distúrbios cerebrais agudos (como acidente vascular cerebral, convulsão, trauma cerebral, entre outros) e crônicos (depressão, demência vascular, doenças crônicas neurodegenerativas, entre outros).

Nosso grupo demonstrou previamente que os efeitos dos nucleotídeos da guanina sobre a união do acido caínico (não acoplado a proteínas G) e sobre a atividade da adenilato ciclase poderiam estar dissociados (Souza and Ramirez, 1991). Este efeito sugeriu que a inibição da ligação do acido caínico pelos nucleotídeos da guanina não é dependente de sistemas mediados por proteínas G. Além disso, outros grupos relataram que o efeito inibitório dos nucleotídeos da guanina sobre a união do glutamato e de ligantes glutamatérgicos de receptores ionotrópicos à membrana plasmática apresentava várias inconsistências (Baron et al., 1989; Butcher et al., 1986; Hood et al., 1990; Monahan et al., 1988; Paas et al., 1996; Sharif and Roberts, 1981). Nesse contexto, estudos subseqüentes do nosso grupo reforçaram a hipótese de que os nucleotídeos da guanina podem antagonizar a neurotransmissão glutamatérgica por atuarem em sítios localizados na superfície extracelular da membrana plasmática (Paz et al., 1994; Ramos et al., 1997; Rotta et al., 2004; Rubin et al., 1997b; Tasca et al., 1995).

Concomitante ao estudo sobre a interação entre os nucleotídeos da guanina e os receptores glutamatérgicos, nós iniciamos a investigação sobre o potencial efeito neuromodulador e/ou neuroprotetor dos nucleotídeos da guanina em modelos envolvendo glutamato, tanto *in vitro* (Burgos et al., 1998; Regner et al., 1998; Rubin et al., 1997a; Tasca et al., 1995; Tasca et al., 1998;

Tasca et al., 1999; Tasca and Souza, 2000), quanto *in vivo* (Malcon et al., 1997; Roesler et al., 2000; Saute et al., 2006; Schmidt et al., 2000; Schmidt et al., 2005; Soares et al., 2004). Dessa maneira, observamos que a administração intraperitoneal de GMP, mas também de guanosina (nucleosídeo da guanina que não inibe a união dos ligantes glutamatérgicos), foi capaz de prevenir convulsões causadas pelo ácido quinolínico (Schmidt et al., 2000).

Adicionalmente, trabalhos seguintes do nosso grupo reportaram que alguns dos efeitos promovidos pelo GMP sobre parâmetros que envolvem o sistema glutamatérgico dependem de sua conversão a guanosina, como o aumento da captação de glutamato por astrócitos em cultura e os efeitos amnésicos, antinociceptivos e anticonvulsivantes em roedores (Frizzo et al., 2003; Saute et al., 2006; Schmidt et al., 2007; Schmidt et al., 2008; Schmidt and Souza, 2010; Soares et al., 2004).

Atualmente, há vários trabalhos na literatura que evidenciam o potencial neuroprotetor da guanosina em modelos experimentais de doenças cerebrais que envolvem excitotoxicidade glutamatérgica. Nosso grupo demonstrou que a administração sistêmica de guanosina além de proteger roedores de convulsões induzidas por agonistas glutamatérgicos (Lara et al., 2001; Schmidt et al., 2005; Schmidt et al., 2007; Schmidt and Souza, 2010; Vinade et al., 2003; Vinade et al., 2005), também é capaz de prevenir a queda da captação de glutamato no hipocampo de ratos jovens submetidos à hipóxia e isquemia (Moretto et al., 2005; Moretto et al., 2009). A administração sistêmica de guanosina também foi capaz de aumentar a sobrevida e diminuir o prejuízo cognitivo em modelo animal de isquemia cerebral in vivo (Chang et al., 2008), proteger contra a morte de células neurais e estimular a proliferação celular em

modelo animal de doença de Parkinson (Su et al., 2009). Os mecanismos moleculares e celulares envolvidos na neuroproteção pela guanosina não são bem conhecidos. O nosso grupo de pesquisa demonstrou que a guanosina é capaz de estimular a captação de glutamato em cultura de astrócitos e em fatias de córtex cerebral (Frizzo et al., 2001; Frizzo et al., 2002; Frizzo et al., 2003; Gottfried et al., 2002).

Como a captação astrocitária de glutamato é o mecanismo mais importante para modular sua ação sináptica e inibir a hiperestimulação dos receptores glutamatérgicos (Danbolt, 1994; Danbolt, 2001; Sheldon and Robinson, 2007; Tzingounis and Wadiche, 2007), a estimulação da captação de glutamato pela guanosina pode ser um dos potenciais mecanismos responsáveis pelos seus efeitos neuroprotetores. Entretanto, o mecanismo pelo qual a guanosina estimula esta captação, e a relação desta com os efeitos neuroprotetores ainda não está totalmente esclarecido.

Desde a constatação do potencial efeito neuroprotetor da guanosina, a busca pela caracterização de um sítio de união da guanosina à membrana plasmática cerebral se tornou uma das principais linhas de pesquisa do grupo. Uma das metodologias amplamente aplicadas para a identificação de um sítio de união de um composto à membrana plasmática é através do estudo da união da molécula alvo marcada radioativamente à preparações enriquecidas com membrana plasmática (Bigott-Hennkens et al., 2008; Bylund and Murrin, 2000). Nosso grupo tem experiência com esta metodologia, principalmente no estudo da união de glutamato (devido principalmente aos trabalhos sobre interação dos derivados da guanina com os receptores glutamatérgicos) (Dalcin et al., 2007; Emanuelli et al., 1998; Martini et al., 2007; Moreira et al.;

Porciuncula et al., 2000; Porciuncula et al., 2002; Porciuncula et al., 2004a; Porciuncula et al., 2004b; Rubin et al., 1997b; Schroder et al., 2000) e/ou em ligantes de receptores para adenosina (Tasca and Souza, 2000). Apesar da ampla utilização desta metodologia em nosso laboratório, não conseguimos obter resultados reprodutíveis com a guanosina.

No ano de 2002, Traversa e colaboradores identificaram um sítio de união para a guanosina em preparação de membranas cerebrais de ratos (Traversa et al., 2002; Traversa et al., 2003). Essa identificação de um sítio, possivelmente extracelular, foi completamente embasada em uma metodologia que fornece uma preparação de membranas com grande contaminação de fração mitocondrial. Considerando que a mitocôndria também apresenta metabolismo purinérgico, o sítio de união para guanosina nessa preparação não pode ser considerado exclusivamente e/ou predominantemente sináptico. Além disso, o protocolo aplicado por Traversa não demonstrou reprodutibilidade em nosso laboratório.

Existem fortes evidências de que exista um possível sítio de união da guanosina à membrana plasmática cerebral, que possivelmente este sítio estaria envolvido no mecanismo neuroprotetor da mesma e, entretanto, ainda não há um protocolo de metodologia padronizado para caracterizá-lo. Assim sendo, este capítulo da tese tem como objetivo principal apresentar os resultados obtidos nos últimos 3 anos sobre a investigação do sítio de união da guanosina a membrana plasmática cerebral de ratos. Os experimentos foram focados na purificação da fração subcelular enriquecida em membrana plasmática, quanto à presença de mitocôndrias e purinas endógenas.

É importante salientar que a busca do grupo por esclarecer este sítio de união remonta à, pelo menos, 16 anos. Começou com a Tese de doutorado de Diogo Lara e a Iniciação Científica de André Schmidt, ambos já Doutores, o Diogo Lara há vários anos. Desde 1994, portanto, além destes dois pesquisadores iniciais, muitos alunos e professores participaram desta grande empreitada de identificar o sítio de união de guanosina em cérebro de roedores. Apesar da participação de inúmeros pesquisadores, mesmo altamente qualificados cientificamente, muito pouco se avançou neste tema.

Assim, a idéia de submeter este tópico nesta forma é propiciar uma excelente oportunidade para o nosso grupo de discutir com uma banca altamente qualificada no assunto, visando compreender melhor os preliminares resultados obtidos e, fundamental, discutir perspectivas para novas abordagens, almejando promissoras interações. É adequado enfatizar que, talvez, a grande contribuição inicial que esta tese trouxe ao tema é ter percebido, e preliminarmente realizado, a “limpeza” de purinas da preparação de membrana originalmente utilizada para investigar o sítio de união de guanosina em membrana plasmática de cérebro de rato.

Reafirmo que a idéia básica da forma escolhida de apresentar este capítulo é propiciar uma intensa discussão com a banca.

2. Materiais e Métodos

2.1. Animais

Ratos Wistar adultos machos (90 – 120 dias, pesando 300-350g) foram mantidos em ciclo claro-escuro de 12 horas, em temperatura constante de 22 ± 1°C. Eles foram abrigados em gaiolas plásticas (5 ratos por gaiola), com água e

ração comercial *ad libitum*. Todos os procedimentos estiveram de acordo com o Guia de Cuidados e Uso de Animais de Laboratório, adotado pelo National Institute of Health (USA) e pela Federação das Sociedades Brasileiras de Experimentação Animal (FESBE), e foram aprovados pelo Comitê de Ética em Pesquisa de Universidade Federal do Rio Grande do Sul.

2.2. Preparação das frações sub-celulares enriquecidas em membrana plasmática (MP) e/ou em mitocôndria (MI) cerebrais

Utilizamos a técnica original de Jones e Matus com pequenas modificações para obter uma fração enriquecida em membrana plasmática (MP) e outra fração enriquecida em mitocôndria (MI) de cérebro de ratos (Jones and Matus, 1974). Para cada preparação, 2 animais foram mortos por decapitação sem anestesia e seus cérebros rapidamente removidos. Todos os procedimentos subseqüentes foram realizados a 4^{o}C. Os cérebros totais (sem bulbo olfatório e cerebelo) foram homogeneizados em sacarose 0,32 M (10% p/v) e centrifugados a 1.000g por 10 minutos. O sobrenadante foi armazenado (S1) e o precipitado foi novamente homogeneizado e centrifugado como anteriormente descrito. O sobrenadante da segunda centrifugação (S2) foi adicionado ao S1 e S1+S2 foi centrifugado a 11.000g por 20 min. O precipitado foi ressuspenso em 30 ml de tampão Tris / HCl 5 mM pH 7,4 por 30 min para lise celular por choque osmótico. Após, o lisado foi centrifugado a 14.000g por 10 min e o precipitado ressuspenso no mesmo tampão Tris/HCl em 1,2 mL de volume total. Após, foi adicionado sacarose 48% (p/v) até formar uma solução 34% (p/v) de sacarose. Uma camada de sacarose 28% (p/v) foi adicionada acima da camada 34% e um pequeno volume de sacarose 10% (p/v) foi

adicionado na superfície. Este gradiente de densidade foi ultracentrifugado a 60.000 g por 2 horas. A fração “membrana plasmática” (MP) foi coletada na interface entre 34% e 28,5% de sacarose e a fração “membrana mitocondrial” (MI) foi coletada no precipitado abaixo da camada 34%. Após, as frações foram lavadas duas vezes em tampão Tris/HCl 5 mM 7,4 a 18.000 g por 15 min. As frações foram imediatamente congeladas a -20^{o}C em microtubos em 1 mL de tampão Tris / HCl 5 mM pH 7,4 por no máximo 1 mês. Antes de serem utilizadas nas outras metodologias, as frações foram descongeladas em temperatura ambiente, após foi adicionado 15 mL de tampão Tris / HCl pH 7.4 gelado e as frações então eram centrifugadas a 18.000 g por 15 minutos. O precipitado foi ressuspenso em tampão apropriado para cada metodologia.

