UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS GRADUAÇÃO EM NEUROCIÊNCIAS

ESTUDO DOS EFEITOS NEUROPROTETORES E NEUROTÓXICOS DOS DERIVADOS DA GUANINA EM FATIAS DE HIPOCAMPO DE RATOS E EM CULTURAS DE CÉLULAS DE NEUROBLASTOMA HUMANO SHSY-5Y

Simone Molz

Florianópolis
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Tese apresentada ao Curso de Pós Graduação em Neurociências da Universidade Federal de Santa Catarina, como requisito parcial à obtenção do grau de Doutor em Neurociências

Simone Molz

Orientadora: Prof.ª Dr.ª Carla Inês Tasca

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“A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original”

(Albert Einstein)

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RESUMO

A morte celular que ocorre devido à hiperativação de receptores N-metil-D-aspartato (NMDA) e/ou à disfunção dos transportadores de glutamato (Glu) tem sido correlacionada com a patogênese de doenças neurodegenerativas. Os derivados da guanina são compostos endógenos que podem modular a atividade do sistema glutamatérgico. Neste estudo demonstramos que a morte celular induzida por Glu envolve a ativação de receptores NMDA e também a reversão de seus transportadores em fatias de hipocampo de ratos. A toxicidade induzida por NMDA foi bloqueada na presença de 1mM de guanosina-5'-monofosfato (GMP). Entretanto, GMP não foi capaz de prevenir a perda de viabilidade celular induzida por Glu. Em contrapartida, guanosina (GUO) (100μM) protege fatias de hipocampo de ratos frente à toxicidade do Glu através da redução da liberação de Glu, ativação da via de sinalização da proteína cinase dependente de fosfatidilinositol (PI3K)/proteína cinase B (Akt), inibição da enzima glicogênio sintase cinase (GSK3β) e redução da expressão da enzima óxido nítrico sintase induzida por Glu. Também demonstramos que, assim como ocorre com os derivados da adenina, altas concentrações de um derivado da guanina (GMP – 5mM) podem causar perda de viabilidade celular em fatias de hipocampo de ratos através da diminuição da captação de Glu e estimulação de receptores NMDA e alfa-amino-3-hidróxi-metilisoxazole-propionato (AMPA) de Glu. Utilizando culturas de células de neuroblastoma humano (SHSY-5Y) demonstramos que GUO (1mM) protege estas células da morte neuronal decorrente da superprodução de espécies reativas de oxigênio induzidas pela co-administração de rotenona e oligomicina A, através da ativação da via de sinalização celular PI3K. Adicionalmente, demonstramos que GUO (1mM) também protege culturas de células SHSY-5Y diferenciadas da toxicidade neuronal induzida pelo peptídeo beta amilóide ($A\beta_{25-35}$). Dessa forma, estamos demonstrando que os derivados da guanina (GMP e GUO) apresentam efeito neuroprotetor frente distintos modelos de neurotoxiciade e que GMP, dependendo da concentração utilizada, também pode ser neurotóxico.

ABSTRACT

Cell death due to N-methyl-D-aspartate (NMDA) receptors activation and/or glutamate (Glu) transporters disfunction is related to the pathogenesis of neurodegenerative diseases. Guanine derivatives are endogenous compounds which modulate glutamatergic system activity. In this study, Glu-induced cell death involves NMDA receptors activation as well as the reversal of Glu transporters in rat hippocampal slices. NMDA-induced toxicity was prevented by guanosine-5'-monophosphate (GMP). However, GMP did not prevent Glu-induced cell viability reduction. Differently, guanosine (GUO) (100μM) protected hippocampal slices from Glu-induced toxicity by a mechanism which involves reduction in Glu-induced Glu release, activation of the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) cell signaling pathway, glycogen synthase kinase (GSK3β) inhibition and reduction of Glu-induced expression of inducible nitric oxide synthase. We also demonstrated that as well as occurs with adenine derivatives, high extracellular concentrations of a guanine derivative (GMP – 5mM) can lead to cell death in rat hippocampal slices due to Glu uptake inhibition and NMDA and alfa-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors activation. In human neuroblastoma cell cultures (SHSY-5Y) we also demonstrated that GUO (1mM) protects these cells from neuronal cell death due to rotenone and oligomycin A-induced reactive oxigen species overproduction by the activation of PI3K signaling pathway. Additionaly, we demonstrated that GUO (1mM) also protects differentiated human SHSY-5Y cells from amyloid beta ($A\beta_{25-35}$)-induced neuronal toxicity. Thus, we are showing that guanine derivatives (GMP e GUO) have neuroprotective effects against different neurotoxic stimuli and that GMP, depending on its concentration, may also be neurotoxic.

APRESENTAÇÃO

Esta Tese de Doutorado está organizada da seguinte forma: Introdução, Objetivos, Artigos científicos publicados e em fase de submissão, Resultados preliminares, Discussão, Conclusões e Referências Bibliográficas.

A **Introdução** contém o embasamento teórico para a realização dessa Tese. Os Materiais e métodos, os Resultados, assim como as Referências Bibliográficas específicas, encontram-se no corpo de cada trabalho, os quais estão apresentados na forma de **Artigos Científicos** em quatro capítulos. O quinto capítulo descreve resultados preliminares e que serão complementados posteriormente.

A seção **Discussão** contém uma interpretação geral dos resultados obtidos nos diferentes trabalhos.

A seção **Conclusões** descreve as conclusões gerais da Tese.

A seção **Referências Bibliográficas** apresenta as referências citadas na Tese.

Os capítulos 1, 2 e 3 dessa Tese foram desenvolvidos no Departamento de Bioquímica da UFSC, no laboratório de Neuroquímica 4, sob coordenação da Prof[a]. Dra Carla I. Tasca. Os capítulos 4 e 5 foram desenvolvidos no Departamento de Farmacologia da Universidade Autônoma de Madri, no laboratório 3 - Instituto Teófilo Hernando, sob coordenação da Prof[a]. Dra Manuela García López.

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LISTA DE ABREVIATURAS

Aβ: peptídeo beta amilóide

Aβ25-35: peptídeo beta amilóide fragmento 25-35

A1: receptor de adenosina tipo 1

$A2_A$: receptor de adenosina tipo 2A

$A2_B$: receptor de adenosina do tipo 2B

A3: receptor de adenosina do tipo 3

ADP: adenosina difosfato

Akt/PKB: proteína cinase B

AMPA: alfa-amino-3-hidróxi-metilisoxazole-propionato

ATP: adenosina trifosfato

ATP: adenosina trifosfato

BDNF: fator neurotrófico derivado do cérebro

Ca^{2+}: íon cálcio

DMSO: dimetil sulfóxido

EAAC: carreador de aminoácidos excitatórios

EAAT: transportador de aminoácidos excitatórios

ERK: proteína cinase regulada por sinal extracelular

GDP: guanosina-5'-difosfato

GLAST: transportador de glutamato e aspartato

GLT-1: transportador de glutamato 1

GMP: guanosina-5'-monofosfato

GSK3β: glicogênio sintase cinase-3 β

GTP: guanosina-5'-trifosfato

GUO: guanosina

K^+: íon potássio

LY 294002: [2-4(-morfolinil)-8-fenil-1-(4H)-benzopiran-4-1-hidrocloreto

MAP-2: proteína associada à microtúbulo 2

MAPK: proteína cinase ativada por mitógenos

MEK: MAP cinase cinase

Mg^{2+}: íon magnésio

MPP^{+}: íon 1-metil-4-fenilpiridinium

Na^{+}/K^{+} ATPase: sódio/potássio ATPase

Na^{+}: íon sódio

NMDA: N-metil-D-aspartato

NO: óxido nítrico

NOS: óxido nítrico sintase

P2X: receptores ionotrópicos de ATP

P2Y: receptores metabotrópicos de ATP

PI3K: proteína cinase de fosfatidilinositol 3

PIP_2: fosfatidilinositol-3,4-bifosfato

PIP_3: fosfatidilinositol-3,4,5-trifosfato

PKC: proteína cinase C

PNP: purina nucleosídeo fosforilase

ROS: espécies reativas de oxigênio

Rot/oligo: rotenona/oligomicina A

SHSY-5Y: células de neuroblastoma humano da linhagem SHSY-5Y

SNC: sistema nervoso central

TNFα: fator de necrose tumoral α

TrKB: receptores tirosina cinase B

INTRODUÇÃO

1. SISTEMA GLUTAMATÉRGICO

O glutamato é um aminoácido que desempenha diversas funções no metabolismo celular, dentre elas estão: constituinte da estrutura de proteínas, fonte de energia celular e neurotransmissão. O glutamato é o neurotransmissor da maioria das sinapses excitatórias do sistema nervoso central (SNC) (WATKINS & JANE, 2006). Uma vez liberado para a fenda sináptica, o glutamato é recaptado pelos astrócitos onde é novamente convertido à glutamina pela enzima glutamina sintetase, e liberado por intermédio de transportadores de glutamina para o meio extracelular. A glutamina liberada pelos astrócitos é captada pelas células neuronais e reconvertida a glutamato fechando assim o ciclo glutamato-glutamina (Figura 1). O glutamato também pode ser sintetizado *de novo* a partir da glicose, via ciclo do ácido cítrico e posterior transaminação do α-cetoglutarato (SCHOUSBOE & WAAGEPETERSEN, 2005).

Glutamato é transportado para o interior de vesículas sinápticas através de um mecanismo dependente de gradiente próton-eletroquímico promovido por uma ATPase vacuolar (NAITO & UEDA, 1985) e subsequentemente liberado por exocitose. O glutamato é liberado das vesículas sinápticas após um estímulo de despolarização promovido pela entrada de íons cálcio (Ca^{2+}). O glutamato também pode ser liberado pelos astrócitos e assim modular a atividade dos neurônios. A liberação astrocitária de glutamato pode ocorrer através dos seguintes mecanismos: (i) reversão dos transportadores de glutamato; (ii) exocitose dependente de íons Ca^{2+}; (iii) trocador cisteína/glutamato; (iv) canais iônicos regulados por alterações no volume celular (VRACS); (v) receptores purinérgicos do subtipo P2X7; (vi) hemicanais (MALARKEY & PARPURA, 2008).

Uma vez liberado, o glutamato interage com seus receptores, presentes nas membranas dos terminais pré e pós sinápticos e também na membrana das células gliais (Figura 1).

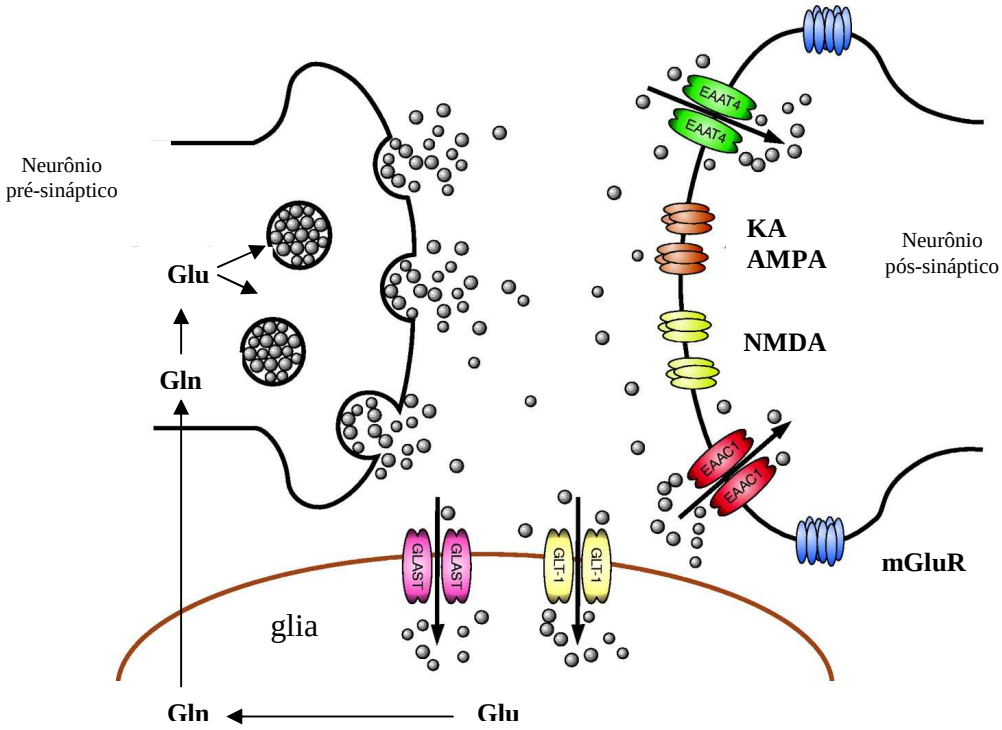

Figura 1. Sinapse glutamatérgica: O glutamato liberado na fenda sináptica interage com seus receptores de membrana (AMPA, KA, NMDA e mGluR). A recaptação do glutamato ocorre através da atividade de seus transportadores de membrana localizados nos astrócitos (GLT-1, GLAST) e nos neurônios (EAAC1 e EAAT4). Nos astrócitos, o glutamato (Glu) pode ser convertido em glutamina (Gln), a qual é liberada dos astrócitos e captada pelos neurônios, onde a Gln é novamente convertida em Glu. O Glu então é armazenado nas vesículas sinápticas (adaptado de GONZÁLEZ & ROBINSON, 2004).

1.1. Receptores de Glutamato

Os receptores de glutamato são classificados em ionotrópicos e metabotrópicos, de acordo com suas características farmacológicas e moleculares. Os receptores ionotrópicos e

metabotrópicos de glutamato geralmente coexistem em uma mesma sinapse (PIN & DUVOISIN, 1995). Os receptores ionotrópicos são canais iônicos dependentes da ativação por ligantes específicos. Distintas famílias de receptores ionotrópicos têm sido identificadas farmacologicamente por suas afinidades com agonistas sintéticos. São eles: alfa-amino-3-hidróxi-metilisoxazole-propionato (AMPA), Cainato e N-metil-D-aspartato (NMDA) (MADDEN, 2002). Estudos de clonagem e expressão gênica têm levado à identificação das subunidades dos receptores ionotrópicos, assim distribuídos: cinco subunidades para receptores NMDA (NR1, NR2A, NR2B, NR2C e NR2D), quatro para receptores AMPA (GluR1, GluR2, GluR3 e GluR4) e cinco para receptores cainato (GluR5, GluR6, GluR7, KA1 e KA2).

A ativação dos receptores AMPA medeia um potencial excitatório rápido e com rápida dessensibilização do receptor. O receptor AMPA é distribuído igualmente pelo SNC sendo ricamente expresso no hipocampo. Ele é permeável aos íons sódio (Na^{+}), porém quando a subunidade GluR2 está ausente na conformação do receptor AMPA, ele se torna permeável a íons Ca^{+2}. O receptor cainato é encontrado no hipocampo, córtex, estriado, cerebelo, amígdala, hipotálamo, medula espinhal e na retina. Os receptores de cainato diferenciam-se dos receptores AMPA pela menor corrente conduzida e pela cinética de desativação mais lenta (HUETTNER, 2003). O receptor NMDA é encontrado em todo o cérebro, sendo localizado principalmente no cérebro anterior e na região CA1 do hipocampo. O potencial de repouso deste receptor é mantido por um íon magnésio (Mg^{+2}). A ativação de receptores NMDA permite o influxo de grandes quantidades de Ca^{+2} extracelular para o interior da célula (OZAWA et al., 1998).

Os receptores metabotrópicos de glutamato são receptores acoplados a proteínas-G. Existem 8 tipos de receptores eles são nomeados de mGluR1 ao 8. Eles foram identificados e classificados em 3 grupos (I, II e III), baseados na homologia da seqüência de aminoácidos, vias de transdução de sinais e seletividade farmacológica (KENNY & MARKOU 2004).

Estes receptores estão localizados nos terminais pré e pós sinápticos e nas células gliais e estão relacionados tanto a efeitos excitatórios quanto inibitórios (OSAWA et al., 1998). O grupo I (mGluR1 e mGluR5) está predominantemente localizado pós-sinapticamente onde se acopla a proteínas G para ativar a fosfolipase C (PLC), que catalisa a produção de inositol (1,4,5)-trifosfato e por meio disso dispara a liberação de íons Ca^{+2} dos estoques intracelulares. O grupo II de receptores metabotrópicos de glutamato (mGluR2 e mGluR3) é encontrado tanto pré como pós-sinapticamente e são acoplados a proteína $G_{i/o}$ modulando a atividade da adenilato ciclase. Por útlimo, o grupo III de receptores mGlu (mGluR4, mGluR6, mGluR7 e mGluR8) está predominantemente localizado no terminal pré-sináptico onde atua como autoreceptor, e também está acoplado a proteínas-G modulando a atividade da adenililato ciclase (KENNY & MARKOU, 2004).

A liberação de glutamato das vesículas sinápticas produz um potencial de ação pós-sináptico excitatório por ativar inicialmente os receptores AMPA. A ligação de glutamato aos receptores AMPA medeia a entrada de Na^{+} para o interior do neurônio, despolarizando-o. Esta despolarização permite a liberação do magnésio e o desbloqueio de receptores NMDA. Uma vez que o receptor NMDA foi desbloqueado, a união de glutamato e de seus co-agonistas, glicina e D-serina, permite a entrada de íons Ca^{2+} (HARA & SNIDER, 2007). As propriedades dos receptores NMDA são responsáveis pelas funções integrativas desencadeadas pela ativação de receptores de glutamato como, por exemplo, a regulação do desenvolvimento neuronal no sistema nervoso do embrião e a potenciação de longa duração (LTP), a qual é responsável pela formação de alguns tipos de memória (IZQUIERDO & MEDINA, 1997).

Como parece não haver nenhuma enzima extracelular capaz de metabolizar o glutamato liberado pelos terminais pré-sinápticos, a única maneira rápida e eficaz de promover a sua retirada do fluido extracelular é através da recaptação feita por carreadores de membrana celular. A retirada do glutamato da fenda sináptica é um importante mecanismo a

fim de diminuir a quantidade desse neurotransmissor e dessa forma prevenir o dano excitotóxico (ANDERSON & SWANSON, 2000).

1.2. Transportadores de Glutamato

A concentração extracelular de glutamato é mantida em níveis fisiológicos devido à presença de transportadores de alta afinidade e dependentes de íons Na^+. Os transportadores de glutamato são responsáveis pela captação do glutamato extracelular, permitindo assim a neurotransmissão excitatória normal e protegendo da excitotoxicidade do glutamato. São identificados cinco subtipos de transportadores de glutamato. Os transportadores de glutamato/aspartato (GLAST) e o transportador de glutamato-1 (GLT-1) descritos em roedores e considerados transportadores gliais (em humanos eles são designados como EAAT1 e EAAT2 – transportadores de aminoácidos excitatórios). Transportadores neuronais também são importantes para manter as baixas concentrações extracelulares de glutamato. O transportador neuronal de glutamato mais amplamente distribuído no cérebro é o carreador de aminoácidos excitatórios 1 (EAAC1 - homólogo humano, EAAT3), encontrado em regiões não-sinápticas. Outros subtipos incluem EAAT4, localizado em células de Purkinje e EAAT5, localizado em neurônios retinianos. O transportador GLT-1 é o transportador mais abundante no SNC e está presente principalmente no córtex e hipocampo, enquanto que a os transportadores GLAST são mais abundantes na glia de Bergmann da camada molecular do cerebelo (DANBOLT, 2001).

O transporte de glutamato ocorre da seguinte forma: uma molécula de glutamato é transportada para o citoplasma juntamente com três íons Na^+ acompanhados da saída de um íon potássio (K^+). Para que esse mecanismo ocorra é necessária a atividade de Na^+/K^+ ATPases para manter o gradiente de concentração de íons Na^+, o que acarreta um grande consumo de ATP (CAMACHO & MASSIEU, 2006; Figura 2). Então, a manutenção dos níveis de ATP intracelular é essencial para o controle da transmissão glutamatérgica e a

captação de glutamato diminui os níveis desse neurotransmissor na fenda sináptica prevenindo assim a excitotoxicidade glutamatérgica (DANBOLT, 2001).

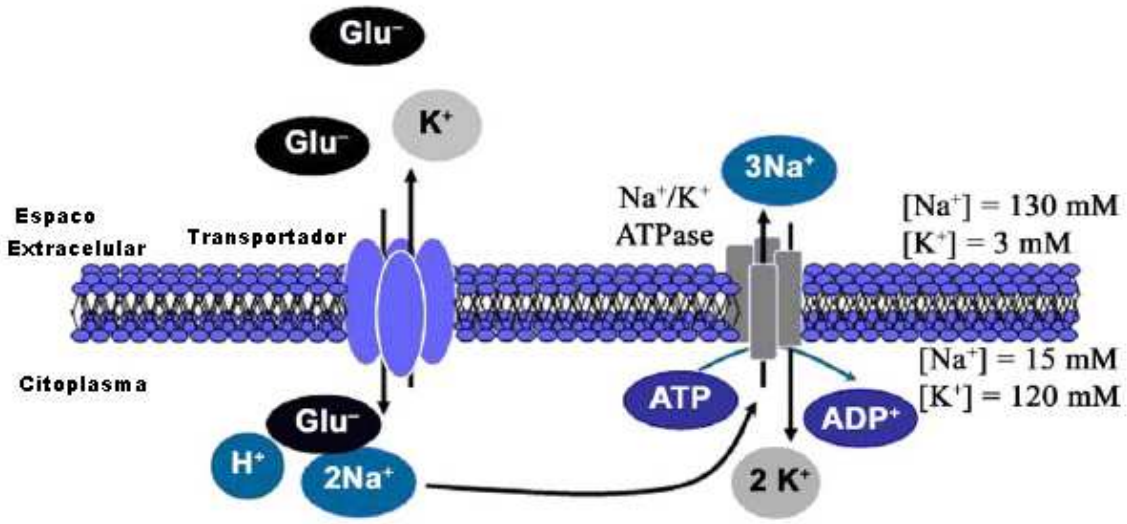

Figura 2. Captação de glutamato através de seus transportadores de membrana celular. A concentração extracelular de glutamato é altamente regulada por proteínas transportadoras localizadas na membrana plasmática de neurônios e glia. A captação é dependente do gradiente eletroquímico de sódio que é mantido pela atividade das Na^+/K^+ ATPases (adaptado de CAMACHO & MASSIEU, 2006).

Em roedores, GLAST e GLT-1 estão presentes no SNC com baixos níveis de expressão em estágios precoces do desenvolvimento e aumentam até a idade adulta. A expressão de GLAST e GLT-1 aumenta drasticamente no período de maior atividade de sinaptogênese (do final da segunda ao final da quarta semana pós-natal), atingindo níveis de adulto após trinta e cinco dias de vida (FURUTA et al., 1997). A expressão do transportador neuronal (EAAC1) também apresenta um padrão dependente de desenvolvimento, sendo que sua expressão é maior nos primeiros estágios do desenvolvimento quando comparado aos níveis encontrados no cérebro adulto (FURUTA et al., 1997; SIMS & ROBINSON, 1999).

Os transportadores de glutamato são regulados por mecanismos transcricionais e pós-transcricionais. A morte neuronal excitotóxica em cultura mista de neurônios e astrócitos resulta na diminuição da expressão de GLT-1 e aumenta a expressão de GLAST nos astrócitos remanescentes (SCHLAG et al., 1998). Alguns dados na literatura têm demonstrado que a via da proteína cinase de fosfatidilinositol (PI3K) está relacionada com o aumento de captação de glutamato e da expressão dos transportadores de glutamato (SIMS et al., 2000; GUILLET et al., 2005). Adicionalmente, há evidências de que a atividade dos transportadores pode ser regulada agudamente (em minutos) de maneira independente da mudança na expressão dos transportadores (DUAN et al., 1999; SHELDON & ROBINSON, 2007). A ativação da via da PI3K/Akt resulta no tráfego dos transportadores de glutamato do citoplasma para a membrana e aumento da captação de glutamato (KRIZMAN-GENDA et al., 2005). A modulação do transporte de glutamato é importante para manter a homeostase da neurotransmissão glutamatérgica e prevenir a excitotoxicidade.

1.3. Excitotoxicidade

O termo excitotoxicidade refere-se à toxicidade causada pelo aumento da concentração de glutamato durante a transmissão sináptica e consequente morte neuronal (MELDRUM 2000). A ação neurotóxica do glutamato está intimamente ligada a um aumento de íons Ca^{+2}. Como já descrito anteriormente, os receptores NMDA são permeáveis a íons Ca^{+2} permitindo que ocorra um grande aumento das concentrações desse íon no interior da célula, portanto, sua hiperativação tem sido considerada a principal responsável pela morte celular devido à excitotoxicidade (STONE & ADDAE, 2002). O aumento de Ca^{+2} intracelular pode ativar proteínas cisteínas proteases, denominadas calpaínas e caspases, que por sua vez podem degradar uma série de substratos como: proteínas de citoesqueleto, receptores de membrana e enzimas. A elevada concentração de Ca^{+2} induz estresse oxidativo (MATTSON,

2003), pertubação do cálcio mitocondrial e metabolismo energético (SMAILI et al., 2003; MATTSON, 2007).

A disfunção mitocondrial e estresse oxidativo são mecanismos envolvidos na morte celular observada em diferentes modelos de citotoxicidade, como por exemplo, a estimulação excessiva de receptores glutamatérgicos (REGO & OLIVEIRA, 2003), toxicidade induzida por H_2O_2 (KIM et al., 2005), doenças neurodegenerativas como Doença de Parkinson, Doença de Alzheimer e isquemia (MATTSON & MAGNUS 2006). O excesso de Ca^{2+} citoplasmático decorrente da ativação de receptores NMDA é captado pela mitocôndria, causando elevação do Ca^{2+} mitocondrial, inibição da fosforilação oxidativa, formação de poros de permeabilidade transitória e colapso bioenergético. O colapso bioenergético mitocondrial pode acarretar na super-produção de espécies reativas de oxigênio (ROS), liberação do Ca^{2+} da mitocôndria e morte celular (RYTER et al., 2007; NICHOLLS, 2008).

Os transportadores de glutamato são capazes de retirar o excesso de glutamato da fenda sináptica, mantendo as concentrações do neurotransmissor em níveis fisiológicos, prevenindo assim, a super-estimulação dos receptores glutamatérgicos. Existe uma correlação direta entre a disfunção dos transportadores de glutamato, aumento da concentração do neurotransmissor na fenda sináptica e desenvolvimento de dano celular excitotóxico tanto em modelos de estudo *in vivo* e *in vitro* (MARAGAKIS & ROTHSTEIN, 2004). Em situações em que ocorre desequilíbrio iônico e diminuição nos níveis de ATP intracelular, existe um acúmulo de Na^{+} no interior das células que pode ocasionar a atividade reversa dos transportadores glutamatérgicos (ROSSI et al., 2000). Quando ocorre esta atividade reversa dos transportadores, o glutamato em vez de ser captado é liberado para o meio, aumentando a sua concentração na fenda sináptica e consequentemente aumentando o dano celular (CAMACHO & MASSIEU, 2006). Além disso, a estimulação de receptores NMDA e o aumento dos níveis de Ca^{2+} contribuem para o aumento da formação de ROS e espécies reativas de nitrogênio (WHITE et al., 2003). Os transportadores de glutamato são vulneráveis

à oxidação, pois apresentam sítios de cisteína regulados pelo estado redox (TROTTI et al., 1998) com isso, o aumento de ROS pode acarretar em uma diminuição da captação de glutamato (TROTTI et al., 1998; BRONGHOLI et al., 2006). A morte neuronal excitotóxica devido à reversão dos transportadores e/ou a hiperativação de seus receptores tem sido correlacionada com a fisiopatologia de doenças neurodegenerativas agudas (hipóxia, isquemia, hipoglicemia, convulsões) e crônicas (Doença de Alzheimer, Doença de Huntington, Doença de Parkinson, esclerose amiotrófica lateral, esquizofrenia) (MATTSON, 2003, MARAGAKIS & ROTHSTEIN, 2004).

A morte excitotóxica pode ocorrer por apoptose ou necrose (ANKARCRONA et al., 1995). Estes dois tipos de morte celular diferem entre si tanto morfologicamente quanto bioquimicamente. A necrose ocorre como resultado de perturbações extremas no ambiente celular, levando à ruptura da membrana plasmática e liberação do conteúdo intracelular. Em contrapartida, a apoptose (ou morte celular programada) é dependente da ativação de vias intracelulares com eventos finamente coordenados e regulados que resultam no “suicídio” celular como, por exemplo, a translocação de proteínas pró-apoptóticas da família Bcl-2 (Bax e Bad) do citosol para a mitocôndria, formação de poros de permeabilidade transitória na membrana mitocondrial, liberação do citocromo c e formação do apoptossoma que promove a ativação da pró-caspase-9. Subsequentemente, a caspase 9 ativa caspases efetoras, como a caspase-3, -6 e -7. A ativação de uma cascata de caspases pode então ativar DNAses que clivam o DNA em fragmentos internucleossomais. A apoptose pode também ocorrer de maneira independente da formação do apoptossoma. A ativação de uma via extrínseca pode ocorrer através da interação de moléculas como Fas e fator de necrose tumoral (TNF) aos seus receptores de superfície, com o recrutamento de moléculas adaptadoras e a ativação direta da caspase-8 e outras caspases (RUPINDER et al., 2007).

1.4. Excitotoxicidade e Vias de Sinalização

Danos físicos, isquemia ou a liberação não apropriada de neurotransmissores são geralmente acompanhadas por eventos como o influxo de Ca^{2+} e o início de uma cascata de eventos que levam à ativação de várias vias de sinalização celular que estão associadas à neurodegeneração. Por outro lado, os danos celulares também podem acarretar a ativação de vias que culminam na produção e ativação de fatores que estão envolvidos na recuperação celular (SAPOLSKY, 2001; BHAT et al., 2004; ZHAO et al., 2006).

Estudos demonstram que há uma correlação entre a ativação de receptores de glutamato e o desenvolvimento de processo inflamatório no SNC (MATUTE, 2007; VESCE et al., 2007). Um dos marcos do processo inflamatório é a ativação da microglia, a qual libera mediadores pró-inflamatórios que incluem a interleucina-1, fator de necrose tumoral α (TNF-α) e óxido nítrico (NO). O NO é uma molécula de sinalização intra e intercelular que desempenha importante papel na regulação do influxo neuronal de Ca^{2+}, plasticidade sináptica e sobrevivência no SNC. O NO é produzido a partir da conversão de L-arginina em NO e L-citrulina, uma reação catalizada pela enzima óxido nítrico sintase (NOS). Existem três isoformas de NOS caracterizadas no SNC: NOS neuronal (nNOS ou NOS1), NOS induzível (iNOS ou NOS2) e a NOS endotelial (eNOS ou NOS3). Na ausência de processo inflamatório, a nNOS é a principal fonte de NO no SNC. A isoforma iNOs não é normalmente expressa no tecido cerebral, porém mediadores inflamatórios como lipopolissacarídeos e citocinas induzem a expressão de iNOS nas células gliais (astrócitos e microglia), sendo a expressão de iNOs uma das principais características da glia “ativada” que acompanha o processo inflamatório no cérebro (KNOTT & BOSSY-WETZEL, 2009). A expressão de iNOS é raramente vista em neurônios, mas a sua expressão pode ocorrer em alguns tipos de células neuronais em resposta à citocinas (SEROU et al., 1999).

A indução de iNOS acarreta em grande produção de NO pelas células gliais, com conseqüente conversão do NO em moléculas altamente reativas como, por exemplo, o

peroxinitrito (SAHA & PAHAN, 2006). NO tem sido correlacionado com a maioria das doenças neurodegenerativas, esclerose múltipla, demência relacionada à AIDS, príon, Doença de Parkinson, Doença de Huntington e Doença de Alzheimer (MURPHY, 2000). Estudos *in vitro* e *in vivo* demonstram o envolvimento da estimulação de receptores de glutamato na indução da expressão da iNOS (CARDENAS et al., 2000; MORO et al., 2004). Estes dados sugerem fortemente o envolvimento da iNOS na morte celular excitotóxica, portanto, o estudo de moléculas que reduzam o excesso de NO produzido em situações patológicas possui importante significado clínico.

A ativação da via de sinalização intracelular PI3K leva à formação de segundos-mensageiros como o fosfatidilinositol-3,4,5-trifosfato (PIP_3) e fosfatidilinositol-3,4-bifosfato (PIP_2). Esses segundos-mensageiros se ligam a um domínio amino-terminal presente na proteína cinase B (PKB ou Akt) promovendo a sua dimerização e translocação para a membrana celular, permitindo que esta seja fosforilada em seus sítios Treonina-308 e Serina-473, resultando em sua ativação. Dentre as funções atribuídas a Akt uma das mais importantes é seu papel na sobrevivência celular. O papel neuroprotetor da Akt frente à excitotoxicidade está bem descrito na literatura. A exposição de culturas de neurônios corticais a concentrações excitotóxicas de glutamato diminui a fosforilação da Akt e causa aumento da ativação de caspase-3 (NISHIMOTO et al., 2008). Após insulto isquêmico ocorre uma drástica diminuição nos níveis de Akt fosforilada e este evento precede a liberação de citocromo-c e ativação de caspases (OUYANG et al., 1999). Em cultura de neurônios hipocampais submetidas à hipóxia, a ativação da via PI3K/Akt preveniu a apoptose através da inibição de genes e proteínas pró-apoptóticas (YAMAGUCHI et al., 2003). Animais submetidos a uma isquemia focal apresentam aumento da expressão de fosfo-AKT na região de penumbra (região que se localiza ao redor do centro isquêmico) no córtex cerebral, e este aumento está relacionado com a proteção de células neuronais (NOSHITA et al., 2001).

Assim, o estudo da via da PI3K/Akt no mecanismo de ação de compostos neuroprotetores é de grande relevância.

A enzima glicogênio sintetase cinase 3β (GSKβ) é uma isoforma da proteína glicogênio sintetase cinase (GSK3). A GSK3 é conhecida há mais de 20 anos e se encontra abundantemente distribuída pelo cérebro e sua função básica é a de fosforilar e inibir a atividade da enzima glicogênio sintetase. Recentemente, foram encontradas outras funções para a enzima GSK-3β que se relacionam com a sinalização e sobrevivência celular, assim como com a sua participação em diversos eventos fisiológicos e também na fisiopatologia de doenças neurodegenerativas (HOOPER et al., 2008). A atividade da GSK3β é controlada mediante fosforilação e defosforilação. Sua atividade pode ser reduzida mediante a fosforilação de uma serina na posição 9 (Ser9), ou pode ser aumentada mediante uma fosforilação em uma tirosina na posição 216 (GRIMES & JOPE, 2001). A proteína Akt modula negativamente a atividade da GSK3β através da fosforilação da serina-9 (CROSS et al., 1995) e a redução da sinalização via PI3K resulta na defosforilação da Ser9 (HETMAN et al., 2000). Entre os alvos ou substratos da enzima GSK-3β estão a proteína precursora do peptídeo β-amilóide e a proteína tau (HOOPER et al., 2008), além de proteínas pró-apoptóticas como caspase-3 e Bax (GRIMES & JOPE, 2001; LINSEMAN et al., 2004). Atualmente, vários trabalhos têm demonstrado que a inibição da enzima GSK-3β poderia ser um alvo interessante para o desenvolvimento de novos fármacos no tratamento de doenças do SNC, principalmente aquelas que envolvem a morte neuronal e a neurodegeneração (HERNÁNDEZ et al., 2009; SELENICA et al., 2007; BHAT et al., 2004).

1.5. Modelos Experimentais de Excitotoxicidade

Modelos experimentais de excitotoxicidade *in vitro* têm sido amplamente utilizados como uma alternativa para experimentação animal, pois reduzem significativamente o número de animais utilizados, uma vez que uma série de experimentos pode ser realizada

simultaneamente; reduzem o tempo de experimentação e tem custo operacional reduzido. A utilização de modelos de estudo *in vitro* também apresenta vantagens experimentais, pois permite um ambiente experimental mais controlado, a avaliação da ação direta de substâncias neurotóxicas ou neuroproteroras sobre as células e está livre de influências externas como variações da pressão arterial e temperatura corporal. Os modelos mais utilizados são culturas de células, culturas organotípicas de tecido e fatias de tecido, geralmente de hipocampo de roedores (GÄHWILLER et al., 1997, MOLZ et al., 2005).

A utilização de fatias de hipocampo de ratos para avaliação de toxicidade apresenta algumas vantagens em relação a outros modelos *in vitro* pelo fato de manter a citoarquitetura do tecido de origem e a manutenção da matriz extracelular, preservando assim as interações neurônio-glia (GÄHWILLER et al., 1997). Por outro lado, a manutenção das fatias de hipocampo é limitado a algumas horas e, por isso, alternativas devem ser estabelecidas para estudos que necessitem a manutenção destas preparações por períodos de tempo mais prolongados.

Em nosso laboratório, verificamos que fatias de hipocampo de ratos jovens podem manter-se viáveis em meio de cultura por até 6 horas (h) sem comprometimento da viabilidade celular. Verificamos que quando as fatias de hipocampo foram submetidas ao glutamato (1 ou 10mM) e posteriormente incubadas por 6 h adicionais em meio de cultura, houve diminuição significativa na viabilidade celular em relação às fatias controle. A diminuição da viabilidade celular induzida por glutamato não foi acompanhada do aumento da liberação de LDH, indicando que a integridade da membrana celular estava preservada. Ainda com relação a esse modelo, verificamos que a morte celular induzida por glutamato ocorreu através de uma via apoptótica, pois o glutamato induziu liberação do citocromo c da mitocôndria, ativação da caspase-3 e fragmentação de DNA em frações internucleossomais. Estes eventos ocorreram 1,5; 3 e 18 h após a incubação com glutamato, respectivamente. Demonstrou-se também que a perda de viabilidade celular induzida por glutamato ocorreu

através da ativação de receptores ionotrópicos e metabotrópicos de glutamato, além da ativação de vias de sinalização das MAP cinases, que ativam a proteína $p38^{MAPK}$ (MOLZ et al., 2008).

Tem sido demonstrado que a susceptibilidade ao dano celular excitotóxico é dependente da idade dos animais, porém, em nosso laboratório, glutamato induz o mesmo padrão de morte celular em ratos jovens e em ratos adultos. Por outro lado, a captação de glutamato em situações basais é maior em ratos jovens que em ratos adultos (THOMAZI et al., 2004).

Muitos estudos utilizam culturas primárias de neurônios para investigar os mecanismos envolvidos na neurodegeneração. Entretanto, a quantidade reduzida de células obtidas a partir de culturas primárias, aliado a problemas éticos devido à utilização de embriões humanos para estudos patofisiológicos associados às doenças neurodegenerativas, torna a utilização de células derivadas de tumores neuronais um sistema amplamente empregado para estudar processos neuronais, incluindo vias de sinalização e mecanismo de morte neuronal.

A utilização de inibidores da cadeia respiratória tem sido empregada no estudo dos mecanismos envolvidos no estresse oxidativo mitocondrial. A utilização de rotenona e oligomicina A (inibidores dos complexos I e V da cadeia de transporte de elétrons, respectivamente) causa o aumento massivo da produção de ROS, perda do potencial de membrana mitocondrial e indução de apoptose, indicando que este é um bom modelo para estudar o estresse oxidativo dependente da mitocôndria (CAÑAS et al., 2007; EGEA et al., 2007). Em estudo recente realizado em culturas organotípicas de hipocampo, Schuh e colaboradores (2008) observaram que a utilização de doses subtóxicas de rotenona e de agonistas glutamatérgicos causa morte celular, sugerindo um mecanismo de ação sinérgico entre estes compostos.

2. NEUROBIOLOGIA DO SISTEMA PURINÉRGICO

As purinas podem ser divididas em purinas derivadas da adenina, representadas pela adenosina-5'-trifosfato (ATP), adenosina-5'-difosfato (ADP), adenosina-5´-monofosfato (AMP) e adenosina; e purinas derivadas da guanina, representadas pela guanosina-5'-trifosfato (GTP), guanosina-5'-difosfato (GDP), guanosina-5'-monofosfato (GMP) e guanosina. Classicamente, as purinas são reconhecidas por seus efeitos intracelulares como: fonte de energia celular, síntese protéica e transdução de sinal. Entretanto, as purinas derivadas da adenina e as purinas derivadas da guanina também atuam como importantes moléculas de sinalização intercelular (CICCARELLI et al., 2001).

Os efeitos da adenosina são desencadeados pela ativação de receptores P1, os quais também podem ser subdivididos em receptores A1, $A2_A$, $A2_B$ e A3. A adenosina age em receptores de membrana acoplados a proteínas G, desencadeando uma variedade de efeitos fisiológicos A ativação de diferentes subtipos de receptores é responsável por mediar os diferentes efeitos da adenosina (FREDHOLM et al., 2007).

Os receptores A1 e $A2_A$ de adenosina estão localizados nas sinapses, principalmente na região pré-sináptica. O principal efeito da adenosina no SNC é regular a liberação de neurotransmissores. O efeito inibitório da adenosina na redução da liberação de glutamato é mediado principalmente pela ativação de receptores A1. Este efeito da adenosina foi demonstrado tanto *in vivo* quanto *in vitro*, principalmente em modelos experimentais de hipóxia/hipoglicemia e modelos de convulsões (CUNHA, 2005). Agindo em receptores A1, a adenosina também hiperpolariza as células reduzindo assim a excitabilidade neuronal e a taxa de disparo dos neurônios (GREENE & HASS, 1991). A ação da adenosina em receptores $A2_A$ está associada ao desenvolvimento de neurotoxicidade, dano neuronal e morte celular. O bloqueio farmacológico dos receptores $A2_A$ ou a inativação genética destes receptores confere neuroproteção em modelos animais de isquemia *in vivo* (CHEN et al., 1999). Adicionalmente, ativação de receptores $A2_A$ facilita a liberação de glutamato em condições normais e durante a

isquemia. Esse aumento extracelular de glutamato pode levar à excitoxicidade e dano neural (CHEN et al., 2007). A ativação de receptores $A2_A$ também modula a morte de neurônios dopaminérgicos em modelos da doença de Parkinson e outras doenças neurodegenerativas (FREDHOLM et al., 2007). Dessa forma, a inativação de receptores $A2_A$ tem sido considerada uma estratégia neuroprotetora em diferentes modelos de doenças neurodegenerativas. Por outro lado, também foi demonstrado que a ativação de receptores $A2_A$ de adenosina protege alguns tipos de neurônios da neurotoxicidade induzida por glutamato (FERREIRA et al., 2001; CHEN et al., 2007).

Os receptores de adenosina também estão presentes nas células gliais (astrócitos, microglia e oligodendrócitos). A ativação de receptores de adenosina nestas células controla a astrogliose e a liberação de diferentes substâncias, contribuindo assim para o controle da atividade neuronal. A ativação de receptores de adenosina também controla a expressão e a liberação de citocinas e a reatividade da microglia, indicando assim que a adenosina também apresenta um papel importante no controle da neuro-inflamação (DI VIRGILIO et al., 2009).

Até o momento, devido à falta de ferramentas farmacológicas seletivas e também devido a baixa expressão de receptores $A2_B$ ou A3 de adenosina no cérebro, pouco se sabe sobre os efeitos da estimulação específica destes receptores no SNC, e efeitos opostos, tanto neuroprotetores quanto neurotóxicos, tem sido atribuídos a estes receptores (CUNHA, 2005).

Os efeitos do ATP são desencadeados pela ativação de receptores P2X e P2Y (BURNSTOCK, 2006). Os receptores P2X são receptores do tipo canal iônico e os receptores P2Y são receptores acoplados a proteínas-G. A ativação por nucleotídeos de receptores do subtipo P2Y podem ativar a fosfolipase-C e desencadear a liberação intracelular de Ca^{2+} ou afetar a atividade da adenilato ciclase alterando os níveis de AMP cíclico (FIELDS & BURNSTOCK, 2006). Ativação de receptores do subtipo P2X7 aumenta a liberação de glutamato e purinas em culturas de astrócitos (DUAN et al., 2003; ANDERSON et al., 2004). Estudos de eletrofisiologia realizados em fatias de hipocampo demonstraram a presença de

dois tipos de resposta à estimulação de receptores P2: a primeira foi a liberação astrocitária de glutamato dependente de cálcio, e a segunda foi a ativação de receptores P2X7 e o efluxo sustentado de glutamato (FELLIN et al., 2006). A co-liberação de ATP com vários neurotransmissores, inclusive glutamato, aponta para a possibilidade de interação entre os componentes purinérgicos e glutamatérgicos durante a transmissão sináptica.

Apesar de seus efeitos neuromoduladores e neuroprotetores, estudos demonstram que os nucleotídeos e nucleosídeos da adenina também podem apresentar efeitos tóxicos (ABBRACCHIO et al., 1995; FRANKE et al., 2006). Alguns tipos de incidentes cerebrais são acompanhados da liberação de grandes quantidades de purinas. Exemplos de situações em que as purinas podem ser liberadas e atingir concentrações neurotóxicas são: hipóxia e isquemia (BRAUN et al., 1998; MELANI et al., 2005); trauma (WANG et al., 2004; FRANKE et al., 2006); condições associadas com aumento da atividade neuronal (convulsões) (WIERASZKO et al., 1989).

Altas concentrações de adenosina induzem apoptose em células tumorais provenientes de astrocitoma de ratos através do envolvimento de vias de sinalização ativadas por receptores do tipo A_1 (SAI et al., 2005). Adenosina também parece mediar apoptose em astrócitos via ativação de receptores A3 (DI IORIO et al., 2002). A aplicação de concentraçãoes tóxicas de ATP induz modificações morfológicas compatíveis com necrose e apoptose em culturas de neurônios cerebelares (AMADIO et al., 2002). Os mesmos autores também demonstraram que a liberação de glutamato induzida por ATP pode contribuir para as os efeitos deletérios das altas doses de ATP. Adicionalmente, ATP também causa morte celular em culturas organotípicas de hipocampo (FRIZZO et al., 2007).

Recentemente, inúmeras evidências presentes na literatura também propõem a presença de um sistema purinérgico baseado nas purinas da guanina (nucleotídeos e guanosina) e apontam estes compostos como novos alvos para a neuroproteção e neuromodulação (SCHMIDT et al., 2007).

3. DERIVADOS DA GUANINA

Os derivados da guanina compreendem os nucleotídeos GTP, GDP, GMP e o nucleosídeo guanosina (GUO). Uma importante função descrita para os derivados da guanina é a modulação da atividade das proteínas-G. As proteínas-G são proteínas de membrana que tem sua atividade determinada pela interação com os nucleotídeos GTP e GDP e atuam como transdutores dos sinais extracelulares, resultando em respostas efetoras específicas. Além de seus efeitos intracelulares, evidências indicam que os derivados da guanina (GTP, GDP e GMP) podem desencadear efeitos extracelulares sem o envolvimento da proteína G (SOUZA & RAMÍREZ, 1991), e exercem efeitos tróficos em neurônios e células gliais, sendo importantes durante o desenvolvimento e em resposta à doenças (CICCARELLI et al., 2001; DECKER et al., 2007).

3.1. Produção, Metabolismo e Transporte

Os nucleosídeos são liberados para o meio extracelular através de transportadores de membrana. Os transportadores de nucleosídeos são uma família de proteínas que possuem diferentes afinidades por substratos, diferentes distribuições em tecidos, são espécie-específicos e sensíveis ao bloqueio por agentes farmacológicos. Os transportadores de nucleosídeos foram caracterizados e classificados em duas categorias: (i) transportadores equilibrativos, os quais transportam nucleosídeos das purinas e pirimidinas de maneira bidirecional, segundo o gradiente de concentração; (ii) transportadores concentrativos, que medeiam o influxo de mucleosídeos acoplado ao gradiente transmembrana de Na^+. No SNC, o transportador do tipo equilibrativo parece predominar (THORN & JARVIS, 1996), contribuindo para a manutenção da concentração dos nucleosídeos dentro e fora da célula.

A liberação de purinas para o meio extracelular já foi demonstrada em culturas de astrócitos. Além disso, foi demonstrado que em situação de hipóxia/hipoglicemia os

derivados da guanina são liberados cerca de 3 vezes mais do que os derivados da adenina, (CICCARELLI et al., 1999),.

A conversão de nucleotídeos em nucleosídeos no meio extracelular também é importante para a manutenção da concentração extracelular das purinas. Em estudo realizado em nosso laboratório, observou-se que nucleotídeo GTP pode ser captado e armazenado em vesículas sinápticas (SANTOS et al. 2006), sugerindo assim um possível mecanismo de liberação desse nucleotídeo para a fenda sináptica. A conversão de GTP em GDP e GMP é realizada por uma família de ectoenzimas (ectoNTDPases), já a conversão de GMP em GUO ocorre pela ação de ecto-5'-nucleotidases, as quais também são responsáveis pela hidrólise dos derivados da adenina (ZIMMERMANN, 1996), contribuindo assim para presença de nucleosídeos no meio extracelular. Estas enzimas são ativas e estão presentes no líquido cerebroespinhal de ratos (PORTELA et al., 2002). Em altas concentrações, a taxa de hidrólise de GDP é maior que a do ADP, possivelmente favorecendo a formação de GMP e, consequentemente, GUO. A presença de derivados da guanina já foi identificada no fluido cérebro-espinhal de humanos e de animais (REGNER et al., 1997; OSES et al., 2007). Recentemente foi demonstrada a presença da enzima purina nucleosídeo fosforilase (PNP) em membranas, a qual pode converter GUO em guanina (RATHBONE et al., 2008). Guanina é metabolizada irreversivelmente até xantina pela enzima guanina deaminase.

3.2. Efeitos Extracelulares dos Derivados da Guanina

Evidências demonstram que os derivados da guanina se unem aos receptores das purinas da adenina com baixa afinidade (MULLER & SCIOR, 1993), sugerindo que estes apresentam sítios de ligação distintos dos derivados da adenina. Estudos que dão embasamento a esta hipótese demonstraram que o GTP une-se a sítios específicos em células de feocromocitoma (PC12) (GYSBERS et al., 2000) e em membranas cerebelares de pintos, sendo que estes sítios não apresentam atividade enzimática (GTPásica) (TASCA et al., 1999).

Estudo realizado por Traversa e colaboradores (2002) demonstrou que a GUO apresenta um sítio de união de alta afinidade em preparações de membranas de cérebros de ratos. Outros estudos demonstram que os efeitos de GUO são possivelmente desencadeados através de um possível receptor de membrana acoplado à proteínas-G (CACIAGLI et al., 2000; TRAVERSA et al., 2002; DI IORIO et al., 2004). Este conjunto de dados indica que a GUO apresentam um sítio de união específico que ainda requer investigação, portanto, antagonistas específicos para seus receptores ainda não estão disponíveis.

Os derivados da guanina apresentam efeitos tróficos em culturas de células gliais e em neurônios (RATHBONE et al., 1999). Os derivados da guanina aumentam a produção de neurotrofinas em culturas de astrócitos e em cultura de neurônios estimulam o crescimento de neuritos (CICCARELLI et al., 2001). Em nosso laboratório foi demonstrado que GMP ou GUO aumentam o número de neurônios cultivados em co-cultura com astrócitos (DECKER et al., 2007). Neste mesmo trabalho foi demonstrado que a adição de GMP ou GUO em culturas de astrócitos altera a organização das proteínas da matriz extracelular, laminina e fibronectina, promovendo a melhor adesão dos neurônios sobre o substrato de astrócitos, o que pode ter um importante papel na neurogênese e na migração neuronal. Adicionalmente, os derivados da guanina também são importantes moduladores da neurotransmissão glutamatérgica.

3.3. Efeitos Neuroprotetores dos Derivados da Guanina: Modulação do Sistema Glutamatérgico

Os nucleotídeos e o nucleosídeo derivados da guanina desempenham importante papel extracelular na modulação da transmissão glutamatérgica: apresentam efeitos sobre a memória e o comportamento e efeitos neuroprotetores frente à excitotoxicidade (SCHMIDT et al., 2007).

GMP modula a transmissão glutamatérgica através da inibição da união de glutamato e agonistas (KA, AMPA e NMDA) a seus receptores sem interagir diretamente com proteínas-G (SOUZA & RAMÍREZ, 1991; PAZ et al., 1994; PAAS et al., 1996; PORCIÚNCULA et al., 2002). Em todos estes estudos, GUO não foi capaz de inibir a união de glutamato e agonistas a seus receptores. Também já foi demonstrado que GMP pode atuar como antagonista competitivo do receptor NMDA (BARON et al., 1989) e Mendieta e colaboradores (2005) demonstraram a existência de um sítio de interação para GMP em receptores AMPA de glutamato. Além de inibir a união de glutamato aos seus receptores, GMP também é capaz de inibir respostas celulares desencadeadas por glutamato ou agonistas tais como: diminuição a forfoforilação de GFAP (TASCA et al., 1995) e aumento de AMP cíclico induzidos por glutamato (TASCA & SOUZA, 2000), bloqueio do influxo de cálcio induzido por NMDA em retinas de pintos (BURGOS et al., 2000).

Em estudos de excitotoxicidade, GMP protege contra a toxicidade induzida por glutamato em fatias de hipocampo em modelos de isquemia (OLIVEIRA et al., 2002; MOLZ et al., 2005, OLESCOVICZ et al., 2008). Além disso, GMP protege fatias corticais submetidas à privação de glicose e oxigênio (FRIZZO et al., 2003). Estudos *in vivo* utilizando o ácido quinolínico como agente indutor de convulsões demonstrou que a administração de GMP protege contra as convulsões e a morte celular induzida por este agonista do receptor NMDA (MALCON et al., 1997; SCHMIDT et al., 2000). Em cultura de neurônios hipocampais e corticais, GMP (mas não GUO) diminui a neurotoxicidade induzida por NMDA e Cainato (MORCIANO, 2004).

Como mencionado anteriormente, GMP é neuroprotetor frente às convulsões induzidas pelo ácido quinolínico (SCHMIDT et al., 2000). Porém, estudos posteriores demonstraram que não somente GMP, mas também a GUO era anticonvulsivante neste modelo (SCHMIDT et al., 2000; VINADÉ et al., 2003; OLIVEIRA et al., 2004). Além disso, estudos demonstraram que o efeito anticonvulsivante do GMP era abolido na presença de um

inibidor da enzima 5´-ectonucleotidase (enzima que converte GMP em GUO) (SOARES et al., 2004). Dessa forma, considerando o modelo de convulsão induzida pelo ácido quinolínico, o efeito anticonvulsicante do GMP dependeu da sua conversão até GUO.

GUO não é capaz de inibir a união de glutamato ou a seus receptores (SOUZA & RAMÍREZ, 1991; MONAHAN et al., 1988), porém apresenta diversos efeitos envolvendo a modulação do transporte de glutamato (SCHMIDT et al., 2007). A retirada do glutamato da fenda sináptica é um importante mecanismo para modular as ações desse neurotransmissor além de manter os níveis de glutamato em concentrações que não sejam neurotóxicas (DANBOLT, 2001). GUO é capaz de aumentar a captação basal de glutamato em culturas de astrócitos e em fatias corticais mantidas em condições basais ou submetidas à privação de glicose e oxigênio (FRIZZO et al., 2002; 2003). GMP também apresentou efeitos sobre a captação de glutamato em astrócitos, porém, este efeito do GMP também dependeu da sua conversão até GUO (FRIZZO et al., 2003).

A administração de GUO previne a diminuição da captação de glutamato em fatias de hipocampo obtidas a partir de animais submetidos ao modelo de convulsão com ácido quinolínico (OLIVEIRA et al., 2004; VINADÉ et al., 2005). Além disso, GUO também previne a diminuição da captação de glutamato em fatias de hipocampo obtidas de ratos previamente submetidos à isquemia neonatal (MORETTO et al., 2005) e também em fatias de hipocampo submetidas à privação de glicose e oxigênio *in vitro* (THOMAZI et al., 2008). Adicionalmente, o ácido quinolínico *in vitro* e *in vivo* estimula a liberação de glutamato em preparações sinaptossomais e inibe a captação astrocitária de glutamato (TAVARES et al., 2000; 2002), o que poderia contribuir para o aumento da liberação de glutamato e indução de convulsões. Esse aumento é revertido na presença de GUO (TAVARES et al., 2005; 2008). Dessa maneira, GUO é considerada uma importante molécula neuroprotetora, pois através da modulação do transporte de glutamato este nucleosídeo contribui para a redução dos níveis

extracelulares de glutamato, prevenindo assim o aumento da concentração de glutamato na fenda sináptica, excitoxicidade e morte celular.

Os efeitos neuroprotetores de GMP e GUO, tanto *in vitro* quanto *in vivo*, não foram inibidos na presença de antagonistas de receptores de adenosina, indicando assim que estes efeitos ocorrem de maneira independente da atuação de GMP ou GUO sobre o sistema adenosinérgico (FRIZZO et al., 2003, LARA et al., 2001).

Astrócitos mantidos em cultura liberam derivados da guanina em condições basais, porém, quando essas culturas são submetidas à hipóxia e hipoglicemia os níveis dos derivados da guanina no meio extracelular aumentam significativamente (CICCARELLI et al., 1999). *In vivo*, os níveis dos derivados da guanina aumentam durante a hipóxia/hipoglicemia e estes níveis permanecem elevados por até uma semana (UEMURA et al., 1991). Em vista disso, estudo recente realizado em nosso laboratório investigou o papel protetor da GUO em um modelo de isquemia cerebral através da privação de glicose e oxigênio em fatias hipocampais de ratos. Neste estudo foi demonstrado que fatias de hipocampo de ratos submetidas a 15 minutos de privação de glicose e oxigênio seguidas de 2 horas de reperfusão, apresentam uma melhora da viabilidade celular quando a GUO é adicionada no período de reperfusão (OLESKOVICZ et al., 2008). Adicionalmente, neste modelo, GUO previne a redução da captação de glutamato induzida pela privação de glicose e oxigênio via ativação de canais de K^+ de alta voltagem ativados por Ca^{2+} e pela via da PI3K (DAL-CIM, 2008).