2.3. Incubação das frações MP e MI

Com o objetivo de analisar a presença de purinas e/ou glutamato endógenos, as frações MP e MI foram incubadas (1,2 mg de proteína/mL) por diferentes tempos em tampão líquido cérebro-espinhal artificial sem fosfato (LCEAsP) contendo (em mM): 137 NaCl; 5.36 KCl 1.26 $CaCl_2$; 0.41 $MgSO_4$; glucose (pH 7.2) a 35^{o}C. Após a incubação as preparações eram centirfugadas a 30^{o}C por 18.000 g e o sobrenadante era imediatamente congelado a -20^{o}C, e mantido assim até ser utilizado para a detecção dos compostos. O tempo 0 significa que as amostras não foram previamente incubadas, porem processadas como descrito acima para a coleta do sobrenadante.

Nos experimentos que utilizamos preparações pré-incubadas por 24 h, as frações eram incubadas por 24 h como descrito acima, após, centrifugadas a 30^{o}C por 18.000 g e ressuspensas no tampão apropriado para cada técnica.

Denominamos frações MP ou MI frescas, quando as amostras não foram incubadas e MP ou MI 24 h quando as frações foram previamente incubadas por 24 h.

2.4. Análise espectrofotométrica da atividade do complexo IV da cadeia respiratória

A atividade da citocromo C oxidase (complexo IV) foi determinada nas preparações MP e MI frescas ou MP e/ou MI 24 h de acordo com (Rustin et al., 1994). A atividade do complexo IV foi calculada em nmol. min-1. mg de proteína-1.

2.5. Análise espectrofotométrica da atividade da Na^+, K^+-ATPase

Para o ensaio da reação de Na^+, K^+-ATPase foi adicionado 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM de tampão Tris/HCl, pH 7,4 e aproximadamente 3 µg de proteína das preparações em um volume de 200 mL. O ensaio enzimático ocorreu a 37 º C durante 5 minutos, iniciando com a adição de ATP dissódico, sem vanádio em uma concentração final de 3 mM. A reação foi interrompida pela adição de 200 µL de ácido tricloroacético 10%. Mg^{2+}-ATPase ouabaína insensível, foi analisada nas mesmas condições, com a adição de 1 mM de ouabaína. A atividade da Na^+, K^+-ATPase foi calculada pela diferença entre os dois ensaios (Tsakiris e Deliconstantinos, 1984). A liberação de fosfato inorgânico (Pi) foi medida pelo método de Chan et al. (1986). As atividades enzimáticas foram calculadas em nmol Pi liberado/min/mg proteína.

2.6. Imunoquantificação do transportador mitocondrial de nucleotídeos da adenina (TNA)

Para análise por Western blotting, as amostras foram homogeneizadas em tampão Hepes (pH 7,4) contendo 0,1% SDS e coquetel de inibidores de protease e fosfatase, e então normalizadas com um tampão de amostra (4% de dodecilsulfato de sódio, 2,1 mM EDTA, 50 mM Tris e 5% mercaptoetanol). As amostras (20µg proteína/poço) foram submetidas à eletroforese e transferidas para uma membrana de nitrocelulose. As membranas foram processadas da seguinte forma: (1) foram bloqueadas com albumina sérica bovina por 1h30 min; (2) incubadas durante uma noite com anticorpo primário anti-TNA (Santa Cruz, Brasil); (3) incubadas com anticorpo secundário conjugado à peroxidase por 1h30 min; (4) a quimioluminescência (ECL) foi detectada por filmes de raio-X. Os filmes foram escaneados e a intensidade das bandas foi analisada usando ImageJ (desenvolvido no US National Institutes of Health e disponível na internet em http://rsb.info.nih.gov/nih-image/).

2.7. Microscopia eletrônica de transmissão

MP frescas e/ou MP 24 h em tampão LCEAsP foram ressuspensas e fixadas em uma solução contendo paraformaldeído 2% e 2,5% de glutaraldeído em tampão fosfato 0,1 M (pH 7,4) durante 60 min. Após, a membrana foi lavada no mesmo tampão, contendo 0,1 M tampão cacodilato (pH 7,4) e pós-fixadas em 1% tetróxido de ósmio, 1,5% de ferricianeto de potássio. Estes procedimentos previnem a formação de camadas de membrana por ligação cruzada aleatoriamente entre membranas em suspensão. Incorporação: todos os precipitados foram tratados com ácido tânico, desidratados e incorporados

em Epon 812 pelo procedimento convencional. As amostras foram seccionadas no eixo vertical (para controle de qualquer estratificação) em secções de 60 nm. As secções foram coradas com acetato de uranila e citrato de chumbo; as secções foram analisadas em microscópio eletrônico de transmissão (JEM 1200 EXII, Japão, CME, UFRGS).

2.7.1. Análise quantitativa

Para o procedimento de amostragem, foram fotografados, no microscópio eletrônico de transmissão, 5 campos aleatórios em 3 preparações de MP frescas e/ou MP 24 h com uma ampliação de 75.000 x. Foram obtidas 50 fotos para cada preparação. Todos os negativos foram digitalizados e submetidos à análise quantitativa utilizando o programa Image J (desenvolvido no National Institutes of Health (USA) e disponível na internet no endereço eletrônico http://rsb.info.nih.gov/nih-image/). Com base nas analises qualitativas, foi calculado e comparado os seguintes parâmetros entre os dois grupos (MP fresca e MP 24 h): número total de vesículas, área das vesículas, terminais pós sinápticos.

2.8. Cromatografia liquida de alta eficiência (CLAE)

2.8.1. Dosagem de purinas:

A determinação das concentrações de purinas foi realizada através da metodologia de CLAE (Schmidt et al., 2009). Foram analisados os seguintes compostos: guanosina 5` trifosfato (GTP), guanosina 5` difosfato (GDP), guanosina 5`monofosfato (GMP), guanosina (GUO), adenosina 5`trifosfato (ATP), adenosina 5`difosfato (ADP), adenosina 5`monofosfato (AMP),

adenosina (ADO), inosina (INO), hipoxantina (HIPOX), xantina (XAN) e acido úrico (AU) no sobrenadante das preparações MP e MI frescas, incubadas por diferentes tempos. As análises foram realizadas pelo sistema de cromatografia Shimadzu Class-VP. O sistema é composto por uma bomba quaternária de gradiente, sistema de desgaseificaçao, autoinjetor modelo Shimadzu SIL-10AF com uma válvula de injeção com capacidade de até 50 µl e detector de ultravioleta onde as detecções foram realizadas em comprimento de onda de 254 nm. A separação das amostras foi feita através de uma coluna modelo Supelco C18 250 mm × 4.6 mm, 5 µm. A dosagem foi realizada em um fluxo de 1.2 mL/min a uma temperatura de 24°C. A composição dos tampões empregados (fase móvel) foi: Solução A contendo150 mmol/l de tampão fosfato pH 6,0 e 150 mmol/L de cloreto de potássio. Solução B composta da solução A com 15% de acetonitrila. O perfil do gradiente foi modificado da seguinte forma de acordo com a solução B: 0% em 0.00 min, 2% em 0.05 min, 7% em 2.45 min, 50% em 10.00 min, 100% em 11.00 min e 0% em 12.40 min. Foi injetada 25 µl de amostra

2.8.2. Dosagem de glutamato:

A análise de glutamato foi realizada por CLAE, em sobrenadantes de MP e MI frescos ou incubados por diferentes tempos, de acordo com Joseph and Marsden, 1986. As amostras foram derivatizadas com o-ftalaldeído, separadas com uma coluna de fase reversa (Supelcosil LC-18, 250 mm x 4.6 mm, Supelco) em um sistema de cromatografia Shimadzu Class-VP. O fluxo da fase móvel foi de 1.4 mL/min a uma temperatura de 24^{o}C. Composição da Solução A: 0,04 mol/L tampão fosfato de sódio monoidratato, pH 5,5, contendo 20% de

metanol. Solução B: 0.01 mol/L de fosfato de sódio monobásico pH 5,5, contendo 80% de metanol O perfil do gradiente foi modificado da seguinte forma de acordo com a solução B: 0% em 0.00 min, 25% em 13.75 min, 100% em 15.00-20.00 min, 0% em 20.01-25.00 min. A absorbância foi medida em um detector de fluorescência Shimadzu onde o comprimento de excitação foi de360 nm e o de emissão de 455 nm. Foi injetado 25 µL de amostra.

2.9. Protocolo da união da [^{3}H] guanosina a MP e MI

A quantificação da união da [^{3}H] guanosina à MP e/ou MI frescas ou MP e/ou MI 24 h foi realizada através da técnica de filtração, usando filtros de fibra de vidro GF/B. Alíquotas contendo de 10 a 50 µg de proteína foram incubadas por diferentes tempos em tampão LCEAsP pH 7.2 (ou Tris 5 mM pH 7.4 quando indicado) a 35^{o}C (ou 4^{o}C, quando indicado) com 200 nM de [^{3}H] guanosina em um volume total de 250 µl. A união inespecífica de [^{3}H] guanosina foi determinada pela adição de 1 mM de guanosina não radioativa no meio, em um ensaio paralelo. A incubação foi iniciada pela adição de [^{3}H] guanosina. A reação foi parada por filtragem a vácuo seguidas de 3 lavagens seqüenciais com 3 ml de tampão LCEAsP gelado. O procedimento durou no máximo até 10 segundos. Os filtros foram colocados para secar por 1h a 40 min e depois os filtros foram transferidos para microtubos de polietileno. Foi adicionado líquido de cintilação nos microtubos e a cintilação incorporada no material biológico foi contada por um contador de cintilação (HIDEX 300 SL). Os experimentos foram realizados em triplicatas.

2.10. Protocolo de união de [^{3}H] Glutamato a MP

A quantificação da união do [^{3}H] glutamato a MP frescas ou 24 h foi realizada nas mesmas condições descritas previamente para a quantificação da união da [^{3}H] guanosina. [^{3}H] glutamato (200 nM) foi incubado com MP frescas ou MP 24 h em tampão LCEAsP por diferentes tempos a 35^{o}C (ou 4^{o}C, quando indicado) e a união inespecífica foi determinada pela adição de 1 mM de glutamato não radioativo no meio em um ensaio paralelo.