Estudos recentes também têm demonstrado um importante papel protetor da GUO em modelos de doenças neurodegenerativas. A utilização de GUO em ratos submetidos a um modelo de doença de Parkinson reduz apoptose e promove neurogênese (SU et al., 2009). A adição de GUO na linhagem de neuroblastoma humano SH-SY5Y protege essas células contra a apoptose induzida pelo peptídeo β-amilóide (PETTIFER et al., 2004) ou MPP^+ (PETTIFER et al., 2007). A GUO é capaz de diminuir a expressão e função de receptores

CD40 (receptores celulares associados a eventos inflamatórios) em culturas de células expostas a agentes pró-inflamatórios e ao peptídeo β-amilóide (D'ALIMONTE et al., 2007). Além disso, a GUO parece possuir um efeito anti-apoptótico através da ativação da via PI3K/Akt em cultura de astrócitos (DI IORIO et al., 2004).

Diante do exposto anteriormente, os derivados da guanina tem apresentado efeitos neuroprotetores importantes em diferentes modelos de neurotoxicidade. Portanto, o estudo dos efeitos protetores dos derivados da guanina frente a distintas situações de neurototoxicidade, bem como os mecanismos envolvidos no efeito desencadeado por estes compostos pode apresentar importante significado terapêutico.

4. OBJETIVOS

4.1. Objetivo geral

Estudar os efeitos neuroprotetores e neurotóxicos dos derivados da guanina (GMP e GUO) em fatias de hipocampo de ratos e culturas de células de neuroblastoma humano (SHSY-5Y).

4.2. Objetivos específicos

- Avaliar o efeito neuroprotetor de GMP frente à excitotoxicidade induzida por glutamato e NMDA em fatias de hipocampo de ratos;
- Avaliar o efeito neuroprotetor da GUO frente à excitotoxicidade induzida por glutamato em fatias de hipocampo de ratos, estudando o envolvimento do transporte de glutamato, da via PI3K/Akt/GSK3β e da modulação da iNOS no mecanismo neuroprotetor da GUO;
- Avaliar o possível efeito neurotóxico do GMP e GUO em fatias de hipocampo de ratos e os mecanismos relacionados a esse efeito;

- Investigar o efeito neuroprotetor da GUO em um modelo de estresse oxidativo mitocondrial em culturas de células de neuroblastoma humano (SHSY-5Y) e os mecanismos envolvidos no efeito da GUO;
- Avaliar a susceptibilidade de culturas de células de neuroblastoma humano (SHSY-5Y) não diferenciadas e diferenciadas à toxicidade do peptídeo β-amilóide (fragmento 25-35) e avaliar o possível efeito protetor da GUO frente à toxicidade do peptídeo β-amilóide nestas células.

CAPÍTULO 1

GMP PREVENTS EXCITOTOXICITY MEDIATED BY NMDA RECEPTOR ACTIVATION BUT NOT BY REVERSAL ACTIVITY OF GLUTAMATE TRANSPORTERS IN RAT HIPPOCAMPAL SLICES

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Research Report

GMP prevents excitotoxicity mediated by NMDA receptor activation but not by reversal activity of glutamate transporters in rat hippocampal slices

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ABSTRACT

Glutamate is the main excitatory neurotransmitter in the mammalian nervous system and is essential for its normal functions. However, overstimulation of glutamatergic system due to hyperactivation of NMDA receptors and/or impairment of glutamate reuptake system has been implicated in many acute and chronic neurological diseases. Regulation of extracellular glutamate concentrations relies on the function of glutamate transporters which can be reversed in situations related to excitotoxicity. Guanosine-5′-monophosphate (GMP), a guanine nucleotide which displays important extracellular roles, such as trophic effects to neurons and astrocytes, behaves as antagonist of glutamate receptors and is neuroprotective in hippocampal slices against excitotoxicity or ischemic conditions. Hippocampal slices exposed to 1 or 10 mM glutamate, or 100 μM NMDA with 10 μM glycine for 1 h and evaluated after 6 or 18 h, showed reduced cell viability and DNA fragmentation, respectively. Glutamate- or NMDA-induced cell death was prevented by 50 μM MK-801, but only NMDA-induced cell damage was prevented by GMP (1 mM). Glutamate-induced cell viability impairment and glutamate-induced L-[^{3}H]glutamate release were both prevented by adding DL-TBOA (10 μM). Otherwise, NMDA-induced cell viability loss was not prevented by 10 μM of DL-TBOA and NMDA did not induce L-[^{3}H] glutamate release. Our results demonstrate that GMP is neuroprotective when acting selectively at NMDA receptors. Glutamate-induced hippocampal slice damage and glutamate release were blocked by glutamate transporter inhibitor, indicating that glutamate-induced toxicity also involves the reversal of glutamate uptake, which cannot be prevented by GMP.

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Abbreviations: DL-TBOA, DL-threo-β-benzyloxyaspartic acid; Glu, Glutamate; GMP, Guanosine-5′-monophosphate; MK-801, (+)–5–methyl–10,11–dihydro–5H–dibenzo[a,d]cyclohepten–5,10-imine maleate; MTT, 3-(4,5-dimethylthiazol-2-yl)-,5-diphenyltetrazolium bromide; NMDA, N-methyl-D-aspartate

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1. Introduction

Besides the well established physiological roles of glutamate in the mammalian nervous system (Meldrum, 2000; Segovia et al., 2001), high concentrations of extracellular glutamate can cause cell death through a process called excitotoxicity (Choi, 1988; Meldrum, 2000).

Glutamate-induced excitotoxicity can be accomplished by its interaction with NMDA subtype of ionotropic glutamate receptors leading to a massive calcium influx and cell death by necrosis or apoptosis (Ankarcrona et al., 1995, Bonfoco et al., 1995, Mattson, 2000), which is generally blocked by the non-competitive NMDA receptor antagonist, MK-801 (Chen and Lipton, 2006). Another important process of the regulation of excitotoxicity relies on the function of glutamate transporters present on astrocytes and, to a lesser extent, in neurons (Anderson and Swanson, 2000). In the rat hippocampus, three glutamate transporters, GLAST, GLT-1 and EAAC1, are present. GLAST and GLT-1 are the most prominent transporters and are localized on astrocytes. These transporters are coupled to Na/K ATPase and normally function to remove excessive glutamate from brain extracellular space (Danbolt, 2001). In situations where membrane Na gradient is disrupted, such as ischemia or hypoglycemia, the increased extracellular glutamate is correlated to activation of glutamate transporters reversal of uptake (Madl and Buegesser, 1993; Rossi et al., 2000; Camacho and Massieu, 2006).

Accumulation of glutamate in the extrasynaptic cleft due to reverse activation of glutamate transporters and/or hyperactivation of glutamate receptors has been implicated not only in acute injuries, as hypoxia, ischemia, hypoglycemia or epileptic seizures, but has also been related to progressive degenerative disorders, as Alzheimer's, Huntington's and Parkinson's diseases, amyotrophic lateral sclerosis, schizophrenia and other psychiatric disorders (Choi, 1992; Segovia et al., 2001; Matute et al., 2002; Mattson, 2003, Maragakis and Rothstein, 2004; Sattler and Rothstein, 2006).

Guanine nucleotides are endogenous compounds known for their intracellular role in modulating G-protein activity, although they can also display fundamental extracellular roles (for a review, see Schmidt et al., 2007). Guanine derivatives can act as trophic factors to neurons and astrocytes (Neary et al., 1996, Ciccarelli et al., 2001, Decker et al., 2007), behave as competitive antagonists of glutamatergic receptors (Baron et al., 1989; Souza and Ramírez, 1991; Dev et al., 1996; Porciúncula et al., 2002 and can also inhibit physiological cell responses due to glutamate receptor activation (Tasca et al., 1995; Tasca et al., 1998; Burgos et al., 2000). Guanosine-5′-monophosphate (GMP), a guanine nucleotide which does not bind to G-proteins, is shown to be released from astrocytes after hypoxia/hypoglycemia (Ciccarelli et al., 1999). Studies from our laboratory demonstrated that GMP is neuroprotective in hippocampal slices against glutamate- or glucose/oxygen deprivation-induced neurotoxicity (Oliveira et al., 2002, Molz et al., 2005, Oleskovicz et al., 2008). GMP has also been reported to prevent NMDA-mediated induction of seizures (Schmidt et al., 2000). Recently, a specific binding site to GMP in GluR2 AMPA receptors was demonstrated (Mendieta et al., 2005).

We have previously reported that millimolar concentrations of glutamate induced hippocampal slice cell death with apoptotic features such as cytochrome c release and caspase-3 activation, culminating in DNA fragmentation (Molz et al., 2008). So, the aim of the present study was to investigate the putative neuroprotective role of GMP against glutamate- and NMDA-induced hippocampal slice injury. We observed that glutamate- and NMDA-induced cell death in hippocampal slices involved DNA fragmentation into internucleosomal fractions which resembles apoptosis, and it was prevented by MK-801. GMP was neuroprotective against the cell viability reduction and DNA fragmentation only when selectively acting as antagonist at NMDA receptors. Furthermore, glutamate-induced hippocampal slice damage and glutamate release were also blocked by a glutamate transporter inhibitor, indicating that glutamate-induced toxicity also involved the reversal of glutamate uptake.

2. Results

2.1. *Glutamate-induced toxicity is not prevented by GMP*

Hippocampal slices were incubated for 1 h with 1 or 10 mM glutamate and were maintained in incubation medium for 6 h to the evaluation of cellular viability. When present, MK-801 (50 μM) or GMP (1 mM) was preincubated for 10 and 30 min, respectively, before glutamate challenge. Glutamate (1 or 10 mM) significantly reduced cell viability when compared to control slices, which was prevented by MK-801, a non-

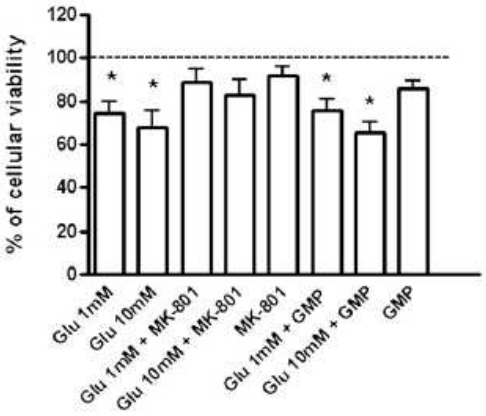

Fig. 1 – Cell viability in hippocampal slices incubated with glutamate in the presence of MK-801 and GMP. Hippocampal slices were incubated for 1 h with glutamate (1 mM or 10 mM). When present, MK-801 (50 μM) or GMP (1 mM) was preincubated for 10 and 30 min, respectively. After this period, incubation medium was withdrawn and replaced for fresh culture medium without glutamate and maintained for additional 6 h. Control group was considered as 100% and represents cell viability of slices incubated only in culture medium. MTT (0.5 mg/ml) was incubated for 20 min at 37 °C and cell viability was accessed at 550 nm. The values represent means ± error deviations of at least 4 experiments carried out in triplicates. *Indicates means significantly different from all other groups; $p < 0.05$.

competitive NMDA receptor antagonist. However, GMP pre-incubation did not alter the reduction in cell viability induced by glutamate (1 or 10 mM) (Fig. 1).

2.2. *Neuroprotective effect of GMP against NMDA-induced toxicity*

Since glutamate induced cell damage was prevented by MK-801 (Fig. 1), NMDA toxicity was also evaluated. NMDA (100 μM) in the presence of glycine (10 μM) a co-agonist of NMDA receptors, significantly reduced cell viability when compared to control slices. NMDA-induced hippocampal slice damage was completely prevented when slices were preincubated for 10 min with 50 μM MK-801 (Fig. 2).

The potential neuroprotective role of GMP against NMDA-induced toxicity in hippocampal slices was tested. GMP (1 mM) completely prevented hippocampal slice toxicity induced by NMDA receptor activation (Fig. 2).

2.3. *GMP prevents NMDA-induced DNA fragmentation in hippocampal slices*

We have previously shown that glutamate-induced toxicity involves apoptotic features, with a slight DNA fragmentation into internucleosomal fractions (Molz et al., 2008). Hippocampal slices were incubated for 1 h with glutamate (1 or 10 mM) or NMDA (100 μM) and glycine (10 μM) and DNA fragmentation was evaluated after 18 h. Fig. 3 shows the eletrophoretic migration pattern of extracted DNA from hippocampal slices

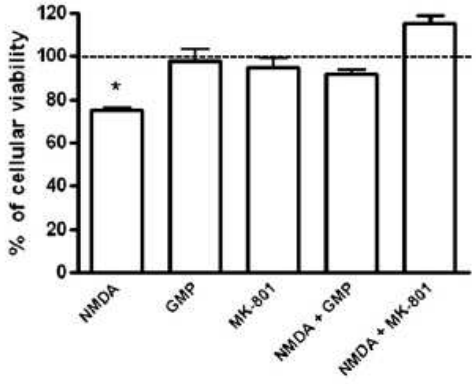

Fig. 2 – Cell viability in hippocampal slices incubated with NMDA in the presence of MK-801 and GMP. Hippocampal slices were incubated for 1 h with 100 μM NMDA and 10 μM glycine. When present, MK-801 (50 μM) or GMP (1 mM) was preincubated for 10 and 30 min, respectively. After this period, incubation media was withdrawn and replaced for fresh culture medium without NMDA and maintained for additional 6 h. Control group was considered as 100% and represents cell viability of slices incubated only in culture medium. MTT (0.5 mg/ml) was incubated for 20 min at 37 °C and cell viability was accessed at 550 nm. The values represent means ± error deviations of at least 4 experiments carried out in triplicates. * Indicates means significantly different from all other groups; $p<0.05$.

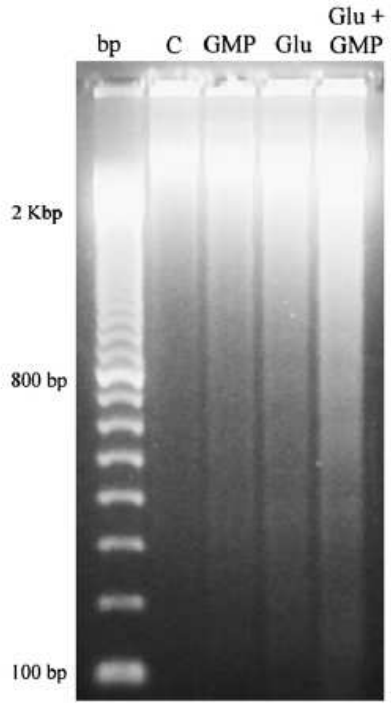

Fig. 3 – Evaluation of DNA fragmentation in internucleosomal fractions in hippocampal slices exposed to glutamate and GMP. Control slices or glutamate-treated hippocampal slices (1 or 10 mM) were incubated for 1 h in the presence or absence of 1 mM GMP. Medium was replaced and slices were maintained for additional 18 h in fresh culture medium without glutamate. DNA was extracted from hippocampal slices and DNA "laddering" visualised in agarose gel electrophoresis with ethidium bromide. The lanes in the gel are: 100 base pair (bp) pattern, Control (C), GMP 1 mM (GMP), Glutamate 10 mM (Glu) and Glutamate 10 mM GMP 1 mM (Glu GMP). A slight DNA fragmentation is observed at lanes Glu and Glu GMP. This gel is representative from 5 individual experiments.

submitted to glutamate (1 or 10 mM) in the presence of GMP (1 mM). GMP did not prevent the slight glutamate-induced DNA fragmentation (Fig. 3).

In the DNA fragmentation analysis following slice exposition to NMDA, we observed that NMDA induced a significant DNA fragmentation (Fig. 4), suggesting the involvement of apoptosis in the mechanism of NMDA receptor-mediated cell damage. The NMDA-induced DNA fragmentation was prevented by slice preincubation with 50 μM MK-801 or 1 mM GMP (Fig. 4), reinforcing the neuroprotective action of GMP against NMDA-induced cell injury.

2.4. *Involvement of reversal uptake in glutamate but not in NMDA-induced toxicity*

Since MK-801 neuroprotection against NMDA-induced hippocampal toxicity was more prominent than the observed

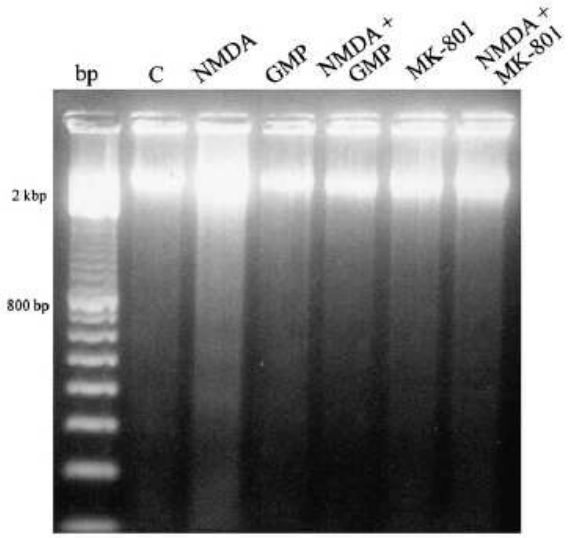

Fig. 4 – Evaluation of DNA fragmentation in internucleosomal fractions in hippocampal slices exposed to NMDA, MK-801 or GMP. Control slices or NMDA-treated hippocampal slices were incubated for 1 h in the presence or absence of 50 μM MK-801 or 1 mM GMP. Medium was replaced and slices maintained for additional 18 h in fresh culture medium without NMDA. DNA was extracted from hippocampal slices and DNA "laddering" visualised in agarose gel electrophoresis with ethidium bromide. The lanes in the gel are: 100 base pair (bp) pattern, Control (C), NMDA 100 μM and glycine 10 μM (NMDA), GMP 1 mM (GMP), NMDA 100 μM and glycine 10 μM plus GMP (NMDA GMP), MK-801 50 μM (MK-801), NMDA 100 μM and glycine 10 μM plus MK-801 (NMDA MK-801). A slight DNA fragmentation is observed at lane NMDA. This gel is representative from 3 individual experiments.

for MK-801 against glutamate-induced toxicity (Fig. 1), and considering GMP was unable to prevent glutamate-induced cell damage, we investigated whether glutamate is acting in another interaction site besides glutamate receptors, such as glutamate transporters. In order to achieve this, we used the non-transportable glutamate transporter inhibitor DL-TBOA in a concentration that does not induce cell death (Anderson et al., 2001; Bonde et al., 2003). We found that preincubation of hippocampal slices for 30 min with 10 μM DL-TBOA *per se* did not induce cell damage, but completely prevented glutamate-induced reduction in cell viability (Fig. 5). Additionally, evaluation of glutamate release showed that glutamate (10 mM) induced an increased amount of released glutamate. This neurotoxic glutamate-induced glutamate release was partially prevented by preincubation with DL-TBOA (Fig. 6), confirming a neurotoxic effect of glutamate also via reversal activity of glutamate transporters. Otherwise, we have found that NMDA-induced toxicity is not prevented by 10 μM DL-TBOA and NMDA did not induce glutamate release (Figs. 5 and 6).

3. Discussion

The present study demonstrated glutamate and NMDA induced cell death with apoptotic features since the reduction of cell viability in hippocampal slices was accompanied by DNA fragmentation. NMDA-induced cell damage was fully prevented by GMP, an endogenous guanine nucleotide acting as a glutamate receptor antagonist. However, GMP did not alter glutamate-induced cell damage. Additionally, we showed that glutamate-induced slice damage can also occur via reversal of the plasma membrane glutamate transporters, since the application of the glutamate transport blocker, DL-TBOA, prevented the loss of cell viability induced by glutamate and glutamate-induced L-[^{3}H]glutamate release. The toxicity via activation of NMDA receptors was not prevented by DL-TBOA and NMDA did not induce glutamate release. Therefore, GMP displays a neuroprotective effect by acting as a glutamate receptor antagonist, but it is not directly involved in the modulation of glutamate transport.

A variety of agents prevents or modulates glutamate receptor overactivation in order to control cell death (Stone and Addae, 2002). MK-801, the non-competitive antagonist of NMDA receptors prevented glutamate- or NMDA-induced cell damage (Figs. 1 and 2) and also prevented NMDA-induced DNA fragmentation (Fig. 4), thus showing that NMDA receptors are involved in glutamate-induced apoptosis in hippocampal

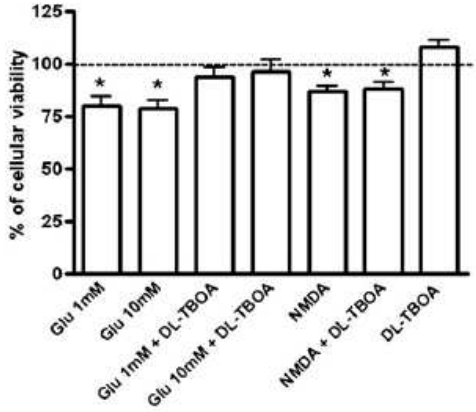

Fig. 5 – Cell viability in hippocampal slices incubated with glutamate or NMDA and the inhibitor of glutamate transport, DL-TBOA. Hippocampal slices were incubated for 1 h with glutamate (1 mM or 10 mM) or NMDA 100 μM and 10 μM glycine in the presence or absence of 10 μM DL-TBOA. When present, DL-TBOA was preincubated for 30 min. After this period, incubation medium was withdrawn and replaced for fresh culture medium without glutamate and maintained for additional 6 h. Control group was considered as 100% and represents cell viability of slices incubated only in culture medium. MTT (0.5 mg/ml) was incubated for 20 min at 37 °C and cell viability was accessed at 550 nm. The values represent means ± error deviations of at least 4 experiments carried out in triplicates. *Indicates means significantly different from all other groups; $p < 0.05$.

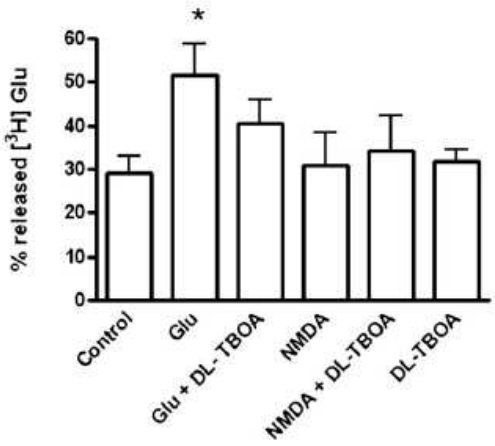

Fig. 6 – Glutamate release in hippocampal slices incubated with glutamate or NMDA and DL-TBOA. Hippocampal slices were incubated for 1 h with 10 mM glutamate or 100 μM NMDA and 10 μM glycine in the presence or absence of DL-TBOA. When present, DL-TBOA (10 μM) was preincubated for 10 min. Glutamate uptake was assessed as described in Experimental procedures. Results were expressed as percentage of total L-[^{3}H]glutamate. The values represent means ± error deviations of at least 7 experiments carried out in triplicates. *Indicates mean significantly different from all other groups; $p<0.05$.

slices. We cannot exclude the possibility that glutamate may also be acting via non-NMDA or metabotropic glutamate receptors. Indeed, we have previously demonstrated that glutamate toxicity in hippocampal slices also involves AMPA and KA receptors as well as metabotropic glutamate receptors (Molz et al., 2008). Moreover, we showed that although glutamate promoted neurotoxicity via NMDA receptor activation, it is also promoting the reversal activity of glutamate transporters, increasing glutamate release which can be blocked by glutamate transport inhibitor (Fig. 6) and therefore providing neuroprotection against glutamate (Fig. 5).

Even though the protective effects of classical glutamate and NMDA receptor antagonists in experimental animal models are well established (Park et al., 1988; Stone and Addae, 2002), the use of current NMDA receptor antagonists in clinical trials have not been successful (Ikonomidou and Turski, 2002; Lipton, 2004; Chen and Lipton, 2006; Muir, 2006). So, searching for new compounds that interact with the glutamatergic system is of great interest. GMP has been shown to be released by cultured astrocytes after hypoxia/hypoglycemia (Ciccarelli et al., 1999) and described to be neuroprotective in oxygen and/or glucose deprivation *in vitro* models (Regner et al., 1998, Oliveira et al., 2002, Molz et al., 2005) as well as *in vivo* models of seizure induction by NMDA (Malcon et al., 1997; Schmidt et al., 2000). GMP is able to displace the binding of NMDA to its receptors (Baron et al., 1989, Porciúncula et al., 2002) and also diminishes NMDA-induced neurotoxicity in neuronal cell cultures (Morciano et al., 2004). We are hereby reporting that GMP counteracted the reduction in cell viability and DNA fragmentation provoked by NMDA (Figs. 2 and 4). Thus, GMP can prevent apoptosis induced by NMDA receptor activation in hippocampal slices *in vitro*.

Considering GMP can be hydrolyzed in the extracellular space by ectonucleotidases (Zimmermann, 1996), we cannot completely rule out the involvement of the nucleoside guanosine as a mediator of GMP-induced neuroprotective effect against glutamate-induced injury. However, guanosine had no (or very little) effect on the binding of glutamate or analogs to glutamate receptors (Souza and Ramírez, 1991; Porciúncula et al., 2002) and evidences support the hypothesis that the neuroprotective effect of guanosine is related to its involvement in modulation of glutamate uptake (Frizzo et al., 2001; 2003) showing it is not directly related to an interaction with glutamate receptors. Moreover, we have recently demonstrated that guanosine-induced neuroprotection against oxygen/glucose deprivation occurs via K-channel activation and depends on extracellular calcium (Oleskovicz et al., 2008). The results showed in this study may point to differential effects of guanine derivatives on the modulation of the glutamatergic system. We can hypothesize that in a brain injury situation, the release of guanine nucleotides from synaptic vesicles (Tasca et al., 2004; Santos et al., 2006) or from astrocytes (Ciccarelli et al., 1999), increasing extracellular GMP levels would be responsible for blocking NMDA receptor activation, whereas after its hydrolysis, the production of the nucleoside, guanosine, may improve glutamate uptake, resulting in neuroprotection.

In a situation of energy deprivation when ATPase stops functioning, transmembrane ion gradient runs down and the transporters will reverse and release glutamate to the extracellular space (Madl and Buegesser, 1993, Camacho and Massieu, 2006). Application of 10 μM DL-TBOA has been found to significantly reduce the reversal of transporters in both astrocytic and neuronal cultures (Anderson et al., 2001), and it is sub-toxic and neuroprotective in slice cultures (Bonde et al., 2003; 2005). DL-TBOA applied before glutamate exposure prevented both glutamate-induced glutamate release and glutamate-induced cell damage (Figs. 5 and 6). Since DL-TBOA counteracted glutamate-induced toxicity (Fig. 5), glutamate uptake reversal and its associated mechanisms, as glutathione depletion (Chen et al., 2000; Re et al., 2003; 2006), are involved in glutamate-induced cell death in hippocampal slices. Differently, NMDA-induced toxicity was not prevented by DL-TBOA and NMDA did not induce glutamate release (Figs. 5 and 6), showing its classical toxicity mechanism via NMDA receptor activation.

In our study, glutamate-induced cell injury was not prevented by GMP (Fig. 2). Studies showing neuroprotective effect of GMP against glucose/oxygen deprivation were previously shown, although in those studies glutamate transport was not evaluated (Regner et al., 1998; Oliveira et al., 2002) or not altered by GMP (Molz et al., 2005).

Glutamate can induce cell death in hippocampal slices via two mechanisms: one involving the binding of glutamate to NMDA receptors leading to receptor-mediated excitotoxicity, which can be blocked by GMP; and other promoted by glutamate-induced reversal of its uptake. This mechanism is not blocked by GMP.

Overactivation of glutamate receptors, loss of energy supply leading to transmembrane ion gradients disruption and altered transport function, are common features of several neurological disorders which involve excitotoxicity such as stroke, amyotrophic lateral sclerosis and epilepsy (Allen et al., 2004). So, understanding the mechanisms involved in glutamate-induced toxicity as well as compounds that can prevent its effects may have therapeutic potential in neurological conditions involving glutamate excitotoxicity. Therefore, our results are contributing to discriminate among the extracellular effects of endogenous guanine derivatives, showing the mono-phosphorylated guanine nucleotide, GMP, can act as a glutamate receptor antagonist, but it does not modulate the glutamate release via reversal of plasma membrane transporters.

4. Experimental procedures

4.1. Animals

Male immature Wistar rats (23–25 days of postnatal age) maintained on a 12-hour light–12-hour dark schedule at 25 °C, with food and water *ad libitum*, were obtained from our local breeding colony. Experiments followed the "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1985) and were approved by the local Ethical Committee of Animal Research (CEUA/UFSC).

4.2. Preparation and incubation of hippocampal slices

Rats were killed by decapitation and the hippocampi were rapidly removed and placed in ice-cold Krebs–Ringer bicarbonate buffer (KRB) of the following composition: 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM D-glucose. The buffer was bubbled with 95% O_2–5% CO_2 up to pH 7.4. Slices (0.4 mm thick) were rapidly prepared using a McIlwain Tissue Chopper, separated in KRB at 4 °C and allowed to recover for 30 min in KRB at 37 °C (Oliveira et al., 2002).

4.3. Slice treatment

After the preincubation time, hippocampal slices were incubated with glutamate (Sigma) (1 or 10 mM) or NMDA (100 μM and 10 μM glycine) for 1 h in KRB. After this period, the medium was withdrawn and replaced by a nutritive culture medium composed of 50% of KRB, 50% of Dulbecco's modified Eagle's medium (DMEM, Gibco), 20 mM of HEPES and 100 μg/ml of gentamicine, at 37 °C in a CO_2 atmosphere (Molz et al., 2008) and slices were maintained for additional 6 or 18 h to evaluate cell viability or DNA fragmentation, respectively.

In order to investigate the mechanisms involved in glutamate- or NMDA-induced toxicity, the slices were preincubated for 10 min with (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801, 50 μM) or for 30 min with DL-threo-β-benzyloxyaspartate (DL-TBOA, 10 μM) before the 1-hour exposure of slices to glutamate or NMDA and maintained during this 1 h of incubation. When present, GMP (1 mM) was also preincubated for 30 min before glutamate or NMDA.

4.4. Evaluation of cell viability

Hippocampal cell viability was evaluated 6 h after glutamate or NMDA exposure. Cell viability was determined through the ability of cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl-diphenyltetrazolium bromide, Sigma) (Mosmann, 1983). Hippocampal slices were incubated with MTT (0.5 mg/ml) in KRB for 30 min at 37 °C. The tetrazolium ring of MTT can be cleaved by active dehydrogenases in order to produce a precipitated formazan. The formazan produced was solubilized by adding 200 μl dimethyl sulfoxide (DMSO), resulting in a coloured compound which optical density was measured in an ELISA reader (550 nm).

4.5. DNA fragmentation analysis

Glutamate- or NMDA-induced DNA fragmentation were evaluated 18 h after glutamate or NMDA exposure. Ten to 20 mg of hippocampal tissue was used for DNA extraction. Tissue was homogenized in a lysis buffer (GenomicPrep™, GE Healthcare) and then incubated for 1 h at 65 °C. Proteins and RNA were digested with proteinase K (Sigma, 100 μg/ml) and RNAse (Sigma, 20 μg/ml), at 55 °C for 12 h and at 37 °C for 1 h, respectively. Proteins and RNA were then precipitated and samples were centrifuged at 16,000 *g* for 15 min at 4 °C. DNA was precipitated for 12 h with 100% isopropanol at 4 °C and 70% ethanol. Centrifuged samples (corresponding to 40 μg DNA) were then submitted to a 2% agarose gel electrophoresis with ethidium bromide (100 μg/ml) and visualised in UV light (Yang and Paul, 1997).

4.6. Glutamate release

After preincubation time in KRB buffer, hippocampal slices were incubated in Hank's balanced salt solution (HBSS), composition in mM: 1.29 $CaCl_2$, 136.9 NaCl, 5.36 KCl, 0.65 $MgSO_4$, 0.27 Na_2HPO_4, 1.1 KH_2PO_4, and 5 HEPES. When present, DL-TBOA (10 μM), was preincubated for 10 min. Glutamate (10 mM) was incubated for 15 min and glutamate uptake was assessed by adding 0.33 μCi/ml L-[^{3}H]glutamate with 100 μM unlabeled glutamate for 7 min and stopped by three ice-cold washes with 1 ml HBSS. The slices were then further incubated for 15 min in 300 μl HBSS and the supernatant was collected in order to measure the amount of released L-[^{3}H]glutamate. Slices were disrupted by overnight incubation with 0.1% NaOH/0.01% SDS and aliquots of lysates were taken for determination of intracellular L-[^{3}H]glutamate content (Tavares et al., 2002; Molz et al., 2005). Intracellular and extracellular [^{3}H]glutamate content were determined through scintillation counting, calculated as nmol of glutamate per milligram of protein per minute and the amount of released glutamate was expressed as percentage of total L-[^{3}H]glutamate.

4.7. Protein measurement

Protein content was evaluated by the method of Lowry et al. (1951). Bovine serum albumin (Sigma) was used as standard.

4.8. Statistical analysis

Comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by Duncan's test if necessary, with $p<0.05$ considered to be statistically significant.

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REFERENCES

Allen, N.J., Káradóttir, R., Attwell, D., 2004. Reversal or reduction of glutamate and GABA transport in CNS pathology and therapy. Pflugers Arch. 449, 132–142.

Anderson, C.M., Swanson, R.A., 2000. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32, 1–14.

Anderson, C.M., Bridges, R.J., Chamberlin, A.R., Shimamoto, K., Yasuda-Kamatani, Y., Swanson, R.A., 2001. Differing effects of substrate and non-substrate transport inhibitors on glutamate uptake reversal. J. Neurochem. 79, 1207–1216.

Ankarcrona, M., Dypburkt, J.M., Bonfoco, E., Zhyvotovsky, B., Orrenius, S., Lipton, S.A., Nicotera, P., 1995. Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron 15, 961–973.

Baron, B.M., Dudley, M.W., McCarty, D.R., Miller, F.P., Reynolds, I.J., Schmidt, C.J., 1989. Guanine nucleotides are competitive inhibitors of *N*-methyl-D-aspartate at its receptor site both *in vitro* and *in vivo*. J. Pharmacol. Exp. Ther. 250, 162–169.

Bonde, C., Sarup, A., Schousboe, A., Gegelashvili, G., Zimmer, J., Noraberg, J., 2003. Neurotoxic and neuroprotective effects of the glutamate transporter inhibitor DL-threo-beta-benzyloxyaspartate (DL-TBOA) during physiological and ischemia-like conditions. Neurochem. Int. 43, 371–380.

Bonde, C., Noraberg, J., Noer, H., Zimmer, J., 2005. Ionotropic glutamate receptors and glutamate transporters are involved in necrotic neuronal cell death induced by oxygen-glucose deprivation of hippocampal slice cultures. Neuroscience 136, 779–794.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., Lipton, S.P., 1995. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insult with *N*-methyl-D-aspartate or nitric oxide in cortical cell cultures. Proc. Natl. Acad. Sci. U. S. A. 92, 7162–7166.

Burgos, J.S., Barat, A., Ramírez, G., 2000. Guanine nucleotides block agonist-driven 45Ca2 influx in chick embryo retinal explants. NeuroReport 11, 2303–2305.

Camacho, A., Massieu, L., 2006. Role of glutamate transporters in the clearance and release of glutamate during ischemia and its relation to neuronal death. Arch. Med. Res. 37, 11–18.

Chen, C.J., Liao, S.L., Kuo, J.S., 2000. Gliotoxic action of glutamate on cultured astrocytes. J. Neurochem. 75, 1557–1565.

Chen, H.S., Lipton, S.A., 2006. The chemical biology of clinically tolerated NMDA receptor antagonists. J. Neurochem. 97, 1611–1626.

Choi, D.W., 1988. Glutamate neurotoxicity and diseases of the nervous system. Neuron 1, 623–634.

Choi, D.W., 1992. Excitotoxic cell death. J. Neurobiol. 23, 1261–1276.

Ciccarelli, R., Di Iorio, P., Giuliani, P., D'Alimonte, I., Ballerini, P., Caciagli, F., Rathbone, M.P., 1999. Rat cultured astrocytes release guanine-based purines in basal conditions and after hypoxia/hypoglycemia. Glia 25, 93–98.

Ciccarelli, R., Ballerini, P., Sabatino, G., Rathbone, M.P., D'Onofrio, M., Caciagli, F., Di Iorio, P., 2001. Involvement of astrocytes in purine-mediated reparative processes in the brain. Int. J. Dev. Neurosci. 19, 395–414.

Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. 65, 1–105.

Decker, H., Francisco, S.S., Mendes-de-Aguiar, C.B., Romão, L.F., Boeck, C.R., Trentin, A.G., Moura-Neto, V., Tasca, C.I., 2007. Guanine derivatives modulate extracellular matrix proteins organization and improve neuron-astrocyte co-culture. J. Neurosci. Res. 85, 1943–1951.

Dev, K.K., Roberts, P.J., Henley, J.M., 1996. Characterisation of the interaction between guanyl nucleotides and AMPA receptors in rat brain. Neuropharmacology 35, 1583–1593.

Frizzo, M.E., Lara, D.R., Dahm, K.C., Prokopiuk, A.S., Swanson, R.A., Souza, D.O., 2001. Activation of glutamate uptake by guanosine in primary astrocyte cultures. NeuroReport 12, 879–881.

Frizzo, M.E., Antunes Soares, F.A., Dall'Onder, L.P., Lara, D.R., Swanson, R.A., Souza, D.O., 2003. Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake. Brain Res. 972, 84–89.

Ikonomidou, C., Turski, L., 2002. Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? Lancet Neurol. 1, 383–386.

Lipton, S.A., 2004. Paradigm shift in NMDA receptor antagonist drug development: molecular mechanism of uncompetitive inhibition by memantine in the treatment of Alzheimer's disease and other neurologic disorders. J. Alzheimer. Dis. 6 (6 Suppl), S61–S74.

Lowry, O.H., Rosembrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Madl, J.E., Buegesser, K., 1993. Adenosine triphosphate depletion reverses sodium-dependent, neuronal uptake of glutamate in rat hippocampal slices. J. Neurosci. 13, 4429–4444.

Malcon, C., Achaval, M., Komlos, F., Partata, W., Sauressing, M., Ramírez, G., Souza, D.O., 1997. GMP protects against quinolinic acid-induced loss of NADPH-diaphorase-positive cells in the rat striatum. Neurosci. Lett. 225, 145–148.

Maragakis, N.J., Rothstein, J.D., 2004. Glutamate transporters: animal models to neurologic disease. Neurobiol. Dis. 15, 461–473.

Mattson, M.P., 2000. Apoptosis in neurodegenerative disorders. Nat. Rev. Mol. Cell Biol. 1, 120–129.

Mattson, M.P., 2003. Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. Neuromol. Med. 3, 65–94.

Matute, C., Alberdi, E., Ibarretxe, G., Sánchez-Gómez, M.V., 2002. Excitotoxicity in glial cells. Eur. J. Pharmacol. 447, 239–246.

Meldrum, B.S., 2000. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J. Nutr. 130, 1007S–1015S.

Mendieta, J., Gago, F., Ramírez, G., 2005. Binding of 5′-GMP to the GluR2 AMPA receptor: insight from targeted molecular dynamics simulations. Biochemistry 44, 14470–14476.

Molz, S., Oliveira, I.J.L., Decker, H., Souza, D.O., Tasca, C.I., 2005. Neurotoxicity induced by glutamate in glucose-deprived rat hippocampal slices is prevented by GMP. Neurochem. Res. 30, 83–89.

Molz, S., Decker, H., Dal-Cim, T., Cremonez, C., Cordova, F.M., Leal, R.B., Tasca, C.I., 2008. Glutamate-induced toxicity in hippocampal slices involves apoptotic features and $p38^{MAPK}$ signaling. Neurochem. Res. 33, 27–36.

Morciano, M., Ortinau, S., Zimmermann, H., 2004. Guanine nucleotides inhibit NMDA and kainate-induced neurotoxicity in cultured rat hippocampal and neocortical neurons. Neurochem. Int. 45, 95–101.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity. J. Immunol. Methods 65, 55–63.

Muir, K.W., 2006. Glutamate-based therapeutic approaches: clinical trials with NMDA antagonists. Curr. Opin. Pharmacol. 6, 53–60.
Neary, J.T., Rathbone, M.P., Cattabeni, F., Abbracchio, M.P., Burnstock, G., 1996. Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. Trends Neurosci. 19, 13–18.
Oleskovicz, S.P., Martins, M.C., Leal, R.B., Tasca, C.I., 2008. Mechanism of guanosine-induced neuroprotection in rat hippocampal slices submitted to oxygen-glucose deprivation. Neurochem. Int. 52, 411–418.
Oliveira, I.J.L., Molz, S., Souza, D.O., Tasca, C.I., 2002. Neuroprotective effect of GMP in hippocampal slices submitted to an *in vitro* model of ischemia. Cell. Mol. Neurobiol. 22, 335–344.
Park, C.K., Nehls, D.G., Graham, D.I., Teasdale, G.M., McCulloch, J., 1988. The glutamate antagonist MK-801 reduces focal ischemic brain damage in the rat. Ann. Neurol. 24, 543–551.
Porciúncula, L.O., Vinadé, L., Wolfchuk, S., Souza, D.O., 2002. Guanine based purines inhibit [^{3}H]glutamate and [^{3}H]AMPA binding at postsynaptic densities from cerebral cortex of rats. Brain Res. 928, 106–112.
Re, D.B., Nafia, I., Melon, C., Shimamoto, K., Kerkerian-Le Goff, L., Had-Aissouni, L., 2006. Glutamate leakage from a compartmentalized intracellular metabolic pool and activation of the lipoxygenase pathway mediate oxidative astrocyte death by reversed glutamate transport. Glia 54, 47–57.
Re, D.B., Boucraut, J., Samuel, D., Birman, S., Kerkerian-Le Goff, L., Had-Aissouni, L., 2003. Glutamate transport alteration triggers differentiation-state selective oxidative death of cultured astrocytes: a mechanism different from excitotoxicity depending on intracellular GSH contents. J. Neurochem. 85, 1159–1170.
Regner, A., Ramirez, G., Belló-Klein, D., Souza, D., 1998. Effects of guanine nucleotides on glutamate-induced chemiluminescence in rat hippocampal slices submitted to hypoxia. Neurochem. Res. 23, 519–524.
Rossi, D.J., Oshima, T., Attwell, D., 2000. Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature 403, 316–321.
Santos, T.G., Souza, D.O., Tasca, C.I., 2006. GTP uptake into rat brain synaptic vesicles. Brain Res. 1070, 71–76.
Sattler, R., Rothstein, J.D., 2006. Regulation and dysregulation of glutamate transporters. Handb. Exp. Pharmacol. 175, 277–303.
Segovia, G., Porras, A., Del Arco, A., Mora, F., 2001. Glutamatergic neurotransmission in aging: a critical perspective. Mech. Ageing Dev. 122, 1–29.
Schmidt, A.P., Lara, D.R., de Faria Maraschin, J., da Silveira Perla, A., Onofre Souza, D., 2000. Guanosine and GMP prevent seizures induced by quinolinic acid in mice. Brain Res. 864, 40–43.
Schmidt, A.P., Lara, D.R., Souza, D.O., 2007. Proposal of a guanine-based purinergic system in the mammalian central nervous system. Pharmacol. Ther. 116, 401–416.
Souza, D.O., Ramírez, G., 1991. Effects of guanine nucleotides on kainic acid binding and on adenylate cyclase in chick optic tectum and cerebellum. J. Mol. Neurosci. 3, 39–45.
Stone, T.W., Addae, J.I., 2002. The pharmacological manipulation of glutamate receptors and neuroprotection. Eur. J. Pharmacol. 447, 285–296.
Tasca, C.I., Wolfchuk, S., Souza, D.O., Ramirez, G., Rodnight, R., 1995. Guanine nucleotides inhibit the stimulation of GFAP phosphorylation by glutamate. NeuroReport 6, 249–252.
Tasca, C.I., Cardoso, L.F., Martini, L.H., Ramírez, G., Souza, D.O., 1998. Guanine nucleotides inhibit cAMP accumulation induced by metabotropic glutamate receptor activation. Neurochem. Res. 23, 183–188.
Tasca, C.I., Santos, T.G., Tavares, R.G., Battastini, A.M., Rocha, J.B., Souza, D.O., 2004. Guanine derivatives modulate L-glutamate uptake into rat brain synaptic vesicles. Neurochem. Int. 44, 423–431.
Tavares, R.G., Tasca, C.I., Santos, C.E., Alves, L.B., Porciúncula, L.O., Emanuelli, T., Souza, D.O., 2002. Quinolinic acid stimulates synaptosomal glutamate release and inhibits glutamate uptake into astrocytes. Neurochem. Int. 40, 621–627.
Yang, G.-M., Paul, S.M., 1997. Cultured cerebellar granule neurons as a model of neuronal apoptosis. In: Poirier, J. (Ed.), Neuromethods, vol. 29. Humana Press Inc., pp. 47–66.
Zimmermann, H., 1996. Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. Prog. Neurobiol. 49, 589–618.

CAPÍTULO 2

THE NEUROPROTECTIVE EFFECT OF GUANOSINE AGAINST GLUTAMATE-INDUCED CELL DEATH IN RAT HIPPOCAMPAL SLICES IS MEDIATED BY PI3K/Akt/GSK3β ACTIVATION AND iNOS INHIBITION

Manuscrito em preparação para ser submetido à Purinergic Signalling

The neuroprotective effect of guanosine against glutamate-induced cell death in rat hippocampal slices is mediated by PI3K/Akt/GSK3β activation and iNOS inhibition

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Running Head: Guanosine is neuroprotective via Akt activation and iNOS inhibition

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Abstract

Excitotoxicity and cell death induced by glutamate is involved in many neurodegenerative disorders. We have previously demonstrated that excitotoxicity induced by millimolar concentrations of glutamate in hippocampal slices involves apoptotic features, as cytochrome c release, caspase-3 activation and DNA fragmentation as well as glutamate-induced glutamate release trough reversal activity of glutamate transporters. Guanosine, an endogenous guanine nucleoside, prevents excitotoxicty by its ability to modulate glutamate transport. In this study, we have evaluated the neuroprotective effect of guanosine against glutamate-induced toxicity in hippocampal slices and the possible mechanism involved in such effect. We have found that guanosine (100 μM) was neuroprotective against 1 mM glutamate-induced cell death and also inhibited glutamate release induced by glutamate. LY294002 (30 μM), an inhibitor of phosphatidylinositol-3 kinase (PI3K), attenuated guanosine-induced neuroprotection and guanosine prevention of glutamate release. In addition, guanosine induced the phosphorylation and thus, activation of PKB/Akt, a downstream target of PI3K. Guanosine also induced the phosphorylation of glycogen synthase kinase 3β, which has been reported to be inactivated by Akt after phosphorylation at Ser9. Glutamate treated hippocampal slices showed increased iNOS expression that was prevented by guanosine. Furthermore, slices pre-incubation with SNAP (a NO donor) inhibited the protective effect of guanosine. Taken together, this observations support the hypothesis that guanosine protects hippocampal slices by a mechanism involving PI3K/Akt/GSK3β^{Ser9} pathway and inhibition of glutamate-induced glutamate release as well as reduction of glutamate-induced iNOS expression in rat hippocampal slices.

Key words: glutamate, guanosine, neuroprotection, Akt, iNOS, hippocampal slices

Abbreviations: Glutamate, Glu; glycogen synthase kinase 3β, GSK3β; guanosine, GUO; [2-(4-morpholinyl)-8-phenyl-4H-1benzo-pyran-4-one hydrochloride, LY 294002; inducible nitric oxide synthase, iNOS; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; phosphoinisitide-3 kinase, PI3K; protein kinase B/Akt; S-nitroso-N-acetyl-l,l-penicillamine, SNAP.

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1. Introduction

Glutamate excitotoxicity is caused by overstimulation of synaptic glutamate receptors and subsequent neuronal injury [1]. Glutamate receptors overstimulation is a common event in many neurodegenerative disorders, including ischemic stroke, Huntington´s disease, amyotrophic lateral sclerosis and Alzheimer´s disease as well as in aging [2]. We have previously reported that excitotoxicity in acute hippocampal slices is induced by millimolar concentrations of glutamate and promotes a reduction of cell viability with apoptotic features, as cytochrome c release, caspase-3 activation and DNA fragmentation, via $p38^{MAPK}$ signalling activation [3]. Glutamate toxicity is also related to reversal activity of glutamate transporters, increasing extracellular glutamate concentration and then promoting excitotoxicty [4].

Excitotoxicity or activated microglia produce superoxide reacting with NO to give peroxynitrite, which in turn causes mitochondrial damage and cell death [5]. Cytokines activate inducible nitric oxide synthase (iNOS) in the glia which then release high levels of nitric oxide (NO). NO augmentation causes glutamate release from astrocytes and neurons resulting in excitotoxicity [6]. Inflammation occurs in the central nervous system (CNS) in response to stimuli such as pathogens or tissue damage. CNS inflammation is in general induced by cytokines and/or bacterial/viral components. The main targets of cytokines are the glial cells, which then release toxic factors to neurons.

Considering that excitotoxicity is related to the major CNS disorders and the lack of effective treatment for such diseases, the study of alternative pharmacological strategies against excitotoxic-induced cell death is of great relevance. The nucleoside guanosine (GUO) and the guanine nucleotides have been implicated in neuroprotection by counteracting glutamate excitotoxicity *in vitro* [7,8,4] and *in vivo* [9,10]. GUO displays extracellular effects as cell modulator or intercellular signaling communication exerting trophic effects to neurons and astrocytes (11,12) and the modulation of the glutamatergic system (for a review see 13). Guanine nucleotides and GUO are released from astrocytic cell cultures under basal or toxic

conditions [14,15]. Alternatively, when nucleotides such as GTP, GDP and GMP are released to the extracellular space and metabolized by ecto-nucleotidases, extracellular GUO levels can increase [15,16]. Our laboratory has demonstrated that GTP is taken up and stored into synaptic vesicles [17] and indirect evidence indicated that GUO could be released from synaptosomes [18].

Astrocytic glutamate uptake is a crucial process for the maintenance of extracellular glutamate concentrations below toxic levels thus preventing excitotoxicity [9]. GUO stimulates glutamate uptake under basal conditions in cultured astrocytes [20] and brain slices [21]. Under excitotoxic situations the modulatory effect of guanosine on glutamate uptake has been directly related to a neuroprotective role [22-24]. *In vitro* GUO also protects rat astrocytes from staurosporine-induced apoptosis [25] and SH-SY5Y cells from β-amyloid-induced apoptosis [26] and MPP^+-induced toxicity [27]. In both cases, the anti-apoptotic effect of GUO was mediated by stimulation of the phosphatidylinositol-3 kinase (PI3K)/Protein kinase B (Akt) cell survival pathway.

The serine/threonine protein kinase Akt is a signaling kinase downstream of PI3K [28]. The PI3K/Akt pathway is a critical transducer for several major survival signals in CNS neurons [29]. Glycogen synthase kinase 3β (GSK3β), which is highly expressed in brain tissue, is one of the key targets of the antiapoptotic signaling mediated by the PI3K/Akt pathway [30]. The activity of GSK3β is negatively regulated by Akt phosphorylation at the N-terminal serine 9 (Ser9) [31]. Reduced PI3K signaling also results in Ser9 dephosphorylation [32].

In the present study, we investigated the putative neuroprotective role of guanosine against glutamate-induced hippocampal slices injury and the role of the PI3K/Akt/$GSK3\beta^{Ser9}$ pathway and iNOS as the possible mechanism involved in the neuroprotection afforded by GUO. The results showed here support the hypothesis that GUO protects hippocampal slices by a mechanism involving PI3K/Akt pathway activation and subsequent reduction of

glutamate-induced glutamate release, as well as increased phosphorylation of GSK3β^{Ser9} and reduction of glutamate-induced iNOS expression in hippocampal slices of young rats.

2. Experimental Procedures

2.1. Animals

Male Wistar rats (23-25 days of postnatal age) maintained on a 12-hour light-12 hour dark schedule at 25º C, with food and water *ad libitum*, were obtained from our local breeding colony. Experiments followed the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) and were approved by the local Ethical Committee of Animal Research (CEUA/UFSC).

2.2. Preparation and Incubation of Hippocampal Slices

Rats were killed by decapitation and the hippocampi were rapidly removed and placed in ice-cold Krebs-Ringer bicarbonate buffer (KRB) of the following composition: 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM D-glucose. The buffer was bubbled with 95% O_2-5% CO_2 up to pH 7.4. Slices (0.4 mm thick) were rapidly prepared using a McIlwain Tissue Chopper, separated in KRB at 4^o C and allowed to recover for 30 minutes in KRB at 37^o C [33] to slices stabilization.

2.3. Slices treatment

After the preincubation period to slices stabilization, hippocampal slices were incubated with glutamate (Sigma) (1 mM) for 1 h in KRB buffer. After this period, the medium was withdrawn and replaced by a nutritive culture medium composed of 50 % of KRB, 50 % of Dulbecco's modified Eagle`s medium (DMEM, Gibco), 20 mM of HEPES and

100 μg/ml of gentamicine, and slices were maintained for additional 6h in a humidified atmosphere 95% air and 5% CO_2 at 37^{o} to evaluate cell viability [3].

When present, GUO was added to incubation medium 30 min before glutamate and maintained during the 1 h of incubation with glutamate. LY294002 (30 μM) or SNAP (1 mM) were added to incubation medium 15 minutes before GUO and maintained during the GUO preincubation period.

2.4. Evaluation of cell viability

Hippocampal cell viability was evaluated 6 h after glutamate exposure. Cell viability was determined through the ability of cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl-diphenyltetrazolium bromide, Sigma) [34]. Hippocampal slices were incubated with MTT (0.5 mg/ml) in KRB for 30 minutes at 37º C. The tetrazolium ring of MTT can be cleaved by active dehydrogenases in order to produce a precipitated formazan. The formazan produced was solubilized by adding 200 μl dimethyl sulfoxide (DMSO), resulting in a coloured compound which optical density was measured in an ELISA reader (550 nm).

2.5. Immunobloting:

In order to evaluate Akt (Ser 473) and GSK3β (Ser 9) phosphorylation, slices were incubated for 90 min under control conditions, or treated with GUO (100 μM) for 30, 60 or 90 min. Then, the slices were homogenized in 100μl of ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4). Proteins (30 μg) from this cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, Chalfont St. Giles, UK). Membranes were

incubated with anti-total-Akt (1:1,000), anti-phospho-Akt (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-GSK3β (1:1,000); anti-total GSK3β (1:1,000); anti-β- actin (1:100,000) (Sigma). Appropriate peroxidase-conjugated secondary antibodies (1:10,000) were used to detect proteins by enhanced chemiluminescence. Different band intensities corresponding to immunoblot detection of protein samples were quantified using the Scion Image program (Scion Corporation, Frederick, MD, USA).

2.6. Glutamate release

Following preincubation period to slices recovery (30 min), hippocampal slices were incubated in Hank's balanced salt solution (HBSS), composition in mM: 1.29 $CaCl_2$, 136.9 NaCl, 5.36 KCl, 0.65 $MgSO_4$, 0.27 Na_2HPO_4, 1.1 KH_2PO_4, and 5 Hepes. When present, GUO was incubated for 30 min and maintained during glutamate exposure. LY 204002 (30 μM) was preincubated for 15 min before guanosine. Glutamate (1 mM) was incubated for 15 min and glutamate uptake was assessed by adding 0.33 μCi/ml D-[^{3}H]aspartate with 100 μM unlabeled aspartate for 7 min and stopped by three ice-cold washes with 1 ml HBSS. D-[^{3}H]aspartate instead of L-[^{3}H]glutamate was used in order to avoid glutamate metabolization in intracellular compartments, although similar results were obtained by using D-[^{3}H]aspartate or L-[^{3}H]glutamate. The slices were then further incubated for 15 min in 300μl HBSS and the supernatant was collected in order to measure the amount of released D-[^{3}H]aspartate. Slices were disrupted by overnight incubation with 0.1% NaOH/0.01% SDS and aliquots of lysates were taken for determination of intracellular D-[^{3}H]aspartate content [4]. Intracellular and extracellular D-[^{3}H]aspartate content were determined through scintillation counting, calculated as nmol of aspartate and the amount of released D-[^{3}H]aspartate was expressed as percentage of total D-[^{3}H]aspartate.

2.7. Statistical analysis

Comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by Duncan's test if necessary, with $p < 0.05$ considered to be statistically significant.

3. Results

3.1. GUO protects hippocampal slices against glutamate-induced cell death

Hippocampal slices exposure to 1 mM glutamate resulted in a significant decrease in cell viability. GUO (100 μM) preincubation significantly prevented the reduction in cell viability induced by glutamate. Such neuroprotection was not observed when slices were preincubated with 30 μM or 300 μM GUO (Fig. 1).

3.2. Involvement of glutamate transport and PI3K/Akt/GSK3β^{Ser9} pathway activation in neuroprotection afforded by GUO

We have previously demonstrated that glutamate excitotoxicity in hippocampal slices involves the reversal activity of glutamate transporters and consequent glutamate release [4]. GUO has been shown to exert neuroprotection against excitotoxicty by its ability to modulate glutamate transport [35]. Therefore, glutamate release was evaluated in the presence of 100 μM of GUO. Figure 2 shows that GUO was able to prevent glutamate-induced D-[^{3}H]aspartate release (i.e. glutamate release) from hippocampal slices.

When LY294002 (30 μM) was added to incubation medium 15 minutes before GUO and maintained during the GUO incubation period, the GUO ability to counteract glutamate release as well as the neuroprotective effect of GUO against glutamate-induced cell viability reduction, were prevented (Figs. 2 and 3).