2.11. Determinação de Proteína

A concentração de proteínas for determinada pelo método do Comassie Blue (Bradford, 1976). Albumina sérica bovina foi usada como padrão.

2.12. Análise estatística

Os dados obtidos são expressos como média ± E.P.M. Foi utilizado teste t de Student quando comparamos somente dois grupos, ANOVA de duas vias para analisar mais do que dois grupos em duas condições distintas, seguido pelo teste de Bonferroni, e ANOVA de uma via com medidas repetidas para analisar o efeito da incubação sobre o conteúdo de purinas. O conteúdo endógeno de glutamato foi avaliado pelo teste t de Student com medidas pareadas. $P<0,05$ foi considerado representar uma diferença significativa.

3. Resultados

3.1. Qualidade das preparações

Para avaliar o enriquecimento das frações MP e MI frescas com membrana plasmática e membrana mitocondrial, respectivamente, nós

realizamos a quantificação da atividade da enzima Na^+K^+ATPase (marcador de membrana plasmática), da atividade do complexo IV (marcador de membrana mitocôndrial) e do imunoconteúdo do TNA mitocondrial. A figura 1 mostra que, comparativamente, a fração MP fresca apresenta maior atividade da enzima NA^+K^+ATPase (A), menor atividade do complexo IV (B) e do imunoconteúdo do ANT mitocondrial (C) comparado com a fração MI fresca ($P<0.05$, teste t de Student).

3.2. Analise da fração MP

3.2.1. Contaminantes endógenos

3.2.1.1. Purinas

Para avaliar a presença de purinas nas frações MP nós incubamos as preparações por diferentes tempos, coletamos o sobrenadante após centrifugação, e dosamos as purinas através de CLAE. A figura 2 mostra que MP frescas contem níveis significativos de todos os nucleosideos das purinas e de seus derivados. Os níveis dos nucleosídeos guanosina e adenosina aumentam significativamente após 30 min de incubação, permanecendo constantemente elevado até 2 h de incubação (2A). Os níveis de inosina, hipoxantina e xantina aumentam constantemente dos 30 min até 6 h de incubação. A hipoxantina e a xantina mantem seus níveis elevados até 24 h de incubação enquanto que os níveis de inosina diminuem (2B). Em nenhum dos tempos analisados foi detectado a presença de nucleotídeos derivados da guanina e da adenina e nem ácido úrico.

A Figura 2C mostra que as frações MP 24 h, quando reincubadas por mais 2 h não apresentaram níveis detectáveis de guanosina no sobrenadante.

3.2.1.2. Glutamato

A Figura 2D mostra que a fração MP fresca possui níveis de glutamato contaminante (0 h) e estes níveis aumentam após 24 h de incubação ($P<0.05$, teste t de Student). Quando a MP 24 h foi reincubada por 2 h, os níveis de glutamato foram indetectáveis.

3.2.2. Efeito da incubação

3.2.2.1. Qualidade da fração MP

Avaliamos os marcadores de membrana plasmática e mitocondrial após a incubação de 24 h das preparações MP. A Figura 3 mostra que a incubação não alterou a atividade da Na^+K^+-ATPase (A) e do complexo IV (B) mas diminuiu significativamente o imunoconteúdo do TNA mitocondrial (C).

3.2.2.2. Avaliação morfológica por microscopia eletrônica

A análise das microfotografias das preparações MP frescas e/ou MP 24 h demonstrou que ambas apresentam o mesmo número de vesículas (13 ± 4 vesículas por fotografia). Não houve diferença significativa no perfil dessas vesículas em relação à área das mesmas. O número de densidades pós-sinápticas foi significativamente maior nas preparações MP frescas (4±1 densidades pós sinapticas por amostra) quando comparadas com MP 24 h (1±1 densidades pós sinápticas por amostra). A Figura 4 mostra imagens representativas das preparações MP fresca (A) e incubada por 24 h (B).

3.2.2.3. União da [^{3}H] guanosina

A união específica da [^{3}H] guanosina nas preparações MP frescas não saturou ao longo do tempo de incubação (5A). Adicionalmente, a união inespecífica (que permaneceu estável em termos absolutos a partir de 30 min) variou em relação ao tempo de incubação entre 75% (30 min) a 23% (5 h) em relação ao total. A pré-incubação das MP por 24 h não só aumentou de forma extremamente significativa comparada com a MP fresca, como tornou a união da [^{3}H] guanosina saturável em relação ao tempo (Fig 5B). A união inespecífica da [^{3}H] guanosina nas preparações MP 24 h foi menor do que 2% em todos os tempos investigados (Fig. 5C). A união da [^{3}H] guanosina foi temperatura dependente (Fig 5D) e a saturabilidade da mesma em relação ao tempo dependeu do tampão utilizado (Fig 5E).

3.2.2.4. União do [^{3}H] glutamato

Avaliamos a união do [^{3}H] glutamato, como controle positivo da qualidade das frações, nas preparações MP frescas e MP 24 h. A união do [^{3}H] glutamato apresentou o mesmo perfil entre as frações MP frescas e/ou MP 24 h (Fig. 6). Entretanto, a união do [^{3}H] glutamato foi maior nas MP 24 h em relação às MP frescas.

3.3. Analise da fração MI

3.3.1. União da [^{3}H] guanosina

MI fresca não apresentou união da [^{3}H] guanosina em 5 min de incubação (dado não mostrado). As frações MI 24 h apresentaram um maior

nível união da [^{3}H] guanosina comparada com a MP 24 h (Fig 7). Adicionalmente, essa união foi sensível à temperatura.

3.3.1.1. Contaminantes endógenos e qualidade da fração após incubação

Conforme a união da [^{3}H] guanosina foi evidenciada nas preparações MI 24 h, nós avaliamos a concentração de guanosina e de glutamato no sobrenadante e a atividade das enzimas Na^+K^+ATPase, do complexo IV e o imunoconteudo do TNA mitocondrial, para avaliar as propriedades das mesmas. Em relação à guanosina endógena, MI fresca apresenta níveis significativos de guanosina e quando incubada por 24h essa concentração é maior. A preparação MI 24 h reincubada novamente por mais 2 h apresenta níveis significativos de guanosina (Fig 8A). MI fresca não apresenta níveis significativos de glutamato, porem constamos níveis significativos de glutamato após 24 h de incubação (Fig 8B). As preparações MI 24 h apresentaram uma significativa concentração de glutamato quando reincubadas novamente por 2 h.

Em relação aos marcadores de membrana plasmática e mitocondrial nas preparações MI, a incubação por 24 h aumentou a atividade da Na^+K^+ATPase (Fig 9A), diminuiu a atividade do complexo IV (Fig 9B) e aumentou o imunoconteudo do TNA mitocondrial (Fig 9C) em relação a MI frescas.

4. Discussão

Os resultados apresentados neste trabalho remetem aos nossos últimos avanços em relação à caracterização do possível sítio de união da guanosina à membrana plasmática cerebral de ratos.

4.1. Qualidade das frações MP e MI

Primeiramente, confirmamos que o nosso processo de subfracionamento celular separa frações enriquecidas em membrana plasmática (MP) de frações enriquecidas em mitocôndrias (MI) (Fig 1). Entretanto, é importante ressaltar que, embora enriquecidas, ambas as frações MP e MI, apresentam contaminação com mitocôndria e membrana plasmática, respectivamente. Essa contaminação se torna relevante pelos dados aqui apresentados, evidenciando que ambas apresentam união da guanosina.

4.2. Contaminação com purinas endógenas na fração MP

Pudemos observar que a preparação de MP apresentou concentração significativa de todos os nucleosideos purínicos e seus derivados analisados (Fig 2). Dessa forma, a concentração das purinas aumentou no sobrenadante da preparação MP ao longo do tempo quando esta foi incubada a 35^{o}C (liberação ou metabolização?). O perfil do aumento da concentração das diferentes purinas e/ou derivados analisados ao longo do tempo variou diferentemente. Em relação ao metabolismo das purinas; guanosina é metabolizada a guanina pela enzima Purina Nucleosídeo Fosforilase (PNP), que por sua vez é degradada a xantina pela enzima Guanase. A adenosina é desaminada pela enzima adenosina deaminase a inosina, a qual é degradada a hipoxantina pela enzima PNP. A hipoxantina é degrada até xantina pela ação da enzima xantina oxidase, a mesma enzima que degrada xantina a ácido úrico. Comparando o perfil de concentração das purinas ao longo do tempo, podemos sugerir que os três nucleosideos são degradados durante a

incubação (antes ou depois da liberação ao sobrenadante?), aumentando assim os níveis de hipoxantina e xantina. Além disso, os resultados sugerem a inatividade da enzima xantina oxidase, pois não aparece ácido úrico.

4.3. Efeito da incubação por 24 h na MP

Os resultados mostram que as preparações MP incubadas por 24 h não sofreram alterações na atividade da Na^+K^+ATPase e do complexo IV mas tiveram o imunoconteúdo do TNA mitocondrial diminuído. Esses resultados indicam que o perfil protéico da preparação foi modificado pela incubação.

A análise das fotografias demonstrou que o perfil morfológico da fração MP não foi alterado pela incubação de 24 h em relação à vesiculação das membranas. Entretanto, é importante salientar a presença de membranas vesículadas, o que pode indicar a captação de guanosina.

4.4. União da [^{3}H] guanosina à MP

Analisando a união da guanosina à MP fresca ou pré-incubada por 24 h, pudemos perceber que a união da guanosina não saturou ao longo do tempo nas MP frescas (Fig 3A) e que MP 24 h apresentaram um nível saturavel e extremamente elevado de união à guanosina (Fig 3B). Esse resultado sugere que a remoção da guanosina contaminante através da incubação elevou o nível de união da guanosina à MP. Como o resultado da análise das purinas sugeriu uma atividade enzimática, não podemos descartar a hipótese de que houve degradação da [^{3}H] guanosina e de que a cintilação quantificada possa representar a união de algum dos produtos de degradação da guanosina à MP. Mais ainda, a união da guanosina foi dependente de temperatura, diminuindo o

nível da união quando o experimento foi realizado a 4^{o}C. Adicionalmente, a união da guanosina a MP não foi saturável em tampão Tris/HCl 5 mM.

4.5. Parâmetros glutamatérgicos na fração MP

Como controle de qualidade das frações, investigamos parâmetros do sistema glutamatérgico. Os resultados mostram que as preparações MP apresentam níveis significativos de glutamato. Similar ao perfil das purinas, o nível de glutamato aumenta após 24 h de incubação a 35^{o}C e não foi detectado quando reincubamos as MP 24 h por mais 2 h. Em relação à união do [^{3}H] glutamato à MP, pudemos verificar que a união do mesmo é saturável nas preparações frescas e/ou MP 24 h.