Since inhibition of PI3K/Akt pathway by LY294002 prevented the neuroprotective effect of GUO, GUO ability to induce the activation of Akt (Akt phosphorylation) was also evaluated. Hippocampal slices were incubated with 100 μM GUO for different periods of time (30, 60 and 90 minutes) and cell lysates were then analyzed by immunoblotting. These periods of incubation with GUO were evaluated because they represent the GUO preincubation time (30 min), the intermediate period when slices are in the presence of glutamate plus GUO (60 min) and the total time that slices were incubated in the presence of glutamate plus GUO (90 min). Figure 4A shows a representative immunoblotting of phospho-Akt (p-Akt) and total-Akt (t-Akt). Under basal conditions (slices incubated for 90 min in KRB), Akt phosphorylation was low and incubation for 30 min with GUO was sufficient to increase the amount of p-AKT. The increase of p-Akt induced by GUO was sustained, at least for 60 and 90 minutes (Fig. 4A and B).

Glycogen synthase kinase 3β (GSK3β) is one of the key targets of the signaling mediated by the PI3K/Akt pathway [30]. Akt mediated phosphorylation of GSK3β at Ser9 inactivates GSK3β leading to increased cell survival against glutamate excitotoxicity [36]. In the present study, hippocampal slices incubation with GUO also showed a significant increase in phosphorylation of Ser9 site of GSK3-β, thus showing GUO inhibition of GSK3β activity was observed 30 minutes after GUO exposure, whereas its phosphorylation levels returns to basal levels after 60 or 90 minutes of GUO exposure (Fig. 5A and B).

3.3. GUO prevents glutamate-induced iNOS expression

Several studies have demonstrated that excitotoxicity involves the activation of cellular pathways that lead to iNOS induction [5]. Studies also demonstrate that activation of the PI3K/Akt pathway may diminish the induction of iNOS [37]. Therefore, the involvement of iNOS in glutamate-induced cell damage to hippocampal slices and the possible prevention of this effect by GUO were investigated. We have found increased levels of iNOS after

hippocampal slices challenge with 1 mM glutamate. This effect was completely prevented when slices were preincubated 100 μM GUO (Fig. 6).

Additionally, preincubation of hippocampal slices with SNAP (1 mM), a NO donor, prior to GUO treatment significantly abolished the protective effect of GUO against glutamate-induced cell damage (Fig. 7). These data point to a neuroprotective mechanism of action of GUO against glutamate excitotoxicity likely involving either the decrease of iNOs expression or NO production in hippocampal slices.

4. Discussion

The present study demonstrates that GUO prevents glutamate-induced hippocampal slices death by a mechanism involving inhibition of glutamate-induced glutamate release trough the activation of the PI3K/Akt pathway and a consequent inhibition of GSK3β, as well as, the reduction of glutamate-induced iNOS expression in rat hippocampal slices.

The regulation of excitotoxicity relies on the function of glutamate transporters present on astrocytes and, to a lesser extent, in neurons [38]. These transporters are coupled to Na^+/K^+-ATPase activity in order to remove excessive glutamate from brain extracellular space thus preventing excitotoxicity [19]. In situations where membrane Na^+ gradient is disrupted, such as after respiratory chain inhibition due to Ca^{2+} overload and oxidative stress, glutamate can be released to the synaptic cleft due to reversal of glutamate transporters [39,40]. In a previous study, we demonstrated that glutamate-induced toxicity in hippocampal slices was due to its ability to trigger glutamate release by reversed glutamate transport, since DL-TBOA, a glutamate transport inhibitor, prevented the loss of cell viability induced by glutamate and glutamate-induced glutamate release [4].

In the present study, preincubation of hippocampal slices with GUO (100 μM) prevented glutamate-induced cell damage, as well as glutamate-induced glutamate release

from hippocampal slices (Figs. 1 and 2), indicating that the neuroprotective effect of GUO is due to its ability to prevent glutamate-induced reversal of glutamate transport, leading to reduced glutamate release thus preventing excitotoxicity. The protective effect of GUO against glutamate-induced cell viability decrease was partially abolished by LY294002, a PI3K inhibitor (Fig 3). Furthermore, the GUO ability to reduce glutamate release was fully inhibited in the presence of LY294002 (Fig 2), and hippocampal slices treated with GUO showed increased levels of p-Akt (Fig 4). Taken together, these results indicate that GUO prevents glutamate release and protects hippocampal slices trough a mechanism that involves activation of PI3K/Akt pathway. Literature data indicate that activation of the PI3K/Akt pathway can stimulate glutamate uptake as well as glutamate transporters trafficking and expression in the cellular membrane [41-43]. The partial protective effect of LY294002 against glutamate-induced toxicity may be due to the involvement of other protective pathways in neuroprotection afforded by GUO, as previously shown [25, 11]. In agreement with our findings that GUO modulates glutamate transport, other studies had demonstrated that GUO stimulates glutamate uptake under basal or physiological [20] as well as excitotoxic conditions [44, 35, 24].

PI3K/Akt signaling pathway has been shown to act as an upstream mechanism of GSK3β activity regulation, since PI3K/Akt system might directly phosphorylate Ser9 of GSK3β, leading to GSK3β inactivation. It has been reported that GSK3β activation may be involved in several apoptotic signaling pathways that lead to activation of caspase-3 [45,46]. Glutamate toxicity in hippocampal slices is also mediated by caspase-3 activation [3]. In astrocytes cultures, GUO prevented the apoptotic effects of staurosporine by inhibiting caspase-3 and activating PI3K/Akt pathway, with subsequent inhibition of GSK3-β [25]. Here we are also demonstrating that preincubation of hippocampal slices with GUO (30 minutes) is sufficient to protect hippocampal slices against subsequent glutamate-induced cell death by a mechanism which involves inhibition of GSK3β activity trough the activation of PI3K/Akt

pathway. GUO-induced Akt phosphorylation was observed at 30 minutes and sustained until 90 minutes (Fig. 4). Otherwise, increased GSK3β phosphorylation at Ser9 was observed at 30 minutes incubation with GUO and then returned to basal levels (Fig. 5). GSK3β phosphorylation at Ser9 is very dynamic, as a number of pathways and kinases converge at GSK3β and its dephosphorylation is regulated by protein phosphatase 1 [47-48].

One of the hallmarks of the inflammatory process in the CNS is the expression of iNOS by activated glia. Glutamate can induce proinflamatory cytokines that in turns can trigger iNOS expression [49-50]. In this study, we demonstrated that glutamate increases iNOS induction in hippocampal slices. One of the proteins that can mediate iNOS induction is $p38^{MAPK}$, in fact, we have previously demonstrated that glutamate-induced toxicity involves the activation of $p38^{MAPK}$ [3]. The glutamate-induced iNOS induction was prevented by GUO (100 μM) (Fig. 6). It has been shown that GUO inhibits CD40 expression and function induced by proinflamatory cytokines and β-amyloid in mouse microglia cells [51]. In the present study, SNAP (a NO donor) abolished the protective effect of GUO against glutamate-induced toxicity (Fig. 7). NO produced by activated glia, at concentrations where it inhibits respiration, causes acute (within seconds) glutamate release from synaptosomes and neurons which has been attributed either to vesicular release or reversal of glutamate transporters [52, 6]. Astrocytes or C6 glioma cells treatment with a PI3K inhibitor lead to increased iNOS expression in response to LPS or cytokines, thus demonstrating that the activity of PI3K pathway is important to counteract iNOS expression [37]. Similarly, in our study, acute stimulation of PI3K/Akt pathway by GUO may account for reduction in iNOS expression.

Taken together, this observations support the hypothesis that GUO protects against neurodegeneration related to inflammatory process by a mechanism which involve the blockade of iNOS expression as well as PI3K/Akt pathway activation and inactivation of GSK3β, with subsequent inhibition of glutamate-induced glutamate release in hippocampal slices of young rats.

Inflammation, impairment of glutamate transport and excitotoxiciy are all processes involved in pathogenesis of neurodegenerative diseases in the CNS [53, 5]. Therefore, GUO protective effects which normalize the activity of glutamate transporters counteracting excitotoxicity may be considered an important neuroprotective strategy to the CNS degeneration.

5. References

1. Lipton SA., Rosemberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 330, 613−622.
2. Mattson MP, Magnus T (2006) Ageing and neuronal vulnerability. Nat Rev Neurosci. 7, 278-94
3. Molz S, Decker H, Dal-Cim T, Cremonez C, Cordova FM, Leal RB, Tasca CI (2008a) Glutamate-induced toxicity in hippocampal slices involves apoptotic features and p38 MAPK signaling. Neurochem Res. 33, 27-36.
4. Molz S, Dal-Cim T, Decker H, Tasca CI (2008b) GMP prevents excitotoxicity mediated by NMDA receptor activation but not by reversal activity of glutamate transporters in rat hippocampal slices. Brain Res 1231, 113–120.
5. Brown GC, Bal-Price A (2003) Inflammatory Neurodegeneration Mediated by Nitric Oxide, Glutamate, and Mitochondria. Molecular Neurobiology 27, 325–355, 2003.
6. Bal-Price A, Brown GC (2001) Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. J Neurosci. 21, 6480–6491.
7. Molz S, Decker H, Oliveira IJL, Souza DO, Tasca CI (2005) Neurotoxicity induced by glutamate in glucose-deprived rat hippocampal slices is prevent by GMP. Neurochem. Res. 30, 83-89.
8. Oleskovicz SP, Martins WC, Leal RB, Tasca CI (2008) Mechanism of guanosine-induced neuroprotection in rat hippocampal slices submitted to oxygen-glucose deprivation. Neurochem Int. 52, 411-418.
9. Schmidt AP, Lara DR, Maraschin JF, Perla AS, Souza DO (2000) Guanosine and GMP prevent seizures induced by quinolinic acid in mice. Brain Research 864, 40-43.

10. Schmidt AP, Ávila TT, SouzaDO (2005) Intracerebroventricular guanine-based purines protect against seizures induced by quinolinic acid in mice. Neurochem Res 30, 69−73.

11. Decker H, Francisco SS, Mendes-de-Aguiar CB, Romão LF, Boeck CR, Trentin AG, Moura-Neto V, Tasca CI (2007) Guanine derivatives modulate extracellular matrix proteins organization and improve neuron-astrocyte co-culture. Journal of Neuroscience Research 85, 1943-1951.

12. Rathbone MP, Pilutti L, Caciagli F, Jiang S. **(**2008) Neurotrophic effects of extracellular guanosine. Nucleosides, Nucleotides, and Nucleic Acids, 27, 666–672.

13. Schmidt AP, Lara DR, Souza DO (2007) Proposal of a guanine-based purinergic system in the mammalian central nervous system. Pharmacology & Therapeutics 116, 401-416.

14. Ciccarelli R, Di Iorio P, Giuliani P, D'Alimonte I, Ballerini P, Caciagli F, Rathbone MP (1999) Rat cultured astrocytes release guanine-based purines in basal conditions and after hypoxia/hypoglycemia. Glia 25, 93-8.

15. Ciccarelli R, Ballerini P, Sabatino G, Rathbone MP, D'Onofrio M, Caciagli F, Di Iorio P (2001) Involvement of astrocytes in purine-mediated reparative processes in the brain. Int. J. Dev. Neurosci. 19, 395-414.

16. Caciagli F, Di Iorio P. Giulani P, Middlemiss PJ, Rathbone MP (2000) The neuroprotective activity of guanosine involves the production of trophic factors and the outflow of purines from astrocytes. Drug Deve. Res. 50, 32(Abstr. R02-02).

17. Santos, TG, Souza DO, Tasca CI (2006) GTP uptake into rat brain synaptic vesicles. Brain Res 1070, 71-6.

18. Fredholm, BB, Vernet L (1979) Release of 3H-nucleosides from 3Hadenine labelled hypothalamic synaptosomes. Acta Physiol Scand 106, 97−107.

19. Danbolt, NC (2001) Glutamate uptake. Prog. Neurobiol. 65, 1-105.

20. Frizzo ME, Lara DR, Dahm KCS, Prokopiuk AS, Swanson RA, Souza DO (2001) Activation of glutamate uptake by guanosine in primary astrocyte cultures. Neuroreport 12, 1-3.

21. Frizzo ME, Lara DR, Prokopiuk AS, Vargas CR, Salbego CG, Wajner M, Souza D.O. (2002) Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. Cell. Mol. Neurobiol. 22, 353-363.

22. Gottfried C, Tramontina F, Goncalves D, Goncalves CA, Moriguchi E, Dias RD et al. (2002). Glutamate uptake in cultured astrocytes depends on age: a study about the effect of guanosine and the sensitivity to oxidative stress induced by H2O2. Mech Ageing Dev 123, 1333−1340.

23. Frizzo, ME, Schwalm FD, Frizzo, JK, Soares FA, Souza DO (2005) Guanosine enhances glutamate transport capacity in brain cortical slices. Cell Mol Neurobiol 25, 913−921.

24. Thomazi AP, Boff B, Pires TD, Godinho G, Battú CE, Gottfried C, Souza DO, Salbego C, Wofchuk ST (2008) Profile of glutamate uptake and cellular viability in hippocampal slices exposed to oxygen and glucose deprivation: Developmental aspects and protection by guanosine. Brain Res. 1188, 233–240.

25. Di Iorio P, Ballerini P, Traversa U, Nicoletti F, D'Alimonte I, Kleywegt S, Werstiuk ES, Rathbone MP, Caciagli F, Ciccarelli R (2004) The antiapoptotic effect of guanosine is mediated by the activation of the PI 3-kinase/AKT/PKB pathway in cultured rat astrocytes. Glia. 46, 356-368.

26. Pettifer KM, Kleywegt S, Bau CJ, Ramsbottom JD, Vertes E, Ciccarelli R, Caciagli F, Werstiuk ES, Rathbone MP (2004) Guanosine protects SH-SY5Y cells against beta-amyloid-induced apoptosis. NeuroReport 15, 833-836.

27. Pettifer KM, Jiang S, Bau C, Ballerini P, D'Alimonte I, Werstiuk ES, Rathbone MP (2007) MPP^{+}-induced cytotoxicity in neuroblastoma cells: Antagonism and reversal by guanosine. Purinergic Signalling 3, 399–409

28. Cantley LC (2002) The phosphoinositide 3-kinase pathway. Science 296:1655–1657.

29. Datta SR, Dudek H, Tao X et al (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91:231–241.

30. Pap M, Cooper GM (1998) Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase/Akt cell survival pathway. J Biol Chem 273, 19929–19932.

31. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789.

32. Hetman M, Cavanaugh JE, Kimelman D, Xia Z (2000) Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal. J Neurosci 20, 2567–2574.

33. Oliveira IJL, Molz S, Souza DO, Tasca CI (2002) Neuroprotective effect of GMP in hippocampal slices submitted to an in vitro model of ischemia. Cellular and Molecular Neurobiology 22, 335-344.

34. Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity. Journal of Immunological Methods 65, 55-63.

35. Moretto MB, Arteni NS, Laviski D, Netto CA, Rocha JBT, Souza DO, Wolchuk S (2005) Hipoxic-ischemic insult decreases glutamate uptake by hippocampal slices from neonatal rats: Prevention by guanosine. Exper. Neurob. 195, 400-406.

36. Nishimoto T, Kihara T, Akaike A, Niidome T, Sugimoto H (2008) α-amino-3-hydroxy-5-methyl-4-isoxazole propionate attenuates glutamate-induced caspase-3 cleavage via regulation of glycogen synthase kinase 3β. J. Neurosci Res. 86, 1096–1105.

37. Pahan, K, Raymond JR, Singh I (1999) Inhibition of phosphatidylinositol-3-kinase induces nitric oxide synthase in lipopolysaccharide- or cytokine-stimulated C6 glial cells. *J. Biol. Chem* 274, 7528-7536.

38. Anderson, CM and Swanson, RA (2000) Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32, 1−14.

39. Rossi, DJ, Oshima, T, Attwell, D (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature 403, 316-321.

40. Camacho A, Massieu L (2005) Role of glutamate transporters in the clearance an release of glutamate during isquemia its relation to neuronal death. Arch. Med. Research. 37, 11-18.

41. Sims KD, Straff DJ, Robinson MB (2000) Platelet-derived growth factor rapidly increases activity and cell surface expression of the EAAC1 subtype of glutamate transporter through activation of phosphatidylinositol 3-kinase. J.Biol Chem. 275, 5228-5237.

42. Krizman-Genda, E, González, MI, Zelenaia O, Robinson MB (2005) Evidence that Akt mediates platelet-derived growth factor-dependent increases in activity and surface expression of the neuronal glutamate transporter, EAAC1. Neuropharmacol. 49, 872-882.

43. Guillet BA, Velly LJ, Canolle B, Masmejean FM, Nieoullon AL, Pisano P (2005) Differential regulation by protein kinases of activity and cell surface expression of glutamate transporters in neuron-enriched cultures. Neurochem Int. 46, 337-46.

44. Oliveira DL, Horn JF, Rodrigues JM, Frizzo ME, Moriguchi E, Souza DO (2004) Quinolinic acid promotes seizures and decreases glutamate uptake in young rats: reversal by orally administered guanosine. Brain Res 1018, 48−54.

45. Grimes CA, Jope RS **(**2001) The multifaceted roles of glycogen synthase kinase in cellular signalling. Prog Neurobiol. 65, 391-426.

46. Bhat, RV, Budd Haeberlein SL, Avila J (2004) Glycogen synthase kinase 3: a drug target for CNS therapies. J Neurochem. 89, 1313-7.

47. Bennecib M, Gong CX, Grundke-Iqbal I, Iqbal K (2000) Role of protein phosphatase-2A and -1 in the regulation of GSK-3, cdk5 and cdc2 and the phosphorylation of tau in rat forebrain. FEBS Lett 485, 87–93.

48. Zhang F, Phiel CJ, Spece L, Gurvich N, Klein PS (2003). Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3. J Biol Chem 278, 33067–33077.

49. Cardenas A, Moro MA, Hurtado O, Leza JC, Lorenzo P, Castillo A, Bodelon OG Bosca L, Lizasoain I. (2000) Implication of glutamate in the expression of inducible nitric oxide synthase after oxygen and glucose deprivation in rat forebrain slices. J. Neurochem. 74, 2041-2048.

50. Moro MA, Cárdenas A, Hurtado O, Leza JC, Lizasoain I (2004) Role of nitric oxide after brain ischemia. Cell calcium 36, 265-275.

51. D'Alimonte I, Flati V, D'Auro M, Toniato E, Martinotti S, Rathbone MP, Jiang S, Ballerini P, Di Iorio P, Caciagli F, Ciccarelli R. (2007) Guanosine inhibits CD40 receptor expression and function induced by cytokines and beta amyloid in mouse microglia cells. J Immunol. 178, 720-31. Erratum in: J Immunol. (2007) 178, 5399.

52. McNaught KSP, Brown GC (1998) Nitric oxide causes glutamate release from brain synaptosomes following inhibition of mitochondrial function. J. Neurochem. 70, 1541–1546.

53. Maragakis NJ, Rothstein JD (2004) Glutamate transporters: animal models to neurologic disease. Neurobiol. Dis. 15, 461-473.

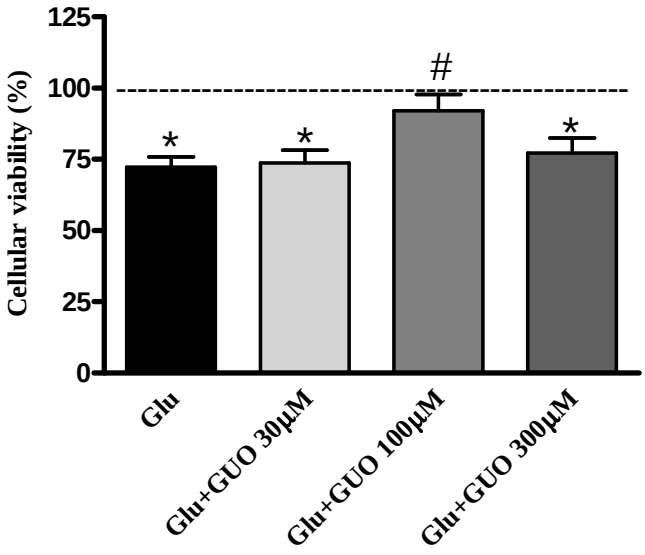

Figure 1. Cell viability in hippocampal slices submitted to glutamate in the presence of GUO. Hippocampal slices were incubated for 1 h with 1mM glutamate (Glu). When present, guanosine (GUO 30, 100 and 300µM) was preincubated for 30 min. After this period, incubation medium was withdrawn and replaced for fresh culture medium without glutamate and maintained for additional 6 h. Control group was considered as 100 % and represents cell viability of slices incubated only in culture medium. MTT (0.5 mg/ml) was incubated for 20 min at 37º C and cell viability was accessed at 550 nm. The values represent means ± error deviations of at least 6 experiments carried out in triplicates. * Indicates means significantly different from control group (100 %) and # indicate mean different from Glu; $p < 0.05$.

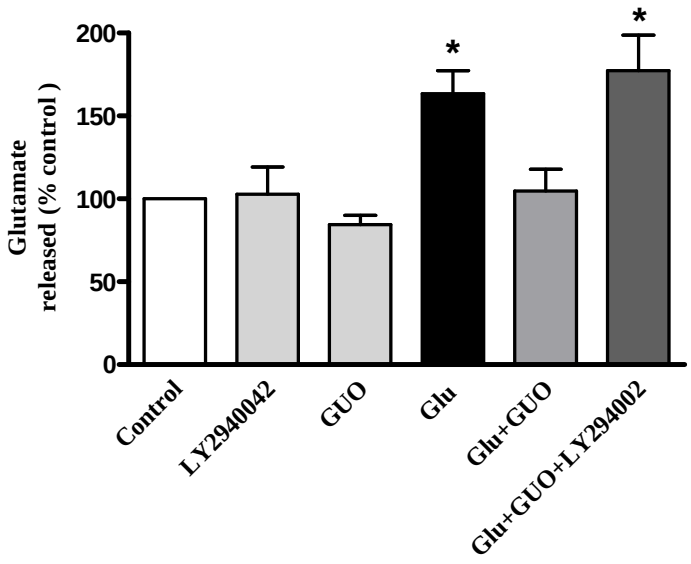

Figure 2. GUO reduces glutamate-induced glutamate release by PI3K/Akt pathway activation. Hippocampal slices were incubated for 15 min with 1mM glutamate (Glu) in the presence or absence of 100μM guanosine (GUO). When present, GUO was preincubated for 30 min before the addition of glutamate. LY294002 (30μM) was added to incubation medium 15 min before GUO and maintained during the GUO preincubation period. Control group was considered as 100 % and represents glutamate released from slices incubated only in HBSS. The values represent means $\pm$ error deviations of at least 4 experiments carried out in triplicates. * Indicates means significantly different from control group (100 %) and Glu+GUO; $p < 0.05$.

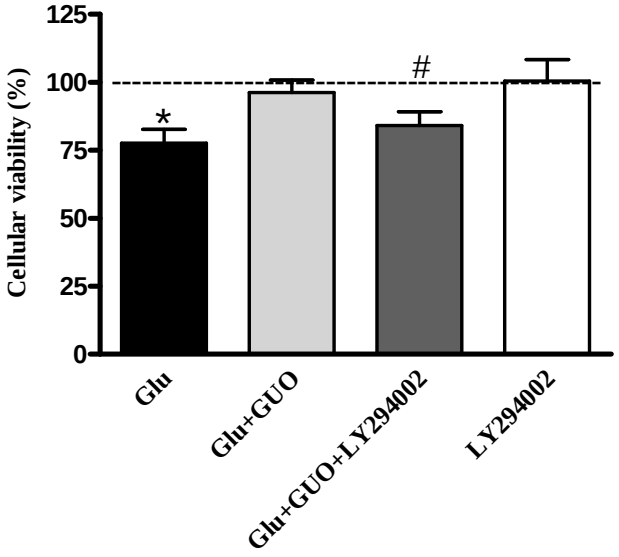

Figure 3. LY294002 prevents the neuroprotective effect of GUO against glutamate-induced cell death. Hippocampal slices were incubated for 1 h with 1mM glutamate (Glu) in the presence or absence of 100μM guanosine (GUO), preincubated for 30 min before the addition of glutamate. LY294002 (30μM) was added to incubation medium 15 min before GUO and maintained during the GUO preincubation period. After this period, incubation medium was withdrawn and replaced for fresh culture medium without glutamate and maintained for additional 6 h. Control group was considered as 100 % and represents cell viability of slices incubated only in culture medium. MTT (0.5 mg/ml) was incubated for 20 min at 37º C and cell viability was accessed at 550 nm. The values represent means ± error deviations of at least 4 experiments carried out in triplicates. * Indicates means significantly different from control group (100 %) and Glu+GUO, # indicate mean different from control; $p < 0.05$.

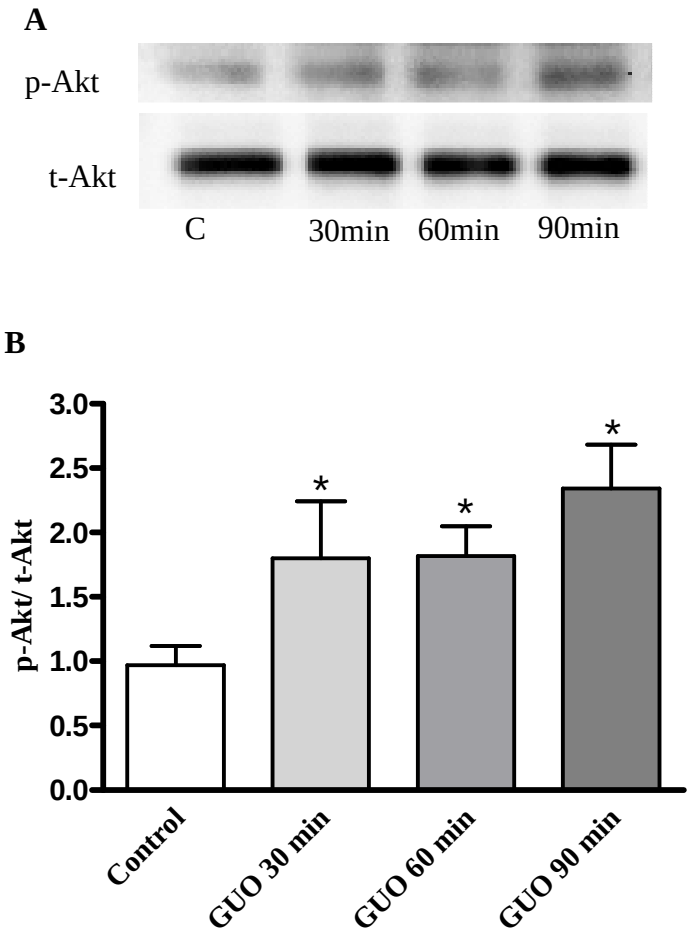

Figure 4. Immunodetection of phosphorylated and total Akt in hippocampal slices treated with GUO. Whole cells lysates were subjected to Western blotting analysis to phosphorylated (p-Akt) and total (t-Akt) Akt detection as described in Methods. **(A)** Representative western blot of phosphorylated Akt (p-Akt) and total Akt (t-Akt) in hippocampal slices exposed to GUO 100μM for 30, 60 or 90 min. **(B)** Quantitative analysis of p-Akt/t-Akt in optical density (O.D.). The control values were normalized to 1, and other treatments were expressed in relation to this value. The values represent means $\pm$ error deviations of 4 independent experiments. * Indicates means significantly different from control group; $p < 0.05$.

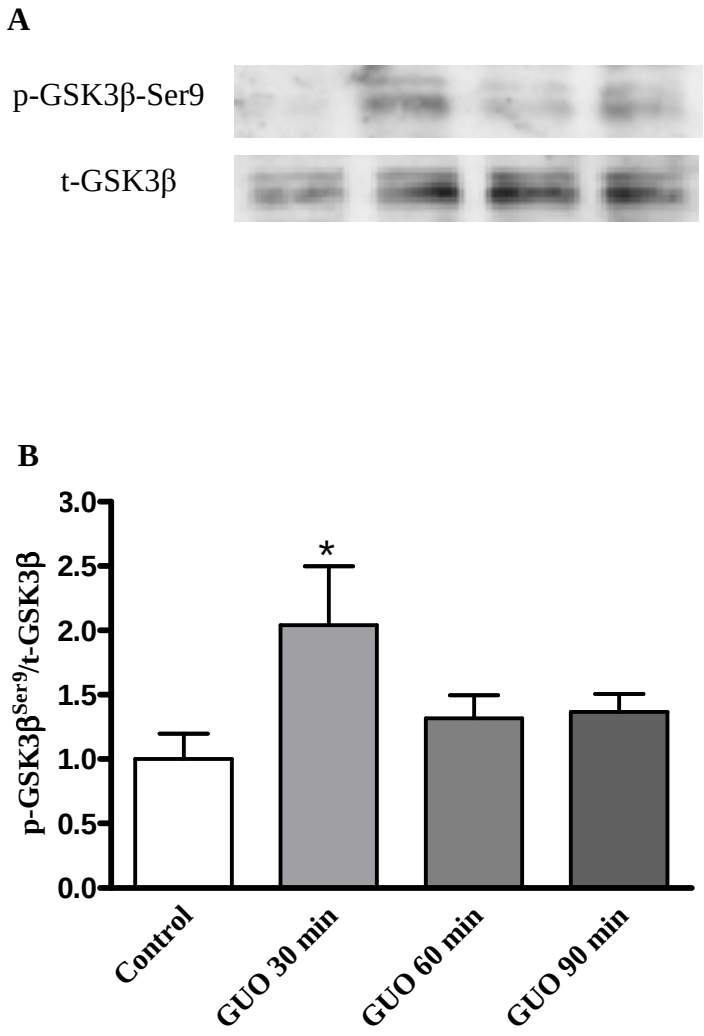

Figure 5. Immunodetection of phosphorylated Ser9-GSK3β and total GSK3β in hippocampal slices treated with GUO. Whole cells lysates were subjected to Western blotting analysis to phosphorylated (p-GSK3β^{Ser9}) and total (t-GSK3β) as described in Methods. **(A)** Representative western blot of phosphorylated GSK3β at Ser9 (p- GSK3β^{Ser9}) and total GSK3β (t- GSK3β) in hippocampal slices exposed to GUO 100μM for 30, 60 or 90 min. **(B)** Quantitative analysis of p-GSK3β^{Ser9}/t-GSK3β in optical density (O.D.). The control values were normalized to 1, and other treatments were expressed in relation to this value. The values represent means ± error deviations of 6 independent experiments. * Indicates means significantly different from control group; $p < 0.05$.

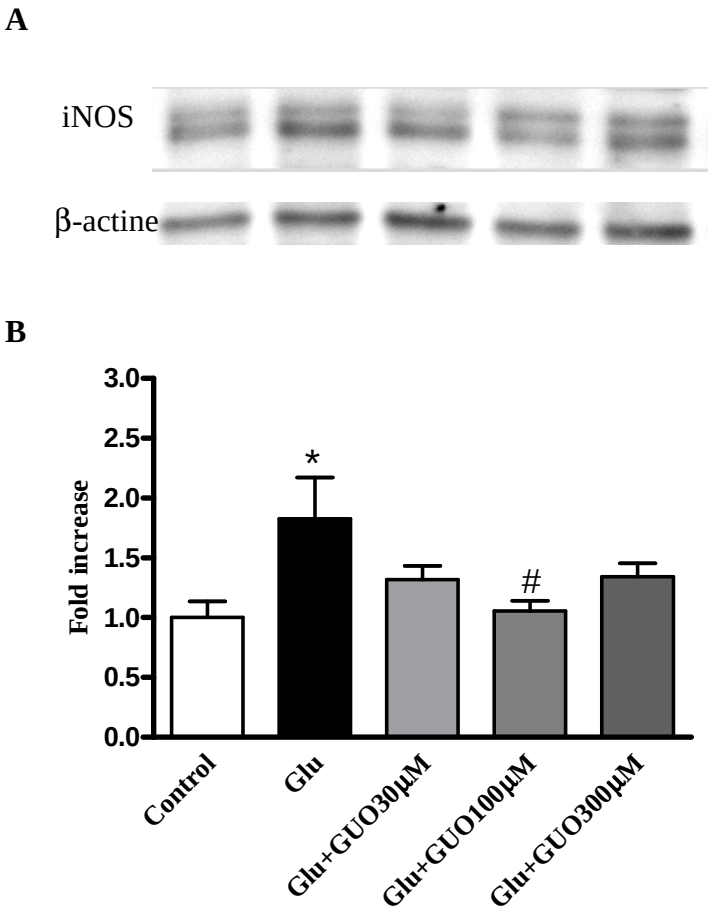

Figure 6. Immunodetection of iNOs in hippocampal slices submitted to glutamate in the presence of GUO. Untreated (Control, C) or 1 mM glutamate (Glu)-treated hippocampal slices were incubated for 1 h in culture medium. Slices were maintained for additional 6 h in fresh culture medium without glutamate. When present, guanosine (GUO 30, 100 or 300μM) was preincubated for 30 min. Whole cells lysates were subjected to Western blot analysis to iNOS detection as described in Methods. **(A)** Representative western blot of iNOS in hippocampal slices submitted to Glu or Glu+GUO. **(B)** Quantitative analysis by optical density of iNOS expression related to β-actine. The control values were normalized to 1, and other treatments were expressed in relation to this value. The values represent means $\pm$ error deviations of 4 independent experiments. * Indicates means significantly different from control group and # indicates means significantly different from Glu; $p < 0.05$.

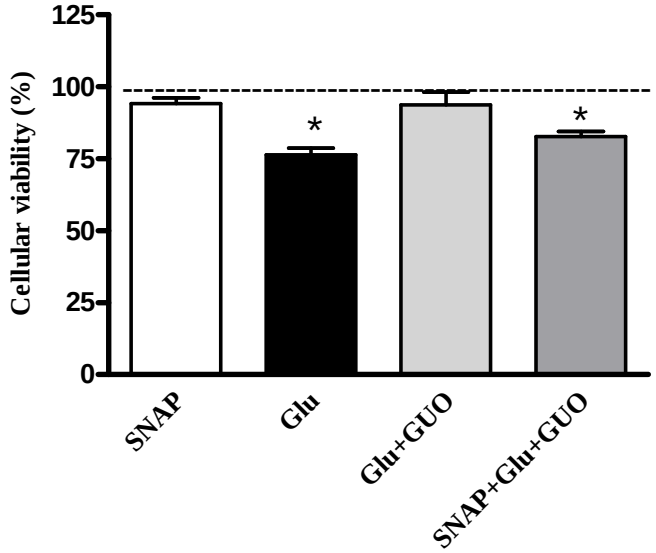

Figure 7. Guanosine neuroprotection against glutamate-induced cell death is prevented by a NO donor. Hippocampal slices were incubated for 1 h with 1mM glutamate (Glu) in the presence or absence of 100μM guanosine (GUO). When present, GUO was preincubated for 30 min before the addition of glutamate. SNAP (1mM) was added to incubation medium 15 min before GUO and maintained during the GUO incubation period. After that, incubation medium was withdrawn and replaced for fresh culture medium without glutamate and maintained for additional 6 hours. Control group was considered as 100 % and represents cell viability of slices incubated only in culture medium. MTT (0.5 mg/ml) was incubated for 20 min at 37º C and cell viability was accessed at 550 nm. The values represent means $\pm$ error deviations of at least 4 experiments carried out in triplicates. * Indicates means significantly different from control group (100 %) and Glu+GUO; $p < 0.05$.

CAPÍTULO 3

GUANOSINE-5'-MONOPHOSPHATE INDUCES CELL DEATH IN RAT HIPPOCAMPAL SLICES VIA IONOTROPIC GLUTAMATE RECEPTORS ACTIVATION AND GLUTAMATE UPTAKE INHIBITION

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Guanosine-5′-monophosphate induces cell death in rat hippocampal slices via ionotropic glutamate receptors activation and glutamate uptake inhibition

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ABSTRACT

Guanine derivatives modulate the glutamatergic system through displacement of binding of glutamate to its receptors acting as antagonist of glutamate receptors in moderate to high micromolar concentrations. Guanosine-5′-monophosphate (GMP) is shown to be neuroprotective against glutamate- or oxygen/glucose deprivation-induced neurotoxicity and also against NMDA-induced apoptosis in hippocampal slices. However, in this study we are showing that high extracellular GMP concentrations (5 mM) reduced cell viability in hippocampal brain slices. The toxic effect of GMP was not blocked by dipyridamole, a nucleoside transport inhibitor, nor mimicked by guanosine, suggesting an extracellular mode of action to GMP which does not involve its hydrolysis to guanosine. GMP-dependent cell damage was not blocked by P1 purinergic receptor antagonists, neither altered by adenosine A_1 or A_{2A} receptor agonists. The blockage of the ionotropic glutamate receptors AMPA or NMDA, but not KA or metabotropic glutamate receptors, reversed the toxicity induced by GMP. GMP (5 mM) induced a decrease in glutamate uptake into hippocampal slices, which was reversed by DL-TBOA. Therefore, GMP-induced hippocampal cell damage involves activation of ionotropic glutamate receptors and inhibition of glutamate transporters activity.

1. Introduction

Purines have several intracellular roles as energy source to cellular work and signal transduction modulation. However, adenine-based purines and guanine-based purines can also be released to the extracellular space and act as important intercellular signaling molecules (Neary et al., 1996; Ciccarelli et al., 2001; Fields and Burnstock, 2006; Decker et al., 2007).

In the Central Nervous System (CNS), adenosine and ATP act as neurotransmitters and neuromodulators, where they can mediate trophic effects to neurons and astrocytes (Fields and Burnstock, 2006). These adenine-based purines also regulate neuronal cell death during CNS development (Rathbone et al., 1999; Di Iorio et al., 2002) and under pathologic conditions (Amadio et al., 2002; Caveliere et al., 2005).

Astrocytes are the main cerebral source of extracellular adenine- and guanine-based purines (Ciccarelli et al., 1999) and express specific purinergic receptors belonging to both adenosine and ATP receptors (Burnstock, 2006). Guanine derivatives (GD) bind with low affinity to adenosine receptors (Muller and Scior, 1993) suggesting that GD may have different targets than the already known purinergic receptors. Indeed, evidences for GD binding sites have been shown in cultured astrocytes (Decker et al., 2007), in PC12 cells (Gysbers and Rathbone, 2000; Bau et al., 2005), and in chick cerebellar (Tasca et al., 1999) and rat brain membranes (Traversa et al., 2002). Recently, a specific binding site to GMP in GluR2 AMPA receptors was also demonstrated (Mendieta et al., 2005).

A relevant extracellular action displayed by GD is their role as modulators of the glutamatergic system (for a review, see Schmidt et al., 2007). GMP displaces the binding of glutamate and ionotropic agonists (kainic acid, NMDA and AMPA) to its receptors without interacting with G-proteins (Souza and Ramírez, 1991; Paz et al., 1994; Dev et al., 1996; Paas et al., 1996; Porciúncula et al., 2002). GMP acts as competitive antagonist of glutamate receptors in moderate to high micromolar concentrations (Baron et al., 1989) and it can also inhibit physiological cell responses induced by

Abbreviations: CPA, cyclopentyl adenosine; CGS21680, 2-[[2-[4-(2-carboxymethyl)phenyl] ethyl]amino]-N-ethylcarboxamidoadenosine; GAMS, γ-D-glutamylaminomethyl sulfonate; Glu, glutamate; GMP, guanosine-5′-monophosphate; DL-TBOA, DL-threo-β-benzyloxyaspartic acid; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DNQX, dinitroquinoxaline dione; MCPG, (RS)-α-methyl-4-carboxyphenylglycine; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cycloheptene-5,10-imine maleate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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glutamate receptors activation (Tasca et al., 1995, 1998; Burgos et al., 2000). Studies from our laboratory demonstrated that 1 mM GMP is neuroprotective against glutamate- or oxygen/glucose deprivation-induced neurotoxicity (Oliveira et al., 2002; Molz et al., 2005; Oleskovicz et al., 2008) and also against NMDA-induced apoptosis in hippocampal slices (Molz et al., 2008a).

The extracellular concentration of GD is determined by efflux to the extracellular space and fast acting ecto-nucelotidases activity, or by a bidirectional nucleoside transport system (Zimmermann, 1996). Recently, an observation from our laboratory shown that GTP (like ATP) is taken up and stored into neuronal synaptic vesicles (Santos et al., 2006), indicating that this GD may also be released from synaptic vesicles and may act as a neurotransmitter.

Cell injury is thought to cause remarkable outflow of purines, which are rapidly catabolised to monophosphate nucleotides and nucleosides accumulating within the traumatic tissue, reaching high concentrations in the extracellular space (Zimmermann et al., 1998). GD are released in amounts three-fold greater than their adenine derivative counterparts (Ciccarelli et al., 1999). High extracellular concentrations of adenosine or adenosine analogues can induce apoptosis in cultured astrocytes (Abbracchio et al., 1995; Rathbone et al., 1999; Di Iorio et al., 2002) and in rat astrocytoma cells (Sai et al., 2006). ATP is also related to CNS neuronal injury under pathologic conditions (Amadio et al., 2002; Caveliere et al., 2005; Franke and Illes, 2006) and induces apoptosis in hippocampal organotypic cultures (Morrone et al., 2005). Until now, a putative involvement of GD in nervous system cell damage has not been demonstrated.

Considering the evidences for protective as well as degenerative effects of purines in the CNS, the aim of the present study was to determine the potential neurotoxicity of high extracellular concentrations of GMP in rat hippocampal slices and the mechanism by which it could be exerting such toxicity. High extracellular GMP concentrations (5 mM) reduced cell viability in hippocampal brain slices. The toxic effect of GMP was not blocked by a nucleoside transport inhibitor, or mimicked by guanosine, suggesting an extracellular mode of action to GMP which does not involve its conversion to guanosine. GMP-dependent cell damage did not involve P1 purinergic receptors, but modulation of ionotropic glutamate receptors and glutamate transporters.

2. Experimental procedures

2.1. Animals

Male immature Wistar rats (23–25 days of postnatal age) maintained on a 12-h light–12-h dark schedule at 25 °C, with food and water *ad libitum*, were obtained from our local breeding colony. Experiments followed the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) and were approved by the local Ethical Committee of Animal Research (CEUA/UFSC).

2.2. Reagents

Cyclopentyl adenosine (CPA), 2-[[2-[4-(2-carboxymethyl)phenyl] ethyl]amino]-N-ethylcarboxamidoadenosine (CGS21680), dipyridamole, 8-cyclopentyl-1,3-dipropylxanthine (DPCX), ZM 241385, γ-D-glutamylaminomethyl sulfonate (GAMS), glutamate, GMP were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). DL-Threo-β-benzyloxyaspartate (DL-TBOA), 6,7-dinitroquinoxaline-2,3-dione (DNQX), (RS)-α-methyl-4-carboxyphenylglycine (MCPG), (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) were purchased from Tocris (Ellisville, MO, USA). Stock solutions were prepared by solubilization in ultra pure water or DMSO and stored at −20 °C. Solutions at concentrations used in the experiments were dissolved in Krebs–Ringer bicarbonate buffer.

2.3. Preparation and incubation of hippocampal slices

Rats were killed by decapitation and the hippocampi were rapidly removed and placed in ice-cold Krebs–Ringer bicarbonate (KRB) buffer of the following composition: 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM D-glucose. The buffer was bubbled with 95% O_2–5% CO_2 up to pH 7.4. Slices (0.4 mm thick) were rapidly prepared using a McIlwain Tissue Chopper, separated in KRB at 4 °C and allowed to recover for 30 min in KRB at 37 °C (Oliveira et al., 2002).

2.4. Slices treatment

After preincubation time (30 min), hippocampal slices were incubated with GMP (0.1–10 mM) (Sigma) for 1 h in KRB. After this period, the medium was withdrawn and replaced by a nutritive incubation medium composed of 50% of KRB, 50% of Dulbecco's modified Eagle's medium (DMEM, Gibco), 20 mM of HEPES and 100 μg/ml of gentamicine, at 37 °C in a CO_2 atmosphere (Molz et al., 2008b).

The involvement of P1-purinergic (adenosinergic) system in GMP-induced toxicity was assessed by preincubating hippocampal slices for 30 min with adenosine receptor antagonists, ZM241385 (50 nM) or DPCPX (100 nM), adenosine receptors agonists, CPA (100 nM) or CGS21680 (100 nM) or a nucleoside transporter inhibitor, dipyridamole (10 μM) (Oleskovicz et al., 2008). The involvement of glutamatergic system in GMP-induced damage was determined by preincubating the slices for 30 min with glutamate receptor antagonists, DNQX (50 μM), GAMS (50 μM), MK-801 (50 μM) or MCPG (500 μM) (Molz et al., 2008b), or with an inhibitor of glutamate transporters, DL-TBOA (10 μM) (Molz et al., 2008a). Slices were then exposed to 5 mM GMP for 1 h in the presence of these drugs. After this period, the medium was removed and slices were maintained in fresh incubation medium for additional 6 h in order to evaluate cell viability.

2.5. Evaluation of cell viability

2.5.1. MTT reduction

Hippocampal cell viability was evaluated 6 h after GMP exposure. Cell viability was determined through the ability of cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl-diphenyltetrazolium bromide, Sigma) (Mosmann, 1983). Hippocampal slices were incubated with MTT (0.5 mg/ml) in KRB for 30 min at 37 °C. The tetrazolium ring of MTT can be cleaved by active dehydrogenases in order to produce a precipitated formazan. The formazan produced was solubilized by adding 200 μl dimethyl sulfoxide (DMSO), resulting in a coloured compound whose optical density was measured in an ELISA reader (550 nm).

2.5.2. Propidium iodide (PI) uptake

Neuronal degeneration was monitored and quantified by densitometric measurement of the cellular uptake of PI (Sigma), a polar compound that only enters dead or dying cells with a damaged cell membrane. Inside the cells, PI binds to nucleic acid and emits a red fluorescence (630 nm) when excited with green light (495 nm). In the present experiments, hippocampal slices subjected to 5 mM GMP were incubated with 7 μg/ml PI in KRB for 30 min at 37 °C and then washed with KRB for analysis on a standard inverted microscope (Olympus IX 71) by using a rhodamine filter set. The pictures were taken with an Olympus C5060 camera. The PI uptake was quantified by densitometric analysis with Scion Image software (http://www.scioncorp.com). The area where PI fluorescence (in pixels) was detectable above the background (damaged area of hippocampal slices) was analyzed by using the "density slice" option of Scioncorp software through the division of PI fluorescence by the total area of the slice (Boeck et al., 2004).

2.6. L-[^{3}H]Glutamate uptake

L-[^{3}H]Glutamate uptake was evaluated as previously described (Molz et al., 2005). After preincubation in KRB buffer, hippocampal slices were incubated for 1 h with 5 mM GMP. When present, 10 μM DL-TBOA was preincubated for 10 min. Hippocampal slices were then washed for 15 min at 37 °C in a Hank's balanced salt solution (HBSS), composition in mM: 1.29 $CaCl_2$, 136.9 NaCl, 5.36 KCl, 0.65 $MgSO_4$, 0.27 Na_2HPO_4, 1.1 KH_2PO_4, and 5 HEPES. Uptake was assessed by adding 0.33 μCi/ml L-[^{3}H]glutamate with 100 μM unlabelled glutamate in a final volume of 300 μl. Incubation was stopped immediately after 7 min by discarding the incubation medium and slices were submitted to two ice-cold washes with 1 ml HBSS. Slices were solubilized by adding a solution with 0.1% NaOH/0.01% SDS and incubated overnight. Aliquots of slice lysates were taken for determination of the intracellular content of L-[^{3}H]glutamate by scintillation counting. Sodium-independent uptake was determined by using choline chloride instead of sodium chloride in the HBSS. Unspecific sodium-independent uptake was subtracted from total uptake to obtain the specific sodium-dependent glutamate uptake. Results were obtained in nmol of L-[^{3}H]glutamate taken up per mg of protein per minute and expressed as percentage of L-[^{3}H]glutamate uptake related to control slices.

2.7. Protein measurement

Protein content was evaluated by the method of Lowry et al. (1951). Bovine serum albumin (Sigma) was used as standard.

2.8. Statistical analysis

Comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by Duncan's test if necessary, with $p < 0.05$ considered to be statistically significant.

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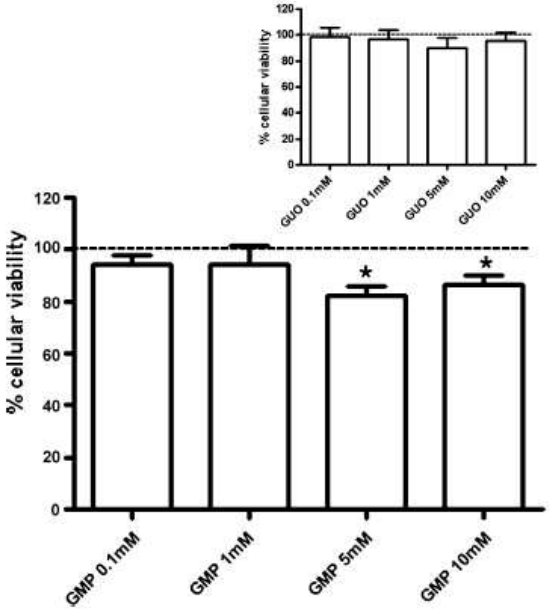

Fig. 1. Cell viability in hippocampal slices incubated with GMP: hippocampal slices were incubated for 1 h with GMP 0.1, 1, 5 or 10 mM. After this period, incubation medium was withdrawn and replaced for fresh culture medium without GMP and maintained for additional 6 h. Inset: Cell viability in hippocampal slices incubated with Guanosine: hippocampal slices were incubated for 1 h with guanosine 0.1, 1, 5 or 10 mM. Control group was considered as 100% and represents cell viability of slices incubated only in culture medium. MTT (0.5 mg/ml) was incubated for 20 min at 37 °C and cell viability was accessed at 550 nm. The values represent means ± standard error of at least 4 experiments carried out in triplicates. *Means significantly different from control group (100%); $p < 0.05$.

3. Results

3.1. GMP induces cell death in hippocampal slices

Hippocampal slices were incubated for 1 h with different concentrations of GMP (0.1–10 mM) and cell viability was evaluated 6 h after GMP exposure by the MTT reduction assay. GMP (5 or 10 mM) significantly reduced cell viability when compared to control slices (Fig. 1). Since GMP-induced toxicity might be indirectly related to its hydrolysis by 5′-ectonucletidases to the guanine nucleoside guanosine (GUO), we also tested a putative induction of cell injury by guanosine (0.1–10 mM). Differently from the observed with GMP, guanosine did not alter cellular viability at any concentration tested (Fig. 1, inset).

Considering both 5 and 10 mM GMP were able to reduce cellular viability at the same extent (Fig. 1), the lowest toxic GMP concentration (5 mM) was chosen in order to induce cell death and further investigate the mechanisms by which GMP induces toxicity in hippocampal slices. GMP (5 mM) also increased propidium iodide uptake into hippocampal slices (Fig. 2), showing labelled dead or dying cells.

3.2. GMP-induced hippocampal slices toxicity does not involve nucleoside transport or adenosine receptors

To evaluate if GMP-induced neuronal injury was dependent on the nucleoside transport activity, hippocampal slices were incubated for 1 h with 5 mM GMP, with or without dipyridamole, a non-selective nucleoside transport inhibitor, and cell viability evaluated after 6 h. When present, 10 μM dipyridamole was preincubated for 30 min before GMP and maintained in culture medium during GMP exposure. Hippocampal slices incubation with dipyridamole did not prevent cell death induced by GMP. Furthermore, dipyridamole *per se* was toxic to hippocampal brain slices (Fig. 3A).

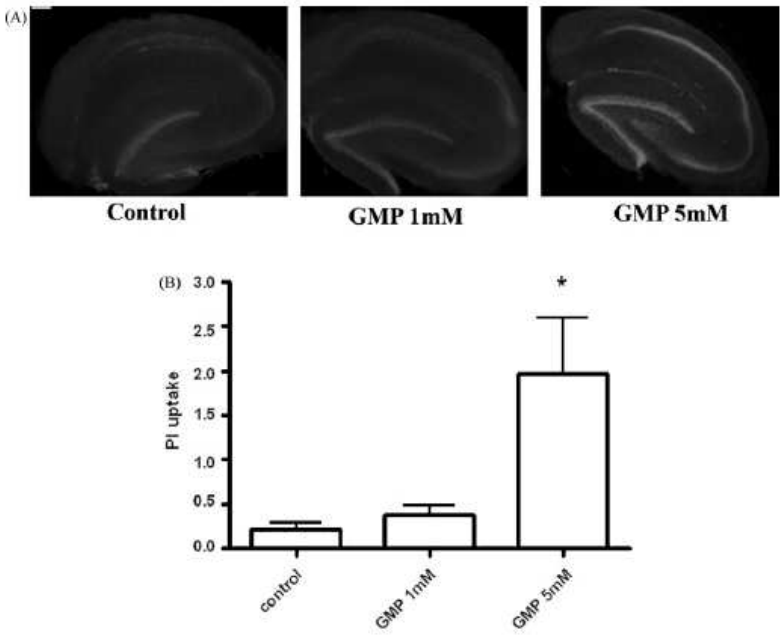

Fig. 2. Effects of GMP on PI uptake in hippocampal slices: hippocampal slices were incubated for 1 h with GMP (1 or 5 mM). After this period, incubation medium was withdrawn and replaced for fresh culture medium without GMP and maintained for additional 6 h. Control group represents PI uptake of slices incubated only in culture medium. (A) Representative images from hippocampal slices incubated under control situation or in the presence of GMP 1 mM or GMP 5 mM. (B) Quantification of PI uptake into hippocampal slices. The values represent means ± standard error of at least 3 experiments carried out in triplicates. *Means significantly different from control group and GMP 1 mM; $p < 0.05$.

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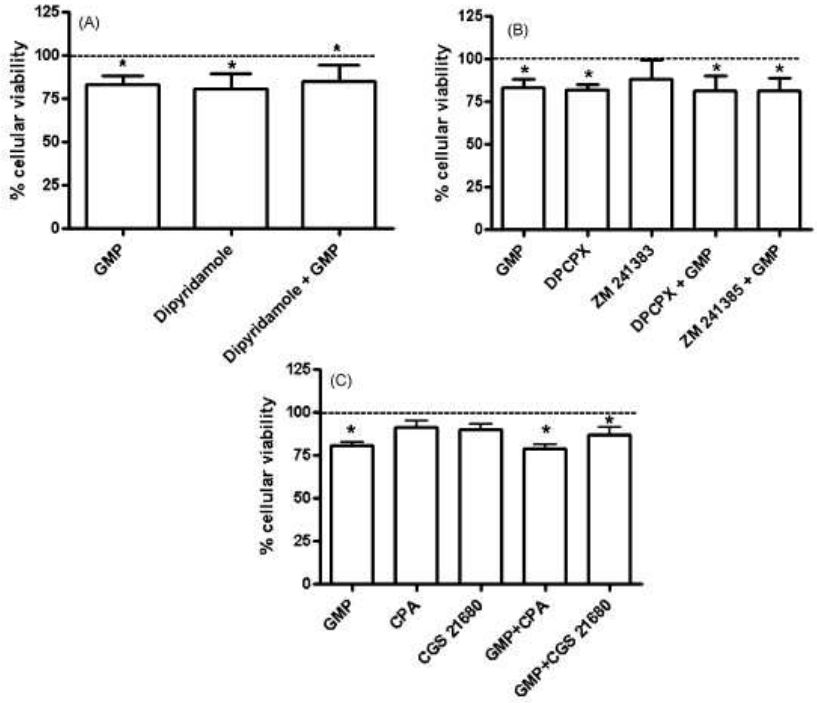

Fig. 3. Evaluation of involvement of nucleoside transport or adenosine receptors in GMP-induced hippocampal slice injury: hippocampal slices were incubated with 5 mM GMP. After this period, incubation medium was withdrawn and replaced for fresh culture medium without GMP and maintained for additional 6 h. (A) Cellular viability in slices incubated with 5 mM GMP for 1 h with or without 10 μM dipyridamole. The values represent means ± error deviations of at least 7 experiments carried out in triplicates. (B) Cellular viability in slices incubated with 5 mM GMP with or without 50 nM ZM241385 or 100 nM DPCPX. The values represent means ± error deviations of at least 7 experiments carried out in triplicates. (C) Cellular viability when slices were incubated with 5 mM GMP with or without 100 nM CPA or 100 nM CGS21680. The values represent means ± standard error of at least 5 experiments carried out in triplicates. All drugs used in these experiments were preincubated for 30 min before adding GMP. Control group was considered as 100% and represents cell viability of slices incubated only in culture medium. MTT (0.5 mg/ml) was incubated for 20 min at 37 °C and cell viability was accessed at 550 nm. *Means significantly different from control group (100%); $p < 0.05$.

Since GD can bind to adenosine receptors (Muller and Scior, 1993), a putative involvement of adenosine receptors in GMP-induced toxicity was also evaluated. Hippocampal slices were preincubated with DPCPX or ZM241385, specific antagonists of adenosine A_1 and A_{2A} receptors, respectively. Fig. 3B shows that preincubation with 100 nM DPCPX or 50 nM ZM241385 was not able to prevent cell viability reduction induced by 5 mM GMP. Additionally, DPCPX *per se* induced a reduction in cell viability in hippocampal brain slices (Fig. 3B).