4.6. Analise da fração MI

Nossos resultados mostraram que não houve união específica da [^{3}H] guanosina nas preparações MI frescas, porém houve um elevado nível de união da [^{3}H] guanosina nas MI 24 h. É importante ressaltar que analisamos a união da guanosina em uma incubação de 5 min e não foi verificada a saturação ao longo do tempo. A união da [^{3}H] guanosina nas preparações MI 24 h foi menor a 4^{o}C quando comparada a 35^{o}C.

Quando analisamos o perfil qualitativo das preparações MI 24 h, nós observamos que a atividade do complexo IV diminuiu significativamente enquanto que o imunoconteúdo do TNA mitocondrial aumentou. Esse resultado sugere que, embora a funcionalidade da membrana mitocondrial tenha sido alterada, a quantidade da mesma aumentou em relação ao conteúdo protéico total. Da mesma forma, a atividade da $Na^{+}K^{+}$ATPase também foi maior quando a fração MI foi incubada por 24 h em relação à preparação fresca.

É importante ressaltar que a incubação de 24 h aumentou a concentração de ambos, guanosina e glutamato, no sobrenadante da preparação MI. Diferentemente da preparação MP, quando foi retirado o sobrenadante após 24 h de incubação da preparação MI, os níveis de guanosina e glutamato foram significativos e até maiores quando comparados a 24 h de incubação. Esses resultados sugerem que a união da [^{3}H] guanosina à MI é maior do que o observado se descontarmos os níveis endógenos restantes da preparação após incubação.

5. Considerações gerais

Embora nós não possamos confirmar de forma satisfatória se há união da guanosina na membrana plasmática com o conjunto de resultados apresentados, eles levantam algumas questões importantes que merecem atenção.

Primeiramente, embora a fração MP, comumente usada em experimentos de união, apresente marcação para membrana plasmática (atividade da enzima Na^+K^+ATPase), ela também apresenta significativa marcação para membrana mitocondrial (complexo IV e TNA mitocondrial). Desde que nossos resultado indicam que possa haver um sítio na MI (Fig 7), seria importante desenvolver novas estratégias para melhor separar a membrana plasmática da membrana mitocondrial nessas preparações.

Como já mencionado, a principal contribuição dos resultados apresentados neste capítulo, é a detecção e a “limpeza” das purinas endógenas presentes nas preparações MP. É extremamente importante observar que o nível de guanosina endógena na preparação MP é maior do

que a quantidade utilizada nos experimentos de união. Concomitante a diminuição dos níveis de nucleosídeo, conseguimos detectar a união da guanosina em MP pré – incubada por 24 h. Desta forma, estes resultados nos possibilitam sugerir que é importante retirar as purinas da fração MP para melhor analisar a união da guanosina na membrana.

Outro resultado que cabe ser salientado é referente às fotografias representativas das preparações (Fig 4 A e B). Estas indicam que as MPs apresentam-se, em grande parte, vesiculadas. Já foi descrito anteriormente que as MP sinápticas vesiculada são capazes de captar purinas (Kraupp and Marz, 1995; Lee and Jarvis, 1988). Diante disso, não podemos descartar a hipótese de estarmos quantificando captação de guanosina e não somente a união da mesma na membrana. Um fato que corrobora esta suspeita é a sensibilidade da união da guanosina à temperatura. Desta forma, novos protocolos experimentais visando diferenciar os componentes 'união' e 'captação' terão de ser desenhados para melhor esclarecer os presentes achados.

Devido à diminuição dos níveis de nucleosideos e o aumento dos níveis de seus derivados ao longo do tempo pela incubação da MP, não podemos excluir a hipótese de estarmos evidenciando a união de outro composto com a MP, possivelmente um derivado da guanosina, e não a mesma. Ainda neste contexto, também há a dúvida se parte dos derivados dos nucleosideos (principalmente xantina e hipoxantina) são liberados da fração MP ou a totalidade vem da metabolização dos nucleosídeos. Para tanto, experimentos utilizando inibidor de transportador e/ou inibidores das enzimas do metabolismo purinérgico cerebral devem ser realizados. Também, pretendemos realizar

experimentos de CLAE com detector de radioatividade para investigar se outras moléculas derivadas da [^{3}H] guanosina apresentam radioatividade.

Outro dado relevante é o fato da união da guanosina à MP não ser saturável, mesmo após 24 h de pré – incubação, quando a união é verificada em tampão Tris/HCL 5 mM (tampão que é comumente utilizado nos experimentos de união). Dados preliminares, mas não mostrados nesse capítulo, indicam que a união da guanosina também não é saturável ao longo do tempo em tampão Hepes 5mM. Esses dados levantam questionamentos sobre a necessidade de um tampão isosmolar e/ou com constituição iônica para a união da guanosina à membrana plasmática ser saturável.

Em resumo, os presentes resultados apontam para a necessidade de uma melhor caracterização da preparação a ser utilizada para a investigação do sítio de união da guanosina à membrana plasmática cerebral de ratos. Por isso, a discussão destes resultados com a banca examinadora será de incontestável importância para avançarmos ainda mais neste objetivo.

Legendas

Figura 1: A fração MP é enriquecida em membrana plasmática e empobrecida de membrana mitocondrial em relação à fração MI. A fração MP apresentou maior atividade da enzima $Na^{+}K^{+}$ATPase (A), menor atividade do complexo IV (B), e menor imunoconteúdo de TNA (C). *P<0,05; teste t de Student. N = 6 preparações de cada fração.

Figura 2: MP apresenta quantidade significativa de purinas endógenas em tempos menores (A) e maiores de incubação (B). (C) Gráfico mostra que MP

pré-incubadas por 24 h, quando reincubadas por mais 2 h, não apresentam nível detectável de guanosina. (D) Gráfico mostra o conteúdo de glutamato nas mesmas situações descrita acima. *P<0,05; Teste ANOVA de uma via com medidas repetidas. N = 6 preparações.

Figura 3: A MP incubada por 24 h apresenta a mesma marcação de membrana plasmática e menor marcação mitocondrial em relação a MP fresca. Não houve alteração da atividade da enzima Na^+K^+ATPase (A), nem do complexo IV (B) pela incubação de 24 h da fração MP. O imunoconteúdo de TNA diminiui pela incubação (C). * P<0,05; teste t de Student. N = 6 preparações de cada fração.

Figura 4: Imagens de microscopia eletrônica de transmissão representativas da fração MP fresca (A) e MP incubada por 24 h (B).

Figura 5: (A) A união da [^{3}H] guanosina na fração MP fresca não é saturável em relação ao tempo. (B) Gráfico mostra a união da [^{3}H] guanosina na fração MP pré-incubada por 24 h é maior e saturável com o tempo qunadocomparada com a união na fração MP fresca. (C) O gráfico C mostra a união total, inespecífica e específica da [^{3}H] guanosina a MP pré-incubada por 24 h. (D) Gráfico mostrando que a união da [^{3}H] guanosina é sensível à temperatura. (E) A união da [^{3}H] guanosina depende do tampão em que a união é avaliada. *P<0,05; teste t de Student. N = 6 preparações de cada fração.

Figura 6: A união do [^{3}H] glutamato na MP pré-incubada por 24 h é maior, porém apresenta um perfil similar à fresca. N = 6 preparações.

Figura 7: A união da [^{3}H] guanosina é maior na fração MI em relação a MP (*), e dependente de temperatura (#). As frações MP e MI foram incubadas por 5 minutos na presença de [^{3}H] guanosina nas diferentes condições investigadas. *,# P<0,05; teste ANOVA de duas vias seguido de pos teste Bonferroni. N = 6 preparações de cada grupo.

Figura 8: (A) MI apresenta quantidade significativa de guanosina endógena. O gráfico A mostra que após 24 h de incubação, quando trocado o meio e reincubado por mais 2 h há um elevado nível de guanosina no sobrenadante. Glutamato não é detectado na fração MI fresca, porém aumenta quando MI é incubada por 24hs e quando reincubada por mais 2 h. * P<0,05; Teste ANOVA de uma via com medidas repetidas. N = 6 preparações.

Figura 9: A incubação por 24 h da fração MI não alterou a marcação de membrana plasmática, porém alterou os marcadores mitocondriais em relação a MI fresca. Gráficos mostram o aumento da atividade da enzima Na^+K^+ATPase (A), a diminuição da atividade do complexo IV(B) e o aumento do imunoconteúdo de TNA (C) na fração MI pré-incubada por 24 h em relação a MI fresca. *P<0,05; teste ANOVA de duas vias seguido de pos teste de Bonferroni. N = 6 preparações de cada fração.

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Figura 1

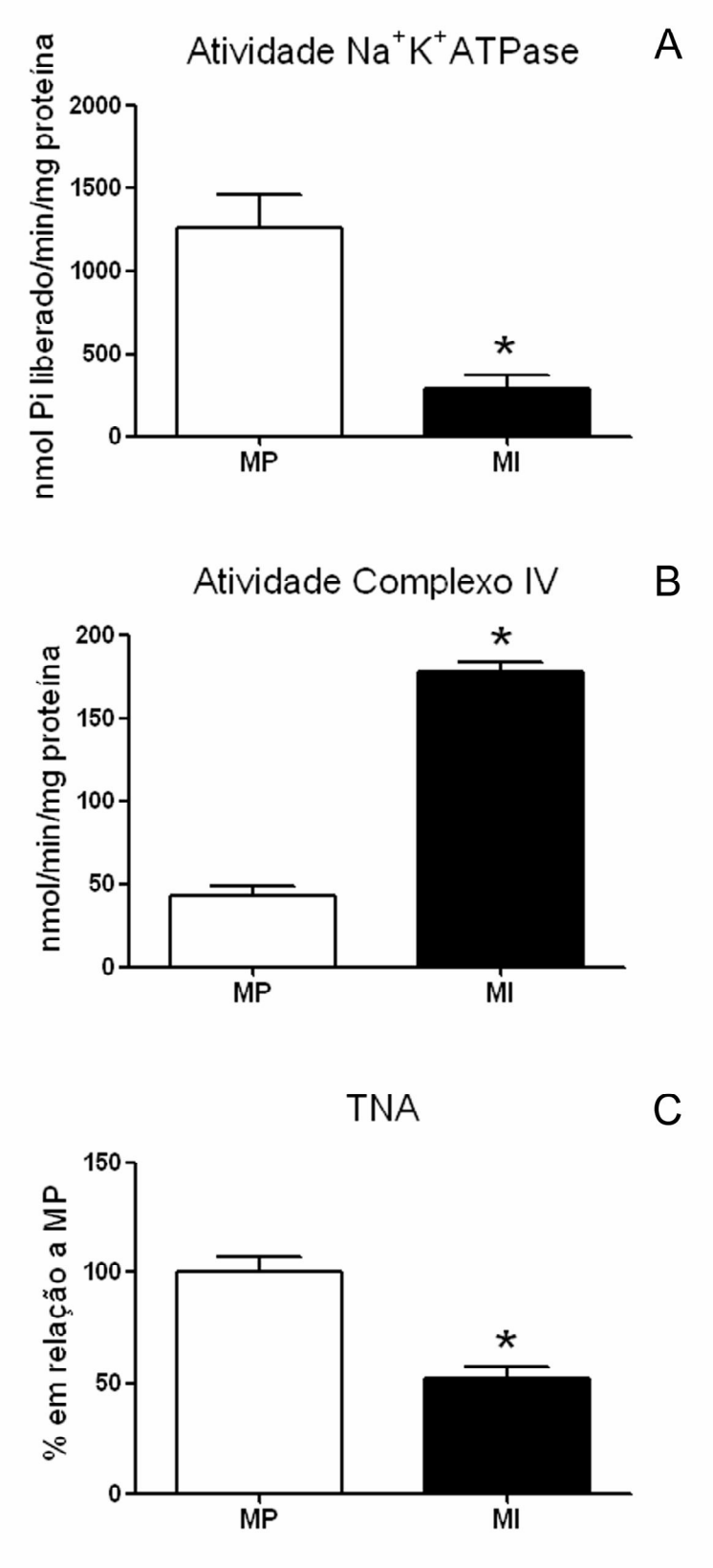

Atividade Na+K+ATPase
A
nmol Pi liberado/min/mg proteína
2000
1500
1000
500
0
*
MP
MI
Atividade Complexo IV
B
nmol/min/mg proteína
200
150
100
50
0
*
MP
MI
TNA
C
% em relação a MP
150
100
50
0
*
MP
MI

Figura 2

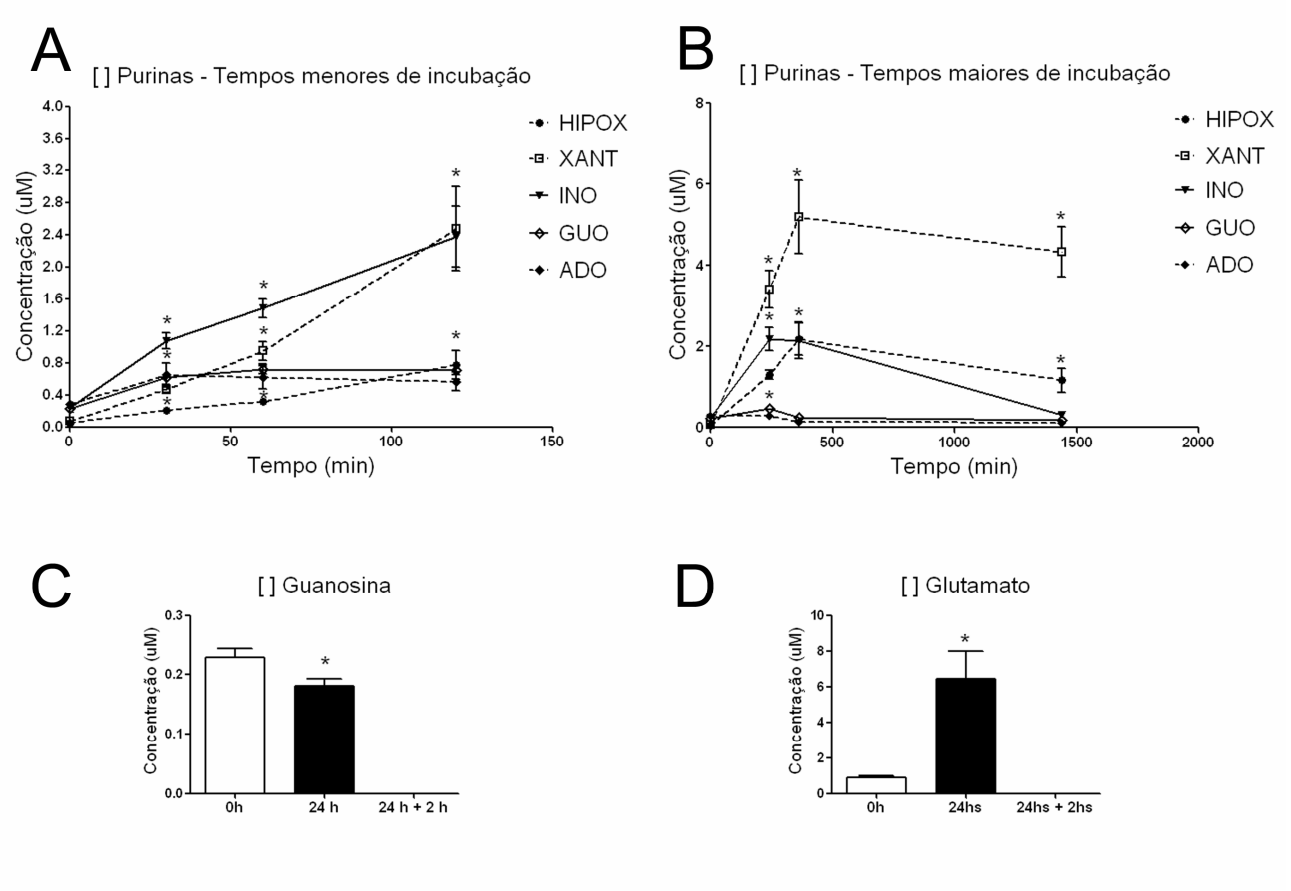

A
[ ] Purinas - Tempos menores de incubação
Concentração (uM)
Tempo (min)
HIPOX
XANT
INO
GUO
ADO
B
[ ] Purinas - Tempos maiores de incubação
Concentração (uM)
Tempo (min)
HIPOX
XANT
INO
GUO
ADO
C
[ ] Guanosina
Concentração (uM)
0h
24 h
24 h + 2 h
D
[ ] Glutamato
Concentração (uM)
0h
24hs
24hs + 2hs

Figura 3

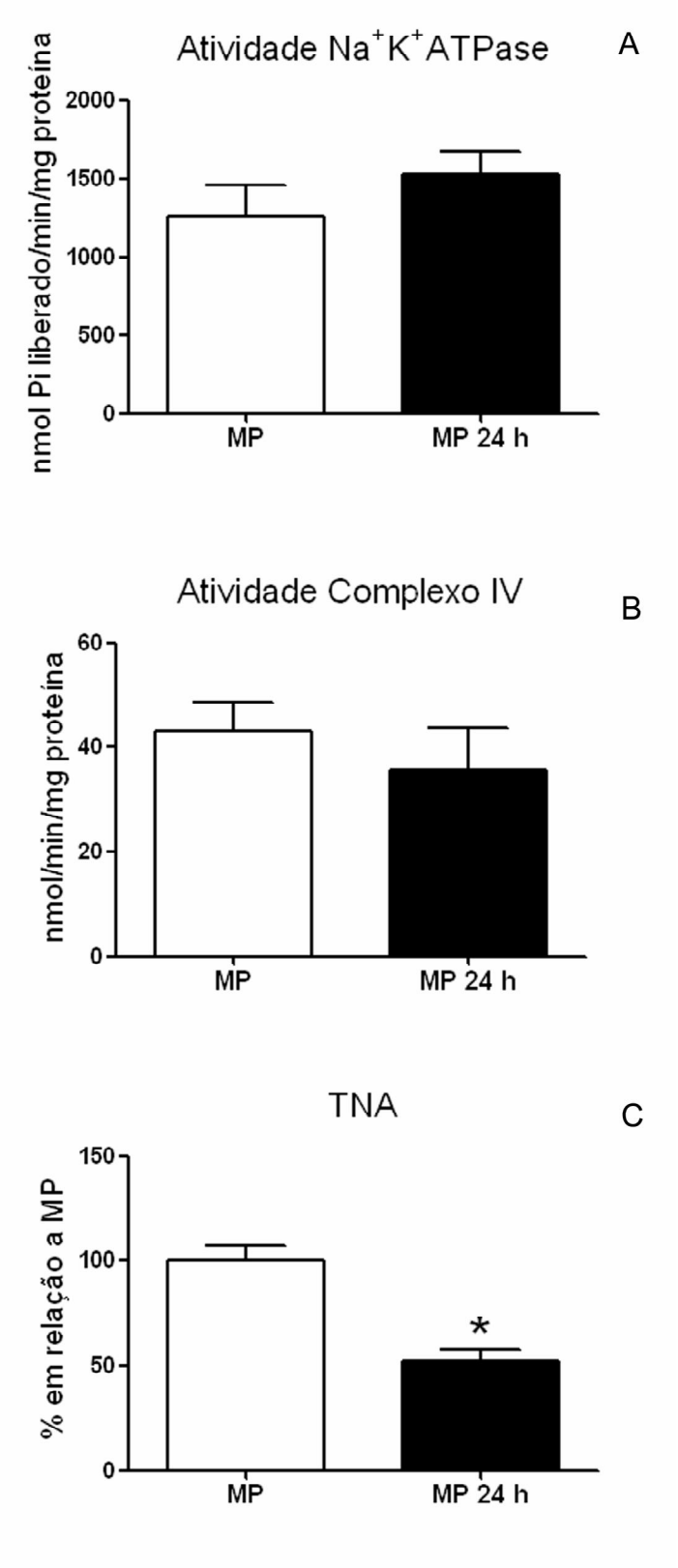
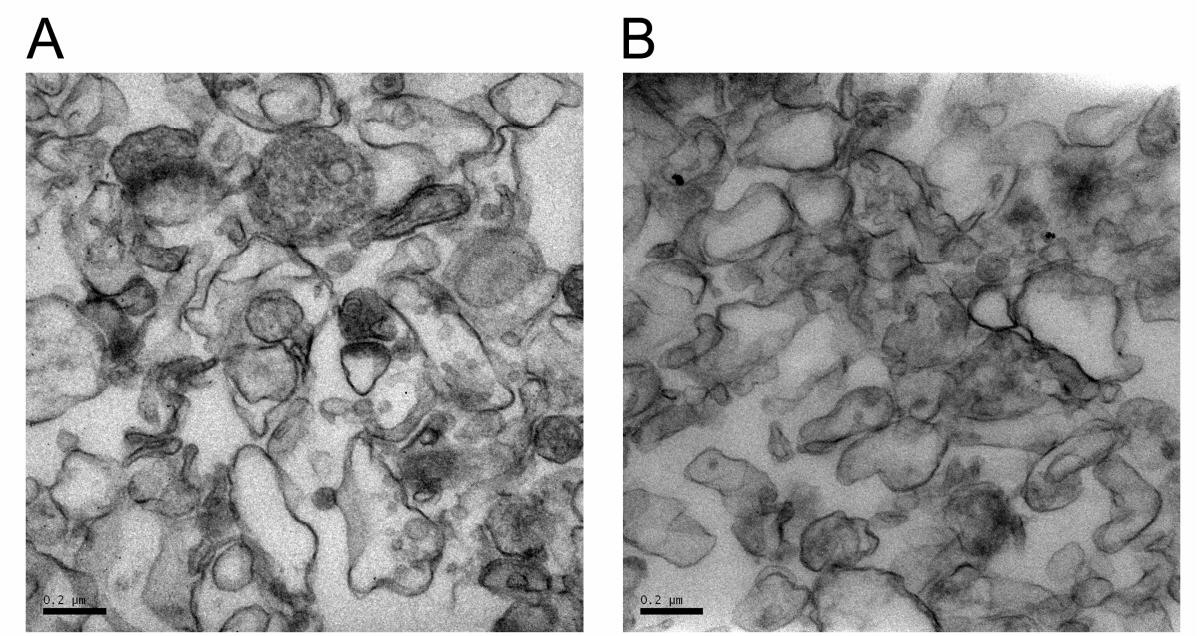