Considering the blockade of adenosine A_1 receptors by DPCPX was toxic to hippocampal slices at the same extent as GMP, we evaluated the possible protective effect of adenosine agonists against GMP-induced slices damage. Hippocampal slices were preincubated with CPA or CGS21680, selective agonists of adenosine A_1 and A_{2A} receptors, respectively. Fig. 3C shows that neither CGS21680 (100 nM) nor CPA (100 nM) was able to counteract GMP-induced cell damage. Furthermore, the activation of A_1 or A_{2A} receptors by CPA or CGS21680 did not induce cell damage (Fig. 3C).

3.3. GMP-induced toxicity involves ionotropic glutamate receptors

Since GMP can displace the binding of glutamate and glutamate agonists to its receptors (Souza and Ramírez, 1991; Porciúncula et al., 2002) we decided to investigate whether glutamate receptor antagonists may block the reduction in hippocampal viability induced by GMP. We then tested the kainic acid receptor antagonist GAMS, the AMPA receptor antagonist DNQX, the non-competitive NMDA receptor antagonist MK-801, and the non-selective metabotropic glutamate receptor antagonist MCPG.

Fig. 4 shows that DNQX (50 μM) and MK-801 (50 μM) prevented cell damage induced by GMP. Otherwise, GAMS (50 μM) and MCPG (500 μM) did not prevent the reduction in cell viability promoted by GMP in hippocampal slices. Slices incubation in the

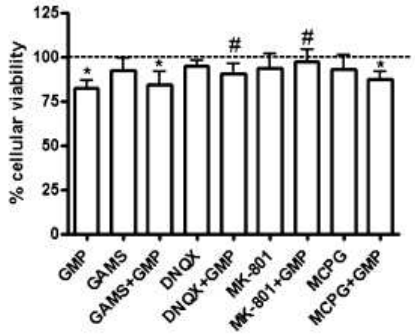

Fig. 4. Cell viability in hippocampal slices incubated with GMP in the presence of glutamate antagonists: hippocampal slices were incubated with 5 mM GMP. When present, GAMS (50 μM), DNQX (50 μM), MK-801 (50 μM) or MCPG (500 μM) were preincubated for 30 min. After this period, incubation medium was withdrawn and replaced for fresh culture medium without GMP and maintained for additional 6 h. Control group was considered as 100% and represents cell viability of slices incubated only in culture medium. MTT (0.5 mg/ml) was incubated for 20 min at 37 °C and cell viability was accessed at 550 nm. The values represent means ± standard error of at least 5 experiments carried out in triplicates. *Means significantly different from control group (100%); $p < 0.05$. #Means significantly different from GMP group; $p < 0.05$.

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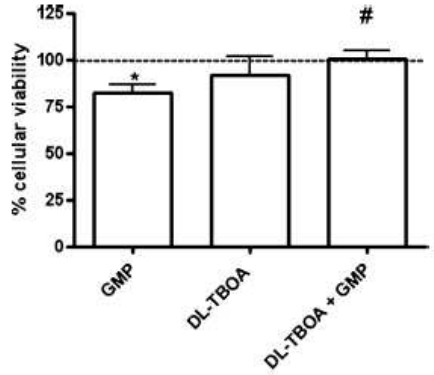

Fig. 5. Cell viability in hippocampal slices incubated with GMP in the presence of glutamate transporter inhibitor (DL-TBOA): hippocampal slices were incubated with 5 mM GMP. When present, DL-TBOA (10 μM) was preincubated for 30 min. After this period, incubation media was withdrawn and replaced for fresh culture medium without GMP and maintained for additional 6 h. Control group was considered as 100% and represents cell viability of slices incubated only in culture medium. MTT (0.5 mg/ml) was incubated for 20 min at 37 °C and cell viability was accessed at 550 nm. The values represent means ± standard error of at least 5 experiments carried out in triplicates. *Means significantly different from control group (100%); $p < 0.05$. #Means significantly different from GMP group; $p < 0.05$.

presence of glutamate receptor antagonists alone did not alter cell viability (Fig. 4).

3.4. Involvement of glutamate transport in GMP-induced toxicity

Since GMP-induced toxicity was prevented by glutamate receptor antagonists, we also looked for GMP effects on glutamate transporters activity. Hippocampal slices were preincubated for 30 min with a glutamate transporter inhibitor (DL-TBOA, 10 μM) in a concentration that it inhibits glutamate reverse transport in hippocampal slices (Anderson et al., 2001; Bonde et al., 2003; Molz et al., 2008a). DL-TBOA did not alter cell viability *per se* and completely prevented GMP-induced reduction of cell viability (Fig. 5).

Additionally, we have evaluated glutamate uptake in slices incubated with 5 mM GMP alone or in combination with 10 μM DL-TBOA. GMP (5 mM) induced a 50% decrease of L-[^{3}H]glutamate uptake in comparison to control uptake into hippocampal slices.

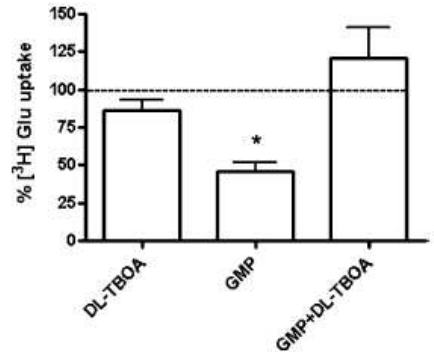

Fig. 6. L-[^{3}H]Glutamate uptake in hippocampal slices incubated with GMP and DL-TBOA. Hippocampal slices were incubated for 1 h with 5 mM GMP in the presence or absence of DL-TBOA. When present, DL-TBOA (10 μM) was preincubated for 10 min. Glutamate uptake was assessed as described in Section 2. Results were expressed as percentage of L-[^{3}H]glutamate uptake related to control slices (100%). The values represent means ± standard error of at least 4 experiments carried out in triplicates. *Mean significantly different from all other groups; $p < 0.05$.

This reduction in L-[^{3}H]glutamate uptake into slices induced by 5 mM GMP was fully prevented by DL-TBOA (Fig. 6). Thus, the neurotoxic effect of GMP is probably occurring via impairment of glutamate transport (Figs. 5 and 6) and glutamate receptors activation (Fig. 4).

4. Discussion

This study showed that high extracellular concentrations of GMP (5 mM) are neurotoxic to hippocampal slices. GMP-induced cell death occurs due to its interaction with the glutamatergic system, since 5 mM GMP decreased L-[^{3}H]glutamate uptake, increasing glutamate concentration in the synaptic cleft. Reinforcing this idea, GMP-induced cell injury was fully prevented by NMDA or AMPA receptor antagonists.

Under pathological conditions as hypoxia, ischemia or mechanical injury, purine nucleotides are thought to be released from damaged cells, and thereby, may reach high concentrations in the extracellular space, as suggested by *ex vivo* and *in vivo* studies (Braun et al., 1998; Ciccarelli et al., 1999; Melani et al., 2005). Adenine-based purines have been shown to exert toxic effects to hippocampal slices when present at millimolar concentrations in the extracellular space (Morrone et al., 2005; Frizzo et al., 2007). In the present study, we demonstrated that a guanine-based purine, GMP, at millimolar levels, decreased MTT reduction and also increased propidium iodide uptake in hippocampal slices (Figs. 1 and 2). The high propidium iodide incorporation observed indicates that GMP may be inducing a necrotic pattern of cellular death. Such observation is reinforced by the absence of GMP-induced DNA laddering in hippocampal slices (data not shown). Similar to our results, 5 mM of GTP or Gpp(NH)p (a non-hydrolyzed GTP analogue) increased glutamate-induced oxidative injury in hippocampal slices (Regner et al., 1998). Therefore, a dysfunction in purines transmission system may lead to a neuronal loss in the hippocampus.

The previously reported protective effects of GMP *in vitro* and *in vivo* have been shown to be dependent on its hydrolysis by ectonucleotidases and its conversion to the nucleoside guanosine (Schmidt et al., 2000; Frizzo et al., 2003; Soares et al., 2004). Hippocampal viability in guanosine-treated slices (0.1–10 mM) was similar to untreated slices, thus guanosine did not promote neurotoxicity at any concentration tested (Fig. 1, inset), showing that GMP-induced cell death does not involve its conversion to guanosine. Thus, the increased GMP concentration evaluated in this study (5 mM), overcomes any protective effect GMP or guanosine may display.

Hippocampal slices incubation with dipyridamole did not prevent cell death induced by GMP, showing GMP-induced toxicity is occurring via interaction with extracellular binding sites to GMP. Otherwise, dipyridamole *per se* was toxic to hippocampal brain slices (Fig. 3a), which implicate that the nucleoside transport is important to maintain cellular viability in hippocampal slices (Fig. 3A).

GD receptors are still not fully characterized but there are some evidences that GD may interact with adenosine receptors (Muller and Scior, 1993; DAlimonte et al., 2007). Blockade of adenosine receptors, mainly A_{2A} receptors, is involved in neuroprotection against different noxious stimulus in the CNS (Dall'Igna et al., 2003; Silva et al., 2007). Otherwise, activation of A_1 receptors is considered neuroprotective under various cell-damaging conditions, including hypoxia, hypoglycemia, ischemia and oxidative stress (Cunha, 2001). Based on these evidences, we looked for participation of the adenosinergic system in GMP-induced toxicity. DPCPX or ZM241385 (A_1 or A_{2A} receptors antagonist, respectively) did not prevent GMP-induced cell injury (Fig. 3). Interestingly, DPCPX *per se* was able to induce cell damage at the same extent of GMP (Fig. 3B). These results and the observation that nucleoside

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transport is important to maintain cellular viability in hippocampal slices (Fig. 3A), indicate that basal activation of A_1 receptors is important in our experimental model to maintain hippocampal cells integrity. These evidences lead us to evaluate the possible protective effect of adenosine receptor agonists against GMP-induced slices damage. Likewise, neither CGS21680 nor CPA was able to counteract GMP-induced cell damage and the activation of A_1 receptors or A_{2A} receptors by CPA or CGS21680 did not induce cell damage (Fig. 3C). These results are consistent with the well-known role of adenosine as an endogenous neuroprotective metabolite that through the activation of A_1 receptors results in reduction of neuronal damage (Cunha, 2001; Gervitz et al., 2002; Wardas, 2002; Almeida et al., 2003), and exclude adenosinergic receptors in the mechanism related to GMP toxicity.

Increased extracellular glutamate levels and subsequent excitotoxicity are thought to be one of the major pathological factors leading to neuronal death upon a variety of neurodegenerative diseases with different origins (Choi, 1992; Segovia et al., 2001; Mattson, 2003; Maragakis and Rothstein, 2004; Sattler and Rothstein, 2006). Several studies have shown a modulatory effect of GD on glutamatergic transmission. GMP displaces the binding of glutamate to ionotropic glutamate receptors (Souza and Ramírez, 1991; Paz et al., 1994; Dev et al., 1996; Paas et al., 1996; Porciúncula et al., 2002) suggesting GMP binding to these receptors. In this study, a selective kainate receptor antagonist (GAMS) or a non-selective metabotropic glutamate receptor antagonist (MCPG) did not afford protection against GMP toxicity. However, NMDA or AMPA receptor antagonists (MK-801 or DNQX, respectively) were both able to prevent cell death induced by GMP (Fig. 4). Therefore, we may conclude that the toxic effect of GMP is due to AMPA and NMDA receptors activation.

Glutamate uptake has been shown to be implicated in neuroprotection (Schousboe and Waagepetersen, 2006) and the reduction of glutamate uptake is associated to neurotoxic insults such as ischemia (Brongholi et al., 2006) and neurodegenerative diseases (Matute et al., 2002; Mattson, 2003; Maragakis and Rothstein, 2004; Sattler and Rothstein, 2006). Neurotoxic concentration of GMP induced a decreased glutamate uptake into hippocampal slices which was prevented by a subtoxic concentration of DL-TBOA (Fig. 6). The blockade of GMP-induced glutamate uptake decrease by DL-TBOA correlates with its ability to prevent GMP-induced cell damage (Fig. 5). Similar to our results, microdialysis studies showed that endogenous ATP facilitated the increase in extracellular glutamate levels (Krügel et al., 2004). There are also evidences for the involvement of glutamatergic mechanisms in purinergic signaling changes occurring after cerebral injury (Franke et al., 2006).

Nevertheless, GD have been implicated in neuroprotection by counteracting glutamate excitotoxicity *in vitro* (Oliveira et al., 2002; Molz et al., 2005, 2008b; Oleskovicz et al., 2008) and *in vivo* (Schmidt et al., 2000, 2005) animal models. Similarly, low NMDA concentrations are neuroprotective against excitotoxic insults, while high NMDA concentrations provoke neuronal death *in vitro* (Valera et al., 2008) or *in vivo* (Vandresen-Filho et al., 2007). Disturbance in purines metabolism have been related to pathological alterations observed in some neurological syndromes (Torres et al., 2007). We here provide evidences that, as previously demonstrated to ATP, high concentrations of GMP could be toxic to hippocampal slices. Therefore, high nucleotide concentrations in the CNS may be related to the pathophysiological alterations observed in neurodegeneration, as glutamate receptors activation or inhibition of glutamate clearance from the synaptic cleft, thus inducing neurotoxicity. Our results reinforce the role of GMP as a modulator of the glutamatergic transmission and contribute to understanding the role of purines in the pathophysiology of degenerative diseases in the brain.

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References

Abbracchio, M.P., Ceruti, S., Barbieri, D., Franceschi, C., Malorni, W., Biondo, L., Burnstock, G., Cattabeni, F., 1995. Biochem. Biophys. Res. Commun. 213, 908–915.
Almeida, C.G., Mendonça, A., Cunha, R.A., Ribeiro, J.A., 2003. Adenosine promotes neuronal recovery from reactive oxygen species induced lesion in rat hippocampal slices. Neurosci. Lett. 339, 127–130.
Amadio, S., D'Ambrosi, N., Cavaliere, F., Murra, B., Sancesario, G., Bernardi, G., Burnstock, G., Volonté, C., 2002. P2 receptor modulation and cytotoxic function in cultured CNS neurons. Neuropharmacology 42, 489–501.
Anderson, C.M., Bridges, R.J., Chamberlin, A.R., Shimamoto, K., Yasuda-Kamatami, Y., Swanson, R.A., 2001. Differing effects of substrate and non-substrate transport inhibitors on glutamate uptake reversal. J. Neurochem. 79, 1207–1216.
Baron, B.M., Dudley, M.W., McCarty, D.R., Miller, F.P., Reynolds, I.J., Schmidt, C.J., 1989. Guanine nucleotides are competitive inhibitors of N-methyl-D-aspartate at its receptor site both in vitro and in vivo. J. Pharmacol. Exp. Therap. 250, 162–169.
Bau, C., Middlemiss, P.J., Hindley, S., Jiang, S., Ciccarelli, R., Caciagli, F., Di Iorio, P., Werstiuk, E.S., Rathbone, M.P., 2005. Guanosine stimulates neurite outgrowth in PC12 cells via activation of heme oxygenase and cyclic GMP. Purinergic Signal. 1, 161–172.
Boeck, C.R., Ganzella, M., Lottermann, A., Vendite, D., 2004. NMDA preconditioning protects against seizures and hippocampal neurotoxicity induced by quinolinic acid in mice. Epilepsia 45, 745–750.
Bonde, C., Sarup, A., Schousboe, A., Gegelashvili, G., Zimmer, J., Noraberg, J., 2003. Neurotoxic and neuroprotective effects of the glutamate transporter inhibitor DL-threo-beta-benzyloxyaspartate (DL-TBOA) during physiological and ischemia-like conditions. Neurochem. Int. 43, 371–380.
Braun, N., Zhu, U., Krieglstein, J., Culmsee, C., Zimmermann, H., 1998. Upregulation of the enzyme chain hydrolyzing extracellular ATP after transient forebrain ischemia in the rat. J. Neurosci. 18, 4891–4900.
Brongholi, K., Souza, D.G., Bainy, A.C., Dafre, A.L., Tasca, C.I., 2006. Oxygen–glucose deprivation decreases glutathione levels and glutamate uptake in rat hippocampal slices. Brain Res. 1083, 211–218.
Burgos, J.S., Barat, A., Ramírez, G., 2000. Guanine nucleotides block agonist-driven $^{45}Ca^{2+}$ influx in chick embryo retinal explants. Neuroreport 11, 2303–2305.
Burnstock, G., 2006. Purinergic signalling. Br. J. Pharmacol. 147, S172–181.
Caveliere, F., Dinkel, K., Reymann, K., 2005. Microglia response and P2 receptor participation in oxygen/glucose deprivation-induced cortical damage. Neuroscience 136, 615–623.
Choi, D.W., 1992. Excitotoxic cell death. J. Neurobiol. 23, 1261–1276.
Ciccarelli, R., Di Iorio, P., Giuliani, P., D'Alimonte, I., Ballerini, P., Caciagli, F., Rathbone, M.P., 1999. Rat cultured astrocytes release guanine-based purines in basal conditions and after hypoxia/hypoglycemia. Glia 25, 93–98.
Ciccarelli, R., Ballerini, P., Sabatino, G., Rathbone, M.P., D'Onofrio, M., Caciagli, F., Di Iorio, P., 2001. Involvement of astrocytes in purine-mediated reparative processes in the brain. Int. J. Dev. Neurosci. 19, 395–414.
Cunha, R.A., 2001. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem. Int. 38, 107–125.
ĎAlimonte, I., Ballerini, P., Nargi, E., Bucella, S., Giuliani, P., Di Iorio, P., Caciagli, F., Ciccarelli, R., 2007. Staurosporine-induced apoptosis in astrocytes is prevented by A1 adenosine receptor activation. Neurosci. Lett. 418, 66–71.
Dall'Igna, O.P., Porciucula, L.O., Souza, D.O., Cunha, R.A., Lara, D.R., Lara, D.R., 2003. Neuroprotection by caffeine and adenosine A_2A receptor blockade of b-amyloid neurotoxicity. Br. J. Pharmacol. 138, 1207–1209.
Decker, H., Francisco, S.S., Mendes-de-Aguiar, C.B., Romão, L.F., Boeck, C.R., Trentin, A.G., Moura-Neto, V., Tasca, C.I., 2007. Guanine derivatives modulate extracellular matrix proteins organization and improve neuron–astrocyte co-culture. J. Neurosci. Res. 85, 1943–1951.
Dev, K.H., Roberts, P.J., Henley, J.M., 1996. Characterisation of the interaction between guanyl nucleotides and AMPA receptors in rat brain. Neuropharmacology 35, 1583–1593.
Di Iorio, P., Ciccarelli, R., Traversa, U., Andrew, C.M., Crocker, C.E., Werstiuk, E.S., Rathbone, M.P., 2002. Mechanism of purine nucleosides induced apoptosis in astrocytes. Glia 38, 179–190.
Fields, R.D., Burnstock, G., 2006. Purinergic signalling in neuron–glia interactions. Nat. Rev. Neurosci. 7, 423–436.

Please cite this article in press as: Molz, S., et al., Guanosine-5′-monophosphate induces cell death in rat hippocampal slices via ionotropic glutamate receptors activation and glutamate uptake inhibition. Neurochem. Int. (2009), doi:10.1016/j.neuint.2009.06.015

Franke, H., Illes, P., 2006. Involvement of P2 receptors in the growth and survival of neurons in the CNS. Pharmacol. Therap. 109, 297–324.
Franke, H., Grummich, B., Härtig, W., Grosche, J., Regenthal, R., Edwards, R.H., Illes, P., Krügel, U., 2006. Changes in purinergic signaling after cerebral injury—involvement of glutamatergic mechanisms? Int. J. Dev. Neurosci. 24, 123–132.
Frizzo, M.E., Soares, F.A., Dall'Onder, L.P., Lara, D.R., Swanson, R.A., Souza, D.O., 2003. Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake. Brain Res. 972, 84–89.
Frizzo, M.E., Frizzo, J.K., Amadio, S., Rodrigues, J.M., Perry, M.L., Bernardi, G., Volonté, C., 2007. Extracellular adenosine triphosphate induces glutamate transporter-1 expression in hippocampus. Hippocampus 17, 305–315.
Gervitz, L.M., Nalbant, D., Williams, S.C., Fowler, J.C., 2002. Adenosine-mediated activation of Akt/protein kinase B in the rat hippocampus in vitro and in vivo. Neurosci. Lett. 328, 175–179.
Gysbers, J.W., Rathbone, M.P., 2000. Neurite outgrowth from PC12 cells is enhanced by guanosine through both cAMP-dependent and -independent mechanisms. Int. J. Dev. Neurosci. 41, 19–34.
Krügel, U., Schraft, T., Regenthal, R., Illes, P., Kittner, H., 2004. Purinergic modulation of extracellular glutamate levels in the nucleus accumbens in vivo. Int. J. Dev. Neurosci. 22, 565–570.
Lowry, O.H., Rosembrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Maragakis, N.J., Rothstein, J.D., 2004. Glutamate transporters: animal models to neurologic disease. Neurobiol. Dis. 15, 461–473.
Mattson, M.P., 2003. Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. Neuromol. Med. 3, 65–94.
Matute, C., Alberdi, E., Ibarretxe, G., Sánchez-Gómez, M.V., 2002. Excitotoxicity in glial cells. Eur. J. Pharmacol. 447, 239–246.
Melani, A., Turchi, D., Vannucchi, M.G., Cipriani, S., Gianfriddo, M., Pedata, F., 2005. ATP extracellular concentrations are increased in the rat striatum during in vivo ischemia. Neurochem. Int. 47, 442–448.
Mendieta, J., Gago, F., Ramírez, G., 2005. Binding of 5′-GMP to the GluR2 AMPA receptor: insight from targeted molecular dynamics simulations. Biochemistry 44, 14470–14476.
Molz, S., Oliveira, I.J.L., Decker, H., Souza, D.O., Tasca, C.I., 2005. Neurotoxicity induced by glutamate in glucose-deprived rat hippocampal slices is prevented by GMP. Neurochem. Res. 30, 83–89.
Molz, S., Dal-Cim, T., Decker, H., Tasca, C.I., 2008a. GMP prevents excitotoxicity mediated by NMDA receptor activation but not by reversal activity of glutamate transporters in rat hippocampal slices. Brain Res. 22, 113–120.
Molz, S., Decker, H., Dal-Cim, T., Cremonez, C., Cordova, F.M., Leal, R.B., Tasca, C.I., 2008b. Glutamate-induced toxicity in hippocampal slices involves apoptotic features and $p38^{MAPK}$ signaling. Neurochem. Res. 33, 27–36.
Morrone, F.B., Horn, A.P., Stella, J., Spiller, F., Sarkis, J.J.F., Sal Bejo, C.G., Lenz, G., Battastini, M.O., 2005. Increased resistance of glioma cell lines to extracellular ATP cytotoxicity. J. Neurooncol. 71, 135–140.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity. J. Immunol. Methods 65, 55–63.
Muller, C.E., Scior, T., 1993. Adenosine receptors and their modulators. Pharm. Acta Helvet. 68, 77–111.
Neary, J.T., Rathbone, M.P., Cattabeni, F., Abbracchio, M.P., Burnstock, G., 1996. Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. Trends Neurosci. 19, 13–18.
Oleskovicz, S.P., Martins, M.C., Leal, R.B., Tasca, C.I., 2008. Mechanism of guanosine-induced neuroprotection in rat hippocampal slices submitted to oxygen–glucose deprivation. Neurochem. Int. 52, 411–418.
Oliveira, I.J.L., Molz, S., Souza, D.O., Tasca, C.I., 2002. Neuroprotective effect of GMP in hippocampal slices submitted to an in vitro model of ischemia. Cell. Mol. Neurobiol. 22, 335–344.
Paas, Y., Devillers-Thiery, A., Changeux, J.-P., Medevielle, F., Teichberg, V.I., 1996. Identification of an extracellular motif involved in the binding of guanine nucleotides by a glutamate receptor. Eur. Mol. Biol. Org. J. 15, 1548–1556.
Paz, M.M., Ramos, M., Ramírez, G., Souza, D.O., 1994. Differential effects of guanine nucleotides on kainic acid binding and on adenylate cyclase activity in chick optic tectum. FEBS Lett. 355, 205–208.
Porciúncula, L.O., Vinadé, L., Wolfchuk, S., Souza, D.O., 2002. Guanine based purines inhibit [^{3}H]glutamate and [^{3}H]AMPA binding at postsynaptic densities from cerebral cortex of rats. Brain Res. 928, 106–112.
Rathbone, M.P., Middlemiss, P.J., Gysbers, J.W., Andrew, C., Herman, M.A., Reed, J.K., Ciccarelli, R., Di Iorio, P., Caciagli, F., 1999. Trophic effects of purines in neurons and glial cells. Prog. Neurobiol. 59, 663–690.
Regner, A., Ramirez, G., Bellô-Klein, D., Souza, D., 1998. Effects of guanine nucleotides on glutamate-induced chemiluminescence in rat hippocampal slices submitted to hypoxia. Neurochem. Res. 23, 519–524.
Sai, K., Yang, D., Yamamoto, H., Fugikawa, H., Yamamoto, S., Nagata, T., Saito, M., Yamamura, T., Nishizaki, T., 2006. A1 adenosine receptor signal and AMPK involving caspase 9/3 activation are responsible for adenosine induced RCR-1 astrocytoma cell death. Neurotoxicology 27, 458–467.
Santos, T.G., Souza, D.O., Tasca, C.I., 2006. GTP uptake into rat brain synaptic vesicles. Brain Res. 1070, 71–76.
Sattler, R., Rothstein, J.D., 2006. Regulation and dysregulation of glutamate transporters. Handb. Exp. Pharmacol. 175, 277–303.
Schmidt, A.P., Lara, D.R., Maraschin, J.F., Perla, A.S., Souza, D.O., 2000. Guanosine and GMP prevent seizures induced by quinolinic acid in mice. Brain Res. 864, 40–43.
Schmidt, A.P., Avila, T.T., Souza, D.O., 2005. Intracerebroventricular guanine-based purines protect against seizures induced by quinolinic acid in mice. Neurochem. Res. 30, 69–73.
Schmidt, A.P., Lara, D.R., Souza, D.O., 2007. Proposal of a guanine-based purinergic system in the mammalian central nervous system. Pharmacol. Therap. 116, 401–416.
Schousboe, A., Waagepetersen, H.S., 2006. Glial modulation of GABAergic and glutamatergic neurotransmission. Curr. Top. Med. Chem. 6, 929–934.
Segovia, G., Porras, A., Del Arco, A., Mora, F., 2001. Glutamatergic neurotransmission in aging: a critical perspective. Mech. Ageing Dev. 122, 1–29.
Silva, C.G., Porciúncula, L.O., Canas, P.M., Oliveira, C.R., Cunha, R.A., 2007. Blockade of adenosine A2A receptors prevents staurosporine-induced apoptosis of rat hippocampal neurons. Neurobiol. Dis. 27, 182–189.
Souza, D.O., Ramírez, G., 1991. Effects of guanine nucleotides on kainic acid binding and on adenylate cyclase in chick optic tectum and cerebellum. J. Mol. Neurosci. 3, 39–45.
Soares, F.A., Schmidt, A.P., Farina, M., Frizzo, M.E., Tavares, R.G., Portela, L.V., Lara, D.R., Souza, D.O., 2004. Anticonvulsant effect of GMP depends on its conversion to guanosine. Brain Res. 1005, 182–186.
Tasca, C.I., Wofchuk, S.T., Souza, D.O., Ramírez, G., Rodnight, R., 1995. Guanine nucleotides inhibit the stimulation of GFAP phosphorylation by glutamate. Neuroreport 6, 249–252.
Tasca, C.I., Cardoso, L.F., Martini, L.H., Ramírez, G., Souza, D.O., 1998. Guanine nucleotides inhibit cAMP accumulation induced by metabotropic glutamate receptors activation. Neurochem. Res. 23, 183–188.
Tasca, C.I., Burgos, J.S., Barat, A., Souza, D.O., Ramirez, G., 1999. Chick kainate binding protein lacks GTPase activity. Neuroreport 10, 1981–1983.
Torres, R.J., Puig, J.G., 2007. Hypoxanthine–guanine phosophoribosyltransferase (HPRT) deficiency: Lesch–Nyhan syndrome. Orphanet J. Rare Dis. 2, 48.
Traversa, U., Bombi, G., Di Iorio, P., Ciccarelli, R., Werstiuk, E.S., Rathbone, M.P., 2002. Specific [^{3}H]-guanosine binding sites in rat brain membranes. Br. J. Pharmacol. 135, 969–976.
Valera, E., Sánchez-Martín, F., Ferrer-Montiel, A.V., Messeguer, A., Merino, J.M., 2008. NMDA-induced neuroprotection in hippocampal neurons is mediated through the protein kinase A and CREB (cAMP-response element-binding protein) pathway. Neurochem. Int. 53, 148–154.
Vandresen-Filho, S., Herculano, B.A., Franco, J.L., Boeck, C.R., Dafré, A.L., Tasca, C.I., 2007. Evaluation of glutathione metabolism in NMDA preconditioning against quinolinic acid-induced seizures in mice cerebral cortex and hippocampus. Brain Res. 1184, 38–45.
Wardas, J., 2002. Neuroprotective role of adenosine in the CNS. Pol. J. Pharmacol. 54, 313–326.
Zimmermann, H., 1996. Biochemistry, localization and functional roles of ectonucleotidases in the nervous system. Prog. Neurobiol. 49, 589–618.
Zimmermann, H., Braun, N., Kegel, B., Heine, P., 1998. New insights into molecular structure and function of ectonucleotidases in the nervous system. Neurochem. Int. 32, 21–25.