A
Atividade Na+K+ATPase
nmol Pi liberado/min/mg proteína
2000
1500
1000
500
0
MP
MP 24 h
B
Atividade Complexo IV
nmol/min/mg proteína
60
40
20
0
MP
MP 24 h
C
TNA
% em relação a MP
150
100
50
0
*
MP
MP 24 h

Figura 4



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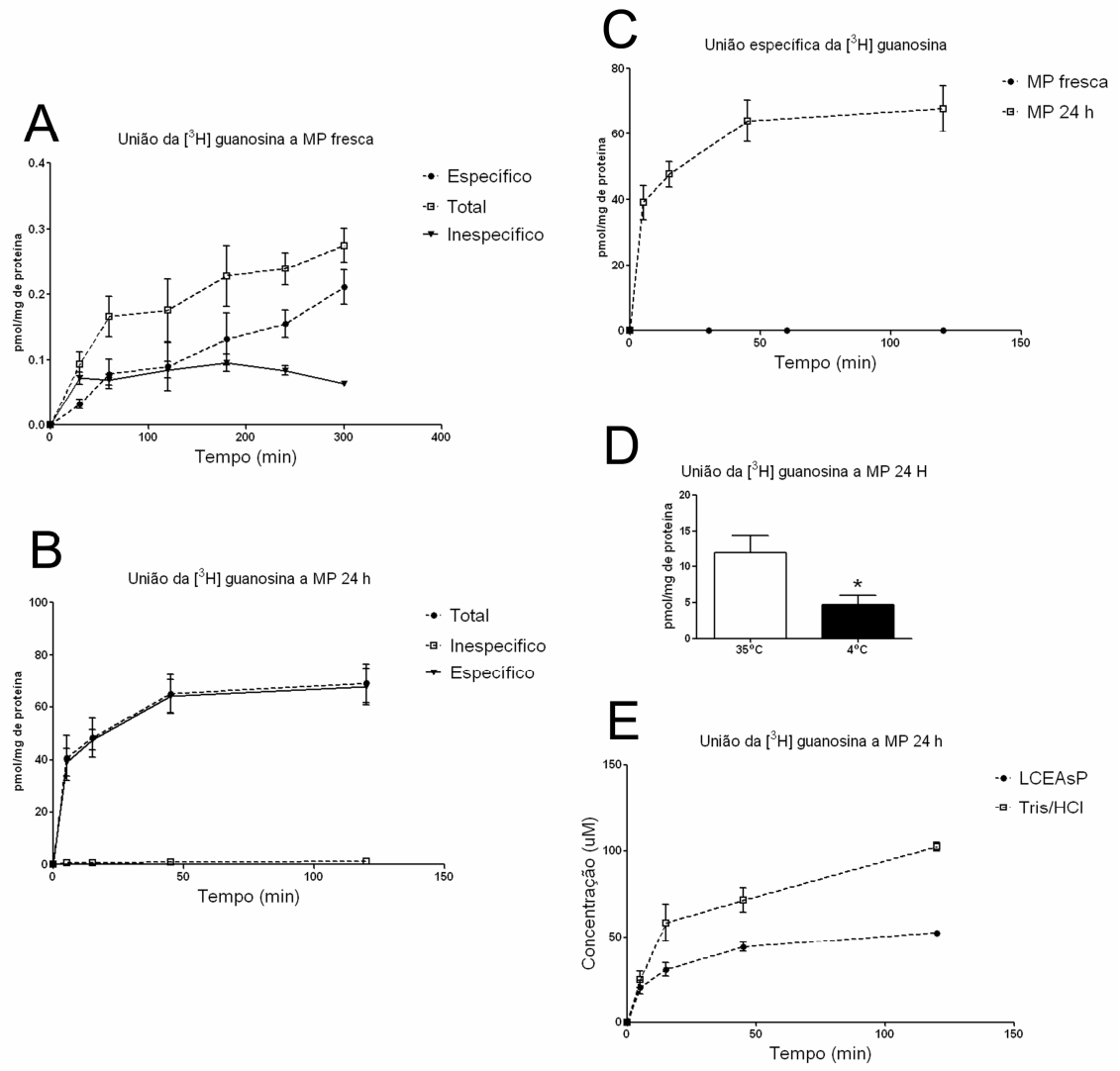

A
União da [3H] guanosina a MP fresca
Especifico
Total
Inespecifico
pmol/mg de proteina
Tempo (min)
B
União da [3H] guanosina a MP 24 h
Total
Inespecifico
Especifico
pmol/mg de proteina
Tempo (min)
C
União específica da [3H] guanosina
MP fresca
MP 24 h
pmol/mg de proteina
Tempo (min)
D
União da [3H] guanosina a MP 24 H
pmol/mg de proteina
35°C
4°C
*
E
União da [3H] guanosina a MP 24 h
LCEAsP
Tris/HCl
Concentração (uM)
Tempo (min)

Figura 6

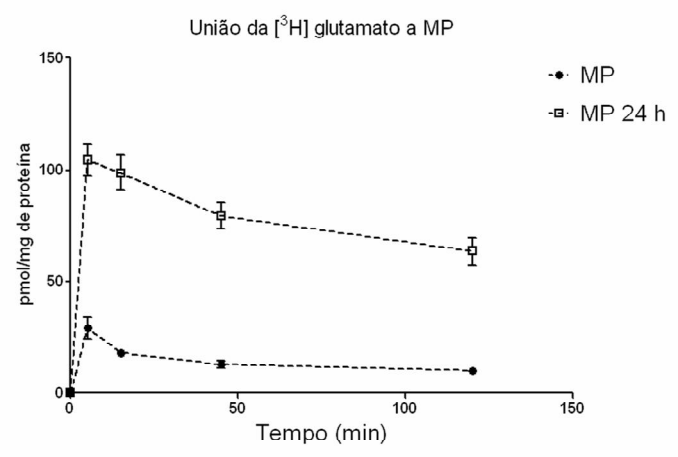

União da [3H] glutamato a MP
MP
MP 24 h
pmol/mg de proteína
150
100
50
0
0
50
100
150
Tempo (min)

Figura 7

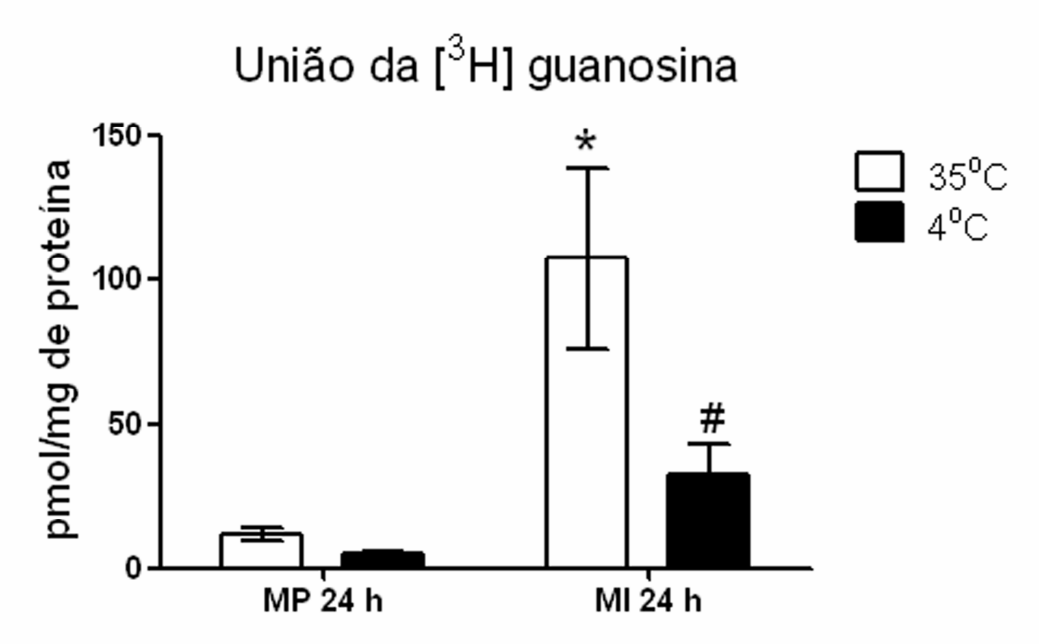

União da [3H] guanosina
pmol/mg de proteína
150
100
50
0
*
#
MP 24 h
MI 24 h
35°C
4°C

Figura 8

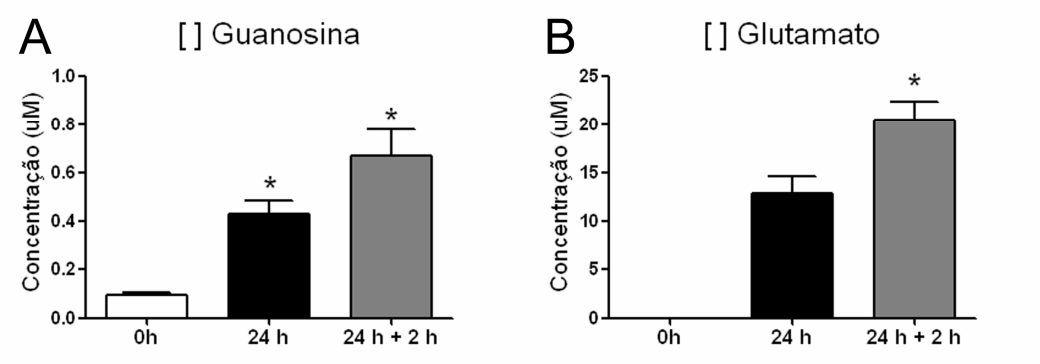
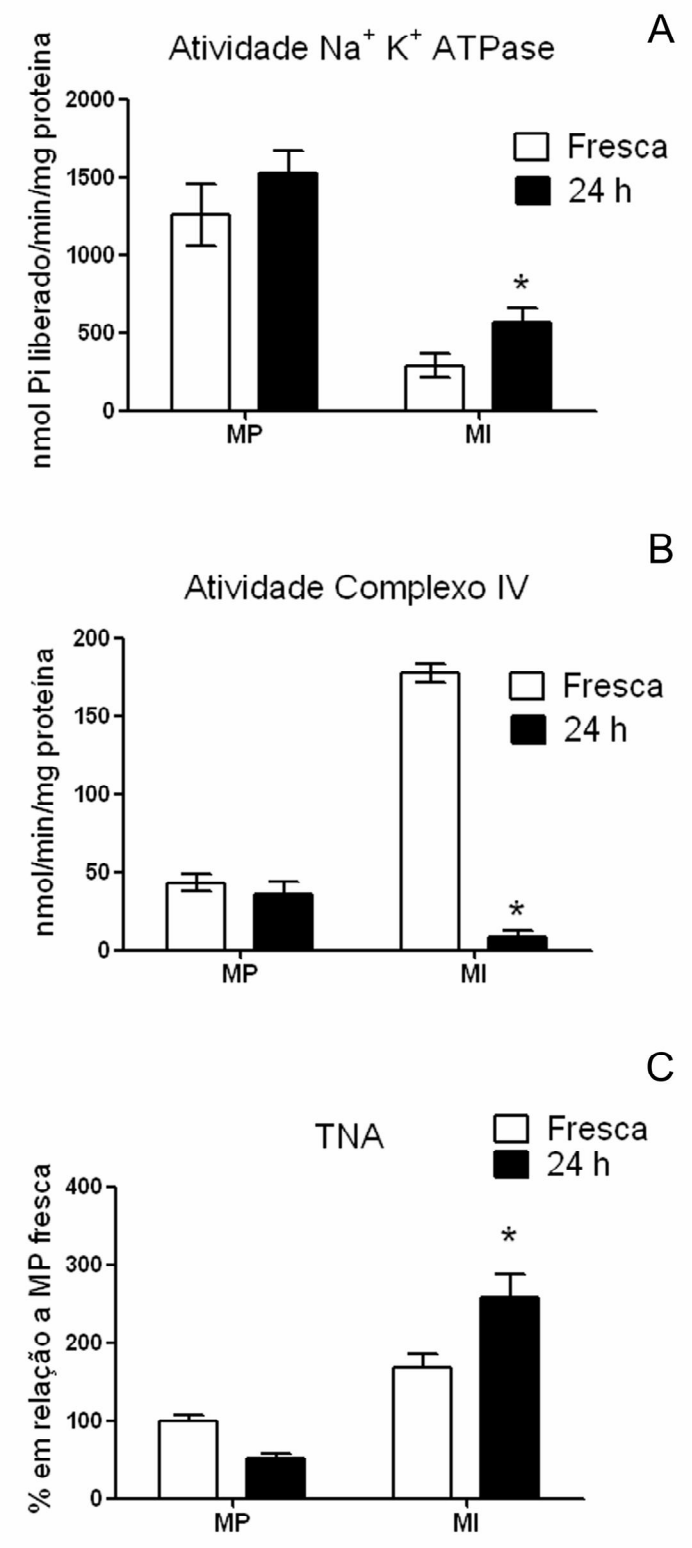

A
[ ] Guanosina
Concentração (uM)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
0h
24 h
24 h + 2 h
B
[ ] Glutamato
Concentração (uM)
25
20
15
10
5
0
*
0h
24 h
24 h + 2 h

Figura 9


A
Atividade Na+ K+ ATPase
nmol Pi liberado/min/mg proteina
2000
1500
1000
500
0
Fresca
24 h
*
MP
MI
B
Atividade Complexo IV
nmol/min/mg proteína
200
150
100
50
0
Fresca
24 h
*
MP
MI
C
TNA
% em relação a MP fresca
400
300
200
100
0
Fresca
24 h
*
MP
MI

Parte III

3. Discussão

Nesta presente tese, nosso objetivo foi investigar parâmetros bioquímicos e comportamentais em modelos experimentais de doenças cerebrais que envolvem o sistema glutamatérgico, além de avaliar o potencial efeito neuroprotetor das purinas derivadas da guanina e da inosina em tais modelos. Por fim, apresentamos os últimos experimentos realizados com o intuito de identificar um possível sítio de união da guanosina a membrana plasmática cerebral de ratos.

No primeiro capítulo, nosso objetivo foi investigar a captação de glutamato em fatias de diferentes estruturas cerebrais de ratas submetidas a um modelo experimental de depressão.

Utilizamos o protocolo do desamparo aprendido, o qual é bem estabelecido em pesquisas neurobiológicas sobre a patogênese molecular e terapias para depressão (Accortt et al., 2008; Duman, 2009; Martin and Brown, 2010). Este protocolo permite identificar individualmente os animais desamparados (comportamento relacionado com a depressão) para estudos neurobiológicos subseqüentes e compará-los com animais que não demonstram tais comportamentos.

Nossos resultados demonstraram que a captação de glutamato é diferente imediatamente após a realização do protocolo, assim como ao longo de 21 dias após, entre os animais desamparados e não desamparados. O hipocampo se mostrou a estrutura cerebral mais sensível a esse protocolo, quando comparado ao córtex e estriado, em relação às diferenças na captação de glutamato entre ratos desamparados e não desamparados. A captação de glutamato em fatias hipocampais de ratas desamparados foi maior do que em

animais não desamparados imediatamente após a sessão de teste, e menor 24 h e 21 dias após. Esta modulação parece estar envolvida com o comportamento de medo, o qual é associado ao comportamento de desamparo (Levenson et al., 2002; Tsvetkov et al., 2004) e a alterações morfológicas, já observadas por outros estudos no hipocampo de animais desamparados (Ferrero et al., 2005).

Portanto, nossos resultados indicaram que uma persistente modulação do mecanismo da captação de glutamato está ocorrendo após o desenvolvimento do comportamento desamparado. Uma melhor compreensão do envolvimento do sistema glutamatérgico pode gerar novas perspectivas em relação ao entendimento da patofisiologia da depressão e a novas abordagens terapêuticas antidepressivas.

No Segundo capítulo, nosso objetivo foi avaliar os efeitos do GMP em modelos comportamentais de ansiedade em ratos.

Investigamos o comportamento ansioso em ratos pelo teste do claro/escuro e labirinto em cruz elevado, os quais são amplamente utilizados para avaliar efeitos ansiolíticos ou ansiogênicos em roedores. Nós também avaliamos o comportamento ansioso na tarefa do campo aberto, medindo o tempo que o animal fica no centro do campo, o qual pode refletir um efeito ansiolítico.

No presente estudo, usamos um grupo controle positivo (droga classicamente ansiolítica – diazepam) em todas as tarefas comportamentais para estabelecer quais parâmetros poderiam indicar um efeito ansiolítico comparado ao grupo controle. Escolhemos diazepam como controle positivo

porque benzodiazepínicos são amplamente utilizados no tratamento da ansiedade em humanos.

O principal resultado do presente trabalho foi que o GMP na dose de 50 mg/kg foi capaz de reproduzir consistentemente os efeitos ansiolíticos do diazepam nos teste do claro/escuro e labirinto em cruz elevado. Na tarefa do campo aberto, nem diazepam ou GMP produziram comportamento ansiolítico. Portanto, parece que há limitações para predizer valores desta tarefa no sentido de testar o efeito ansiolítico destas drogas.

A razão original que nos levou a investigar se o GMP poderia atuar como ansiolítico foi baseado no fato de que o GMP pode diretamente, porém fracamente, antagonizar iGluR (Baron et al., 1989; Mendieta et al., 2005; Paas et al., 1996; Porciuncula et al., 2002; Souza e Ramirez, 1991), e que a administração de antagonistas de iGluR podem induzir comportamento ansiolítico em roedores (Bergink et al., 2004; Chojnacka-Wojcik et al., 2001; Kapus et al., 2008; Kehne et al., 1991; Plaznik et al., 1994). Considerando que as alterações comportamentais observadas 60 minutos após a administração de GMP não foram acompanhadas pelo aumento dos níveis de GMP no LCR, não podemos confirmar que o mecanismo pelo qual o comportamento ansiolítico induzido pelo GMP envolva antagonismo do iGluR.

Um estudo prévio do nosso grupo de pesquisa constatou que GMP aumenta os níveis de guanosina no LCR 30 min após a sua administração, sem alterar os níveis de GMP ou adenosina (Soares et al., 2004). Levando em consideração este dado, não podemos excluir a possibilidade de que os níveis de purinas derivadas da guanina no LCR variaram antes da realização do teste, e nem que os metabólitos do GMP também podem ser os responsáveis pelos

efeitos ansiolíticos observados. Como o GMP é um composto endógeno, aparentemente bem tolerado e com pequena toxicidade, poderia eventualmente ser desenvolvido como uma nova droga para o tratamento da ansiedade.

No terceiro capítulo, nós investigamos o potencial neuroprotetor da administração oral crônica de GMP em camundongos em um modelo *ex vivo* de privação de oxigênio e glicose (POG). Além disso, nós avaliamos alguns parâmetros glutamatérgicos que possivelmente poderiam estar envolvidos com o efeito neuroprotetor observado.

É importante ressaltar que modelos experimentais de isquemia *ex vivo*, como a POG, são importantes para a investigação das respostas fisiopatológicas celulares induzidas pela falha energética (Bosley et al., 1983; Joshi e Andrew, 2001; Matsuda et al., 1992; Oleskovicz et al., 2008; Oliveira et al., 2002; Thomazi et al., 2008). Nossos resultados neste estudo apontaram que a administração de GMP foi capaz de aumentar a resistência das fatias do córtex cerebral de camundongo contra a POG, assim como modular alguns parâmetros do sistema glutamatérgico.

A administração de GMP reduziu a união de glutamato à membrana plasmática do córtex cerebral e o imunoconteúdo das subunidades dos iGLURs, NR2A/B e GluR1, de receptores NMDA e AMPA, respectivamente, além de diminuir o imunoconteúdo da PSD-95, uma proteína implicada na sinalização dos receptores NMDA. Alguns trabalhos da literatura já reportaram que o bloqueio dos iGluRs ou a perturbação de suas vias de sinalização exercem efeitos neuropropetores contra eventos excitotóxicos (Aarts et al., 2002; Arias et al., 1999; Moskowitz et al., 2010; Sun et al., 2008). Desta forma,

podemos especular que a diminuição da via de sinalização glutamatérgica pode estar envolvida na neuroproteção promovida pela administração do GMP.

Em condições de falha energética, como acontece em episódios isquêmicos, há um aumento de glutamato na fenda sináptica, principalmente devido à atividade reversa do transportador neuronal EAAC1 (Hamann et al., 2002; Rossi et al., 2000). Neste trabalho, nós observamos que o tratamento com GMP reduziu os níveis do EAAC1. Este efeito pode ser considerado como uma resposta neuroprotetora/adaptativa que contribui para uma diminuição do acúmulo de glutamato extracelular durante a isquemia.

Embora o EAAC1 desempenhe um papel fundamental durante a isquemia, ele tem uma pequena contribuição para a captação de glutamato fisiológicas total (Danbolt, 2001; Haugeto et al., 1996). Em contraste, o transportador GLT1 não contribui significantemente para a liberação ou remoção do glutamato do espaço extracelular durante a isquemia (Hamann et al., 2002; Rossi et al., 2000), apesar de ser o principal transportador responsável pela captação de glutamato em condições fisiológicas (Danbolt, 2001; Furness et al., 2008). Nossos resultados apontaram que tanto a captação de glutamato nas fatias corticais, como o imunocontéudo cortical de GLT1, diminuiram com a administração de GMP.

Devido ao nosso grupo ter evidenciado que a administração aguda *in vitro* de derivados da guanina estimula a captação de glutamato em cultura de astrócitos e fatias cerebrais de córtex de ratos (Frizzo et al., 2002; Thomazi et al., 2004), e que a administração *in vivo* previne o decréscimo da captação de glutamato apos estímulos excitotóxicos (de Oliveira et al., 2004; Moretto et al., 2005; Moretto et al., 2009), a redução da captação observada pela

administração de GMP pode ser vista de forma inconsistente. No entanto, este foi o primeiro estudo *in vivo* que investigou os efeitos crônicos da administração de GMP na captação de glutamato em fatias corticais de camundongos adultos (que afeta outros parâmetros glutamatérgicos). Desta forma, podemos sugerir que os efeitos dos derivados da guanina na captação de glutamato é dependente dos modelos experimentais utilizados, do derivado da guanina administrado, do tempo e da forma de administração utilizada, dos parâmetros gerais afetados, e do protocolo de estímulo excitotóxico investigado.

Por último, observamos que os níveis de GMP e de seus metabólitos (incluindo guanosina) no LCR aumentaram após 3 semanas de administração de GMP. Desta forma, não podemos descartar a hipótese de que ambos GMP e guanosina estão envolvidos nos efeitos observados na administração de GMP.

No quarto capítulo, nos investigamos o potencial anticonvulsivante da inosina contra convulsões induzidas pela hiper ativação do sistema glutamatérgico. Para isto, nós testamos se uma administração intracerebroventricular de inosina seria capaz de proteger contra a administração de ácido quinolínico, um composto que hiper estimula o sistema glutamatérgico por diversas vias (Stone, 1993; Stone, 2001; Tavares et al., 2002; Tavares et al., 2005).

Nossos resultados demonstraram claramente o efeito anticonvulsivante, dose e tempo de administração dependente, da inosina contra a convulsão induzida por ácido quinolínico em camundongos adultos. Nós também investigamos se o efeito anticonvulsivante da inosina observado envolveu os receptores benzodiazepinicos, uma vez que já foi demonstrado que a inosina

se une aos receptores benzodiazepínicos, e que a ativação destes exerce ação anticonvulsivante no modelo da administração de ácido quinolínico. Neste sentido, o efeito anticonvulivante não foi mediado pela ativação dos receptores benzodiazepinicos, pois a administração prévia de flumazenil (um antagonista benzodiazepinicos), não bloqueou o efeito da inosina enquanto aboliu o efeito anticonvulsivante do diazepam.

Interessante, a atividade anticonvulsivante da inosina pareceu ser mais potente contra crises causadas pela hiper estimulação do sistema glutamatérgico do que as causadas pelo bloqueio do tonus inibitório do SNC observada em outros trabalhos. Ao compararmos os efeitos da inosina contra as convulsões induzidas pelos modelos de acido quinolinico e PTZ, a dose e o tempo da infusão de inosina que promoveram o melhor efeito protetor foram muito semelhantes em ambos os modelos, no entanto, o comportamento convulsivo foi completamente bloqueado quando as convulsões foram promovidas pelo acido quinolínico enquanto somente a latência inicial da convulsão aumentou no modelo de PTZ (Skolnick et al., 1979).

No quinto capítulo, investigamos os efeitos da administração crônica oral com guanosina em ratos submetidos ao modelo de oclusão permanente e bilateral das carótidas comuns (também denominada oclusão de dois vasos - 2VO). Este modelo é utilizado como um modelo de hipoperfusão cerebral crônica (Farkas et al., 2007) que reduz significantemente o fluxo sanguíneo cerebral (Farkas et al., 2007; Marshall et al., 2001; Ni et al., 1994; Ni et al., 1995) e causa um progressivo comprometimento cognitivo e dano neural (Ni et al., 1995; Sarti et al., 2002). 2VO é um modelo animal utilizado para investigação da fisiopatologia de doenças cerebrovasculares crônicas e para a

realização de testes de drogas com potenciais terapêuticos na demência vascular.

Nossos resultados demonstraram que a hipoperfusão cerebral crônica causada pelo modelo de 2VO, prejudicou os processos de aprendizagem e memória espacial, e causou graves danos no hipocampo de 20% dos animais. O dano hipocampal observado neste trabalho foi evidenciado como uma maciça perda neuronal na região hipocampal do CA1, que foi acompanhada com um intenso aumento de GFAP, indicando um processo de astrogliose.

Os efeitos comportamentais não foram afetados pelo tratamento com guanosina, no entanto, nenhum dano hipocampal foi observado nos animais submetidos a 2VO tratados com guanosina.

É importante ressaltar, que no nosso estudo nós não observamos correlação entre o prejuízo cognitivo e dano celular no hipocampo, embora nós não possamos descartar que outros danos celulares não investigados por este trabalho, possam estar presentes no grupo submetido a 2VO. De fato, a relação entre a hipoperfusão cerebral crônica induzida por 2VO e o prejuízo cognitivo ainda não esta bem elucidada. Outros estudos também mostraram a inexistência de uma correlação entre a perda neuronal na região hipocampal do CA1 e prejuízo cognitivo (Institoris et al., 2007; Jaspers et al., 1990; Lyeth et al., 1990).

Neste trabalho, nós também investigamos o niveis do BDNF no LCR e a proteína S100B no soro, como possíveis marcadores de insulto cerebral. Ambas as proteínas são comummente utilizadas como biomarcadores de doenças cerebrais, incluindo doenças cerebrovasculares e doença de Alzheimer (Chaves et al., 2010; Grillo et al., 2007; Haas et al., 2010; Kessler et

al., 2007; Machado-Vieira et al., 2007; Schaf et al., 2005; Zhang et al., 2008). Entretanto, ambos não foram alterados, indicando que pelo menos nas condições analisadas, eles não são bons marcadores de lesão celular.

É importante ressaltar que a guanosina foi administrada oralmente durante 6 semanas, iniciando-se imediatamente após a cirurgia de 2VO. A escolha da forma de administração foi escolhida porque estudos prévios demonstraram que a administração crônica de guanosina foi neuroprotetora contra insultos excitotóxicos (Vinade et al., 2003; Vinade et al., 2005) e a escolha do tempo de início do tratamento com a guanosina foi escolhida porque neste período há uma redução no fluxo sangüíneo cerebral (Ohta et al., 1997; Otori et al., 2003). No entanto, vários estudos têm demonstrado alterações significativas em função do tempo nos marcadores celulares no hipocampo, incluindo as alterações celulares que começam após seis semanas do pós-operatório (Farkas et al., 2007; Schmidt-Kastner et al., 2005). Desta forma, outros protocolos utilizando guanosina em diferentes tempos de administração poderiam ser utilizados para investigar o potencial neuroprotetor da guanosina neste modelo.

No sexto capítulo nós apresentamos os últimos resultados sobre a investigação do possível sítio de união da guanosina na membrana plasmática cerebral de ratos.

Nossos resultados mostraram que as preparações enriquecidas em membrana plasmática obtidas por um protocolo amplamente utilizado de subfracionamento celular, apresentam quantidades significativas de nucleosideos purínicos, incluindo a guanosina. Nós também observamos que,

ao incubar essas preparações por 24 h a 35^{o}C, os níveis das purinas diminuem.

Ao analisarmos a união da guanosina à preparações enriquecidas em membrana plasmática, percebemos que preparações pré incubadas por 24 h apresentaram um nível extremamente elevado e saturável de união da guanosina quando comparada a preparações não incubadas.

Devido à contaminação com membrana mitocondrial constatada nas frações enriquecidas em membrana plasmática, nós também avaliamos a união da guanosina a frações enriquecidas em membrana mitocondrial. Nossos resultados mostraram que houve um elevado nível de união da guanosina nas preparações pré-incubadas por 24 h.

Neste sentido, os resultados obtidos neste capítulo ressaltam a necessidade do desenvolvimento de uma metodologia mais adequada para a investigação da união da guanosina a membrana plasmática cerebral de ratos.

4. Conclusão

Nesta tese evidenciamos que a captação de glutamato, principalmente a captação hipocampal, é diferente em ratas que apresentam comportamento relacionado com a depressão, daquelas que não apresentam tal comportamento.

Buscando avaliar o potencial neuroprotetor das purinas derivadas da guanina e da inosina em modelos animais que envolvem excitotoxicidade glutamatérgica, verificamos que: a administração aguda e sistêmica de GMP induz comportamento ansiolitico em ratos; a administração oral crônica de GMP em camundongos aumenta a resistência do córtex cerebral à privação de oxigênio e glicose além de modular parâmetros glutamatérgicos; a inosina administrada intracerebroventricularmente em camundongos é anticonvulsivante contra convulsões induzidas pelo ácido quinolínico; o tratamento oral crônico com guanosina não melhora o prejuízo cognitivo, mas protege contra o dano hipocampal em ratos submetidos à oclusão das carótidas comum.

Por último, esta tese apresentou os últimos avanços do nosso grupo na tentativa de caracterizar um possível sítio de união da guanosina a membrana plasmática cerebral de ratos. Porém este tópico ainda merece discussão.

5. Perspectivas

1. Avaliar o efeito das purinas derivadas da guanina sobre a queda da captação em ratas que apresentam comportamento relacionado com a depressão.

2. Investigar os mecanismos neuroquímicos pelo qual a administração sistêmica de GMP induz comportamento ansiolítico.

3. Aprofundar os estudos sobre os efeitos da administração oral crônica com GMP sobre o sistema glutamatérgico.

4. Investigar os mecanismos pelo qual a inosina exerce efeito anticonvulsivante frente à administração de ácido quinolínico em camundongos.

5. Avaliar outros protocolos de administração de guanosina sobre o prejuízo cognitivo e dano hipocampal em ratos submetidos à hipoperfusão cerebral crônica.

6. Continuar os estudos sobre a identificação do possível sítio de união da guanosina na membrana plasmática cerebral de ratos.

6. Referências

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