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CAPÍTULO 4

GUANOSINE PROTECTS HUMAN SHSY-5Y NEUROBLASTOMA CELLS AGAINST OXIDATIVE STRESS-INDUCED CELL DEATH VIA PI3K CELL SURVIVAL ACTIVATION

Manuscrito em preparação para ser submetido à Cellular and Molecular Neurobiology

Guanosine protects human SHSY-5Y neuroblastoma cells against oxidative stress-induced cell death via PI3K cell survival activation

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Running Head: guanosine protects SHSY-5Y from oxidative stress

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Key words: guanosine, electron transport chain inhibitors, oxidative stress, ROS, PI3K.

Abbreviations: Akt (protein kinase B); GUO (Guanosine); LY294002 ([2-(4-morpholinyl)-8-phenyl-4H-1benzo-pyran-4-one); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); PD98059 ([2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-4]); PI3K (phosphoinisitide 3 kinase); ROS (reactive oxygen species); Rot/oligo (rotenone plus olygomicin A).

Abstract

Oxidative stress is implicated in cell death induced by distinct neurotoxic situations such as glutamate-, β-amyloid-, or hydrogen peroxide-induced cytotoxicity. In this context, we have investigated damage of undifferentiated human SHSY-5Y neurobastoma exposed to rotenone (30 μM) plus oligomycin A (10 μM) (rot/oligo), an *in vitro* model of mitochondrial ROS production. Moreover, the putative effect of guanosine was evaluated against rot/oligo-induced neurotoxicity. Exposure of neuroblastoma cells to rot/oligo for 24 h induced a 62% decrease in cell viability which was accompanied by an increased ROS production. Guanosine incubated concomitantly with rot/oligo abolished rot/oligo-induced cell death and ROS production in a concentration dependent manner. The neuroprotection afforded by guanosine was not abolished by the MEK inhibitor (PD98059) or by a PKC inhibitor (cheleritrine).

Otherwise, when the PI3K pathway was inhibited by LY294002, neuroprotection as well as guanosine ability to reduce rot/oligo-induced ROS production were abolished, confirming that this pathway plays a role in the neuroprotective effect of guanosine. Our results suggest that guanosine may be an effective pharmacological intervention in diseases which involves oxidative stress-induced cell death, as observed in acute or chronic neurodegenerative diseases.

1. Introduction

Guanosine is a guanine nucleoside which exerts neuroprotective effect in the central nervous system (for a review, see Schmidt et al., 2007 and Rathbone et al., 2008). Guanosine and other guanine nucleotides have been implicated in neuroprotection by exerting trophic effects (Ciccarelli et al., 2001; Decker et al., 2007) as well as by counteracting glutamate excitotoxicity *in vitro* (Molz et al., 2005; Oleskovicz et al., 2008; Molz et al., 2008) and *in vivo* (Schmidt et al., 2000; 2005). Guanosine also protects cultured rat astrocytes from staurosporine-induced apoptosis (Di Iorio et al., 2004) and SH-SY5Y cells from β-amyloid-induced apoptosis (Pettifer et al., 2004). In both cases, the anti-apoptotic effect of guanosine was mediated by stimulation of the phosphatidylinositol-3-kinase (PI3K)/Akt/protein kinase B and mitogen-activated protein kinase (MAPK) cell survival pathways.

Extracellular guanosine may be released from glial cells. Astrocytic cell cultures are able to release guanine nucleotides and guanosine under basal or toxic conditions (Ciccarelli et al., 1999, Ciccarelli et al., 2001). Alternatively, nucleotides such as GTP, GDP and GMP are metabolized by ecto- nucleotidases to extracellular guanosine (Caciagli et al., 2000; Ciccarelli et al., 2001).

Oxidative stress is a common mechanism of cell death in distinct cytotoxic situations such as glutamate (Parfenova et al. 2006), β-amyloid (Tamagno et al. 2006), MPP^+ (Nicotera and Pavrez, 2006), or hydrogen peroxide-induced cytotoxicity (Kim et al. 2005). Oxidative stress is related to neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Mattson and Magnus, 2006) and stroke (Saito et al. 2005). Overproduction of reactive oxygen species (ROS), such as superoxide free radicals or hydrogen peroxide lead to damage of both neurons and astrocytes (Lin and Beal, 2006). In this study, we investigated the possible neuroprotective effect of guanosine against oxidative stress evoked by mitochondrial activity disruption, due to the blockade of mitochondrial complexes I and V with the

combination of rotenone plus oligomycin-A (rot/oligo) (Egea et al., 2007). The putative involvement of MEK/ERK1/2, PKC and PI3K pathways as the possible mechanisms related to the neuroprotection afforded by GUO was also assessed.

We are showing that guanosine in the presence of rot/oligo, prevents oxidative stress-induced cell death and ROS production in SHSY-5Y neuroblastoma cells by a mechanism that involves the activation of the PI3K cell survival pathway.

2. Experimental Procedures

2.1. Materials

Rotenone and oligomycin A were obtained from Sigma (Madrid, Spain). Chelerythrine, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) and [2-(4-morpholinyl)-8-phenyl-4H-1benzo-pyran-4-one hydrochloride (LY294002) were purchased from Tocris (Biogen Científica, Spain). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and penicillin/streptomycin were purchased from GIBCO (Madrid, Spain). 2',7'-dichlorofluorescein diacetate (H_2DCFDA) were obtained from Molecular Probes (Invitrogen, Madrid, Spain).

2.2. Preparation of SHSY-5Y cultures

The neuroblastoma cell line SHSY-5Y was a kind gift from the Centro de Biología Molecular Universidad Autonoma de Madrid/Consejo Superior de Investigaciones Cientificas (Madrid, Spain). SH-SY5Y cells, at passages between 3 and 16 after defreezing, were maintained in a DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, and 50g/ml streptomycin. SH-SY5Y cells were seeded into flasks containing supplemented medium, and they were maintained at 37°C in 5% CO_2, humidified air. Stock cultures were passaged 1:3 twice weekly; i.e., one plate was divided (subcultured or

split) into three plates. This procedure was performed twice a week. For assays, SH-SY5Y cells were subcultured in 48 or 96-well plates at a seeding density of 10^5 cells per well (Cañas et al., 2007). Cells were treated with the drugs in DMEM supplemented with 1% fetal calf serum.

2.3. SHSY-5Y cultures treatment

Cell death was induced by adding the combination of rotenone (30μM) plus oligomycin-A (10μM) (rot/oligo) for 24 h. When present, guanosine was added to SHSY-5Y at the same time as rot/oligo and remained in the culture medium for the duration of the experiment (24 h). In experiments where enzyme inhibitors were tested, SHSY-5Y cells were pre-treated with these agents for 30 min prior to the addition of guanosine, and remained in the incubation medium for the entire duration of the experiment. These treatments included: the potent and selective inhibitor of the PI3K, [2-(4-morpholinyl)-8phenyl-1(4*H*)-benzopyran-4-4hydrochloride] (LY294002; 10μM), or the selective inhibitor of the MAP kinase kinase (MEK), [2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-4] (PD98059; 10μM), or the inhibitor of PKC, cheleritrine (0,1μM). LY294002, PD98059 and cheleritrine were dissolved in and added to the culture medium at a final concentration of 0,01% dimethyl sulfoxide (DMSO).

2.4. Evaluation of cell viability

SHSY-5Y cell viability was evaluated 24 h after rot/oligo or rot/oligo plus guanosine exposure. At the end of each experiment, 3-(4,5-dimethylthiazol-2-yl-diphenyltetrazolium bromide (MTT Sigma Aldrich) was added to each well to a final concentration of 0.5 mg/ml (Mosmann, 1983) ad the plates were incubated for 3 h at 37º C. Then the insoluble formazan

was solubilized by adding dimethyl sulfoxide (DMSO), resulting in a coloured compound which optical density was measured in an ELISA reader (550 nm).

2.5. ROS measurement

To measure cellular ROS, we have used the molecular probe H_2DCFDA (Ha et al. 1997). SHSY-5Y cells were loaded with 10 μM H_2DCFDA which diffuses through the cell membrane and is hydrolyzed by intracellular esterases to the nonfluorescent form dichlorofluorescein (DCFH). DCFH reacts with intracellular H_2O_2 to form dichlorofluorescin (DCF), a green fluorescent dye. Fluorescence was measured in a fluorescence microplate reader (FLUOstar Galaxy). Wavelengths of excitation and emission were 485 and 520 nm, respectively.

2.6. Statistical analysis

Comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by Duncan's test if necessary, with $p < 0.05$ considered to be statistically significant.

3. Results

2.1. Guanosine protects SHSY-5Y neuroblastoma cells against rot/oligo-induced cell death

Treatment of SHSY-5Y neuroblastoma cells for 24 h with 30 μM rotenone plus 10 μM oligomycin A (rot/oligo) induced a decrease of 62% in cellular viability (measured by MTT reduction assay) when compared to cultures incubated under basal conditions. Guanosine *per*

se (0.03-1mM) did not alter cellular viability at any concentration tested (Fig.1A). From now, we have normalized rot/oligo-elicited cell death as 100%.

Guanosine added to the incubation medium at the same time as rot/oligo inhibited rot/oligo-induced cell death in SHSY-5Y neuroblastoma cells in a concentration dependent fashion (Fig.1B). When guanosine 0.3 mM was co-incubated with rot/oligo for 24 h, it significantly reduced cell death induced by rot/oligo, and guanosine 1 mM even more potently inhibited SHSY-5Y neuroblastoma cell death induced by rot/oligo. Thus, we selected the concentration of 1mM, since it caused maximum neuroprotection (40%, $p<0.01$).

2.2. Guanosine protects against rot/oligo-induced cell death by activation of PI3K cell survival pathway

In order to analyze the signaling pathway that could participate in the neuroprotective mechanism of guanosine against rot/oligo-induced cell damage in SHSY-5Y cells, we performed experiments with LY 294002, an inhibitor of the PI3K; PD98059, an inhibitor of MEK; and cheleritrine, an inhibitor of PKC. As shown in Figure 2A, only the inhibitor of PI3K (LY294002, 10μM) partially blocked the neuroprotective effect of guanosine.

Additionally, rot/oligo decreased the number of cells when compared to control cultures and transformed healthy cells, isolated or grouped in clusters of cells, into round chapped cells and many debris. SHSY-5Y neuroblastoma cells co-incubation with 1mM GUO and rot/oligo lead to recovery of initial density and exhibited a healthier appearance. This recovery was prevented by LY204002, as shown in the photomicrographs (Fig. 2B).

2.3. Guanosine prevents rot/oligo-induced ROS production

Since rot/oligo are potent inhibitors of the complex I and V of the respiratory chain, respectively, we measured ROS generation with the fluorescent probe H_2DCFDA. At the end

of the 24 h period with rot/oligo, cells were loaded with 10 μM H_2DCFDA for 20 min. ROS-induced DCF fluorescence was monitored by Spectrophotometric measurement of MTT reduction in order to express the results as percentage of ROS-induced fluorescence in viable cells; The treatment with rot/oligo increased ROS production to 293%. When cells were co-incubated with guanosine and rot/oligo for 24 h, the amount of ROS produced by the cells was reduced to basal levels. LY294002 (10μM) abolished the reduction of ROS elicited by GUO (Fig. 3). These results indicate that guanosine protects SHSY-5Y neuroblastoma cells subjected to mitochondrial oxidative stress trough the activation of PI3K cell survival pathway.

3. Discussion

In the present study we are demonstrating that guanosine, when co-incubated with rot/oligo, prevents oxidative-stress induced cell death and ROS production in SHSY-5Y neuroblastoma cells through the activation of the PI3K survival signaling pathway.

The oxidative stress model used in this study consists in promoting mitochondrial disruption by blocking mitochondrial complexes I and V with the combination of 30 μM rotenone plus 10μM oligomycin-A (rot/oligo), respectively (Egea et al., 2007). As a result of mitochondrial disruption the cell cannot further synthesize ATP, and free radicals are generated beyond the capacity of the cells to buffer them, and, ultimately, the cell dies. Rot/oligo profoundly affected cellular viability of SHSY-5Y neuroblastoma cells, as observed by a significant reduction in cellular viability measured by the MTT reduction assay (Fig. 1A), as well as by morphological alterations consistent with cell death (Fig. 2B). Guanosine inhibited the cellular viability reduction (Fig. 1B) as well as the morphological changes induced by rot/oligo in a concentration-dependent manner (Fig 2B). There are data in the literature demonstrating that guanosine stimulates the prolifieration of astrocytes, oligodendocytes and PC12 cells (Ciccarelli et al., 2001; Jiang et al., 2008). Guarnieri and co-

workers (2009) have recently demonstrated that the effects of guanosine on cell proliferation (measured by the MTT reduction assay) in SHSY-5Y neuroblastoma cells were only observed after 4 days of guanosine (0.03-1mM) exposure. In our study, besides methodological approach is not the standard in order to evaluate cell proliferation (MTT reduction assay) the absence of increasing MTT reduction in the presence of guanosine alone at any concentration tested, should indicate the neuroprotective effect of GUO against rot/oligo-induced SHSY-5Y cell death is not due to its effect on cell proliferation (Fig.1A).

Activation of PI3K, MAPK or PKC cascades are key elements of signal transduction involved on cell proliferation, differentiation and stress response (Cantley, 2002, Cañas et al., 2007). We have previously shown that guanosine-induced trophic effect as the alteration of extracelular matrix protein organization in astrocyte/neuron co-cultures is mediated by the activation of MAPK and PKC (Decker et al., 2007). Based on these evidences, we looked for the involvement of PI3K, MEK/ERK1/2 and PKC, as possible survival pathways involved in the neuroprotective effect of guanosine against rot/oligo-induced reduction in cellular viability. The inhibition of PI3K pathway by LY294002 partially abolished the neuroprotective effect of guanosine (Fig. 2A) as well as the maintenance of initial density and the morphological characteristics of SHSY-5Y neuroblastoma cells in culture elicited by guanosine (Fig. 2B). Otherwise, neither MEK nor PKC inhibition altered the protective effect of guanosine, indicating that these signalling pathways are not involved in the neuroprotective effect of guanosine (Fig. 2A).

Pre-incubation of cultured astrocytes or SHSY-5Y neuroblastoma cells with guanosine can lead to a rapid activation of PI3K/Akt and MAPK cell survival pathways and these play a critical role in protecting these cells against cell death (Di Iorio er al., 2004; Pettifer et al., 2007). However, when guanosine was co-incubated with the toxic agent, only PI3K pathway was involved in the neuroprotective effect of guanosine (Pettifer et al., 2007). Taken together,

these results indicate that PI3K pathway may be the major cell survival pathway evoked by guanosine-induced neuroprotection.

Rot/oligo-induced cell death and ROS production were inhibited by guanosine by a mechanism that involves PI3K cell signalling pathway (Fig. 2 and 3). It has been shown that MPP^+-induced SHSY-5Y cell death (Pettifer et al., 2007) is related to inhibition of the complex I of the electron transport chain in mitochondrial and ultimately causes neuronal cell death by a mechanism that most likely involve oxidative stress (Lin and Beal, 2006), but in the study of Petifer et al (2007) the authors did not looked for the effect of guanosine in ROS production. So, we are demonstrating for the first time that the neuroprotective effect of guanosine is directly mediated by its ability to inhibit ROS production elicited by an impairment of the mitochondrial electron transport, which is a broadly common mechanism of numerous neurodegenerative diseases that affect the central nervous system.

Jurkowitz (1998) and Litzki (1999) have suggested that the neuroprotective effect of guanosine against mitochondrial inhibition was intracellular. Otherwise, posterior studies demonstrated that guanosine-induced neuroprotection was not inhibited in the presence of dypiridamole (a nucleoside transporter inhibitor) indicating that the effect of guanosine was extracellular (Di Iorio et al., 2004; Decker et al., 2007). Guanosine receptors are not fully characterized in the CNS, but evidences showed a specific binding site for guanosine in astrocytes membranes (Traversa et al., 2002). Our present results show that guanosine when co-incubated with rot/oligo-treated neuroblastoma SHSY-5Y cells promoted their survival by inhibiting ROS production and oxidative stress. Oxidative stress is a common mechanism of many neurodegenerative diseases including ischemia, Alzheimer Disease, Parkinson Disease and amyotrophic lateral sclerosis. So, endogenous compounds that protect against oxidative stress can be considered important strategies to protect neurons in central nervous system pathologies.

References

Caciagli, F., Di Iorio, P., Giuliani, P., Middlemiss, M. P., Rathbone, M. P. (2000). The neuroprotective activity of guanosine involves the production of trophic factors and the outflow of purines from astrocytes. Drug. Dev. Res. 50: 32.

Cañas, N., Valero T., Villarroya, M., Montell, E., Vergés J., García, A.G., López M.G. (2007). Chondroitin Sulfate Protects SH-SY5Y Cells from Oxidative Stress by Inducing Heme Oxygenase-1 via Phosphatidylinositol 3-Kinase/Akt. J. Pharmacol .Exp. Ther. 323:946-953.

Cantley, LC. (2002). The phosphoinositide 3-kinase pathway. Science 296:1655–1657.

Ciccarelli, R., Ballerini, P., Sabatino, G., Rathbone, M.P., D'Onofrio, M., Caciagli, F., Di Iorio, P. (2001). Involvement of astrocytes in purine-mediated reparative processes in the brain. Int. J. Dev. Neurosci. 19:395-414.

Ciccarelli, R., Di Iorio, P., Giuliani, P., D'Alimonte, I., Ballerini, P., Caciagli, F., Rathbone, M.P. (1999). Rat cultured astrocytes release guanine-based purines in basal conditions and after hypoxia/hypoglycemia. Glia 25:93-98.

Decker, H., Francisco, S.S., Mendes-de-Aguiar C.B., Romão L.F., Boeck C.R., Trentin A.G., Moura-Neto V., Tasca C.I. (2007). Guanine derivatives modulate extracellular matrix proteins organization and improve neuron-astrocyte co-culture. J. Neurosci. Res. 85:1943-1951.

Di Iorio, P., Ballerini, P., Traversa, U., Nicoletti, F., D'Alimonte, I., Kleywegt, S., Werstiuk, E.S., Rathbone, M.P., Caciagli, F., Ciccarelli, R. (2004). The antiapoptotic effect of guanosine is mediated by the activation of the PI 3-kinase/AKT/PKB pathway in cultured rat astrocytes. Glia 46:356-368.

Egea, J., Rosa, A.O., Cuadrado, A., García, A.G., López, M.G. (2007). Nicotinic receptor activation by epibatidine induces heme oxygenase-1 and protects chromaffin cells against oxidative stress. J. Neurochem. 02:1842-52.

Guarnieri, S., Pilla, R., Morabito, C., Sacchetti, S., Mancinelli, R., Fanó, G., Mariggio, M.A. (2009). Extracellular guanosine and GTP promote expression of differentiation markers and induce S-phase cell-cycle arrest in human SH-SY5Y neuroblastoma cells. Int. J. Dev. Neurosci. 27:135-147.

Ha, H. C., Woster, P. M., Yager, J. D., Casero, R. A. Jr (1997). The role of polyamine catabolism in polyamine analogue-induced programmed cell death. Proc. Natl Acad. Sci. U. S. A. 94:11557-11562.

Jiang, S., Ballerini, P., Buccella, S., Giuliani, P., Jiang, C., Huang, X., Rathbone, M.P. (2008). Remyelination after chronic spinal cord injury is associated with proliferation of endogenous adult progenitor cells after systemic administration of guanosine. Purinergic Signal. 4:61-71.

Jurkowitz, M. S., Litsky, M. L., Browning, M. l., Hohl, C. M. (1998). Adenosine, inosine, and guanosine protect glial cells during glucose deprivation and mitochondrial inhibition: correlation between protection and ATP preservation. J. Neurochem. 71:535-548.

Kim, Y. S., Zhuang, H., Koehler, R. C., Dore S. (2005). Distinct protective mechanisms of HO-1 and HO-2 against hydroperoxideinduced cytotoxicity. Free Radic. Biol. Med. 38:85-92.

Lin, M.T., Beal, M.F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 443:787-795.

Litsky, M. L., Hohl, C. M., Lucas, J. H., Jurkowitz, M. S. (1999). Inosine and guanosine preserve neuronal and glial viability in mouse spinal cord cultures during chemical hypoxia. Brain Res. 821:426-432.

Mattson, M.P., and Magnus, T. (2006). Ageing and neuronal vulnerability. Nat. Neurosci. 7:278-294.

Molz, S., Oliveira, I.J.L., Decker, H., Souza, D.O., Tasca, C.I. (2005). Neurotoxicity induced by glutamate in glucose-deprived rat hippocampal slices is prevented by GMP. Neurochem. Res. 30:83-89.

Molz, S., Dal-Cim, T., Decker, H., Tasca, C.I. (2008). GMP prevents excitotoxicity mediated by NMDA receptor activation but not by reversal activity of glutamate transporters in rat hippocampal slices. Brain Res. 22:113-120.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity. J. Immunol. Met. 65:55-63.

Nicotra, A., Pavrez, S.H. (2000). Cell death induced by MPTP, a substrate for monoamine oxidase B. Toxicology 153:157-166.

Oleskovicz, S.P., Martins, M.C., Leal, R.B., Tasca, C.I. (2008). Mechanism of guanosine-induced neuroprotection in rat hippocampal slices submitted to oxygen-glucose deprivation. Neurochem. Int. 52:411-418.

Parfenova, H., Basuroy, S., Bhattacharya, S., Tcheranova, D., Qu, Y., Regan, R. F. Leffler, C. W. (2006). Glutamate induces oxidative stress and apoptosis in cerebral vascular endothelial cells: contributions of HO-1 and HO-2 to cytoprotection. Am. J. Physiol., Cell Physiol. 290:C1399-C1410.

Pettifer, K.M., Kleywegt, S., Bau, C.J., Ramsbottom, J.D., Vertes, E., Ciccarelli, R., Caciagli F, Werstiuk ES, Rathbone MP. (2004). Guanosine protects SH-sy5y cells against beta-amyloid-induced apoptosis. Neuroreport 15:833-836.

Pettifer, K.M., Jiang, S., Bau, C., Ballerini, P., D'Alimonte, I., Werstiuk, E.S., Rathbone, M.P. (2007). MPP^{+}-induced cytotoxicity in neuroblastoma cells: Antagonism and reversal by guanosine. Purinergic Signal. 3:399-409.

Rathbone, M.P., Pilutti, L., Caciagli, F., Jiang, S. (2008). Neurotrophic effects of extracellular guanosine. Nucleosides Nucleotides Nucleic Acids 27:666-672.

Saito, A., Maier, C.M., Narasimhan, P., Nishi, T., Song, Y.S., Yu, F., Liu, J., Lee, Y.S., Nito, C., Kamada, H., Dodd, R.L., Hsieh, L.B., Hassid, B., Kim, E.E., González, M., Chan, P.H. (2005). Oxidative stress and neuronal death/survival signaling in cerebral ischemia. Mol. Neurobiol. 31:105-116.

Schmidt, A.P., Lara, D.R., Maraschin, J.F., Perla, A.S., Souza, D.O. (2000). Guanosine and GMP prevent seizures induced by quinolinic acid in mice. Brain Res. 864:40-43.

Schmidt, A.P., Avila, T.T., Souza, D.O. (2005). Intracerebroventricular guanine-based purines protect against seizures induced by quinolinic acid in mice. Neurochem. Res. 30:69-73.

Schmidt, A.P., Lara, D.R., Souza, D.O. (2007). Proposal of a guanine-based purinergic system in the mammalian central nervous system. Pharmacol. Ther. 116:401-416.

Tamagno E., Bardini P., Guglielmotto M., Danni O. and Tabaton M. (2006). The various aggregation states of beta-amyloid 1-42 mediate different effects on oxidative stress, neurodegeneration, and BACE-1 expression. Free Radic. Biol. Med. 41:202-212.

Traversa, U., Bombi, G., Di Iorio, P., Ciccarelli, R., Werstiuk, E.S., Rathbone, M.P. (2002). Specific [(3)H]-guanosine binding sites in rat brain membranes. Br J Pharmacol. 135:969-76.

Figure 1A

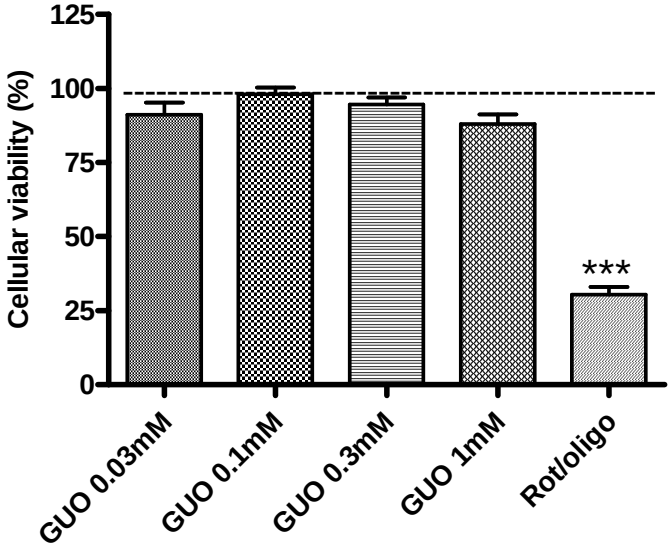

Figure 1B

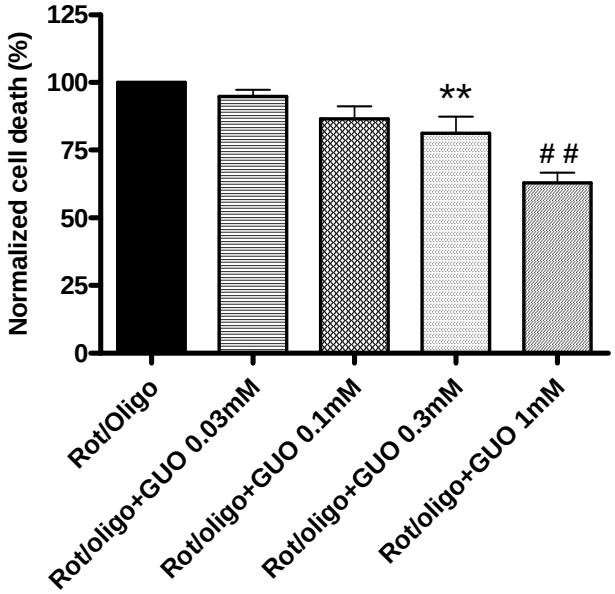

Figure 1. Guanosine protects against cell death induced by Rot/oligo in SHSY-5Y neuroblasmtoma cells: (A) SHSY-5Y neuroblastoma cells were incubated for 24 h with DMEM (basal), with increased concentrations of GUO (0.03-1mM) or with 30 μM rotenone plus 10 μM oligomycin A. Cellular viability was evaluated by the MTT reduction assay and is expressed as percentage of control cells (basal) which represents cells incubated for 24 h in culture medium (100% cellular viability). Data are mean ± SEM of five different cell batches. *** $p < 0.001$, represents means significantly different from control cells. **(B)** Concentration-response curve of guanosine co-incubated for 24 h with Rot/oligo. Cell death was normalized in each individual experiment, as percentage of the maximum cell death (Rot/oligo) that was considered as 100% (black column). Data are mean ± SEM of five different cell batches from five different cultures. **$p < 0.01$ represents means significantly different from Rot/oligo and Rot/oligo+GUO 1mM and ##$p < 0.01$ represents means significantly different from all other groups.

Figure 2A

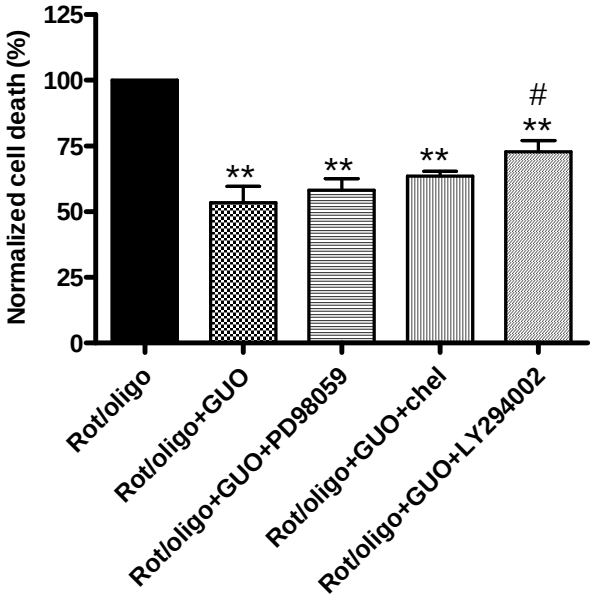

Figure 2B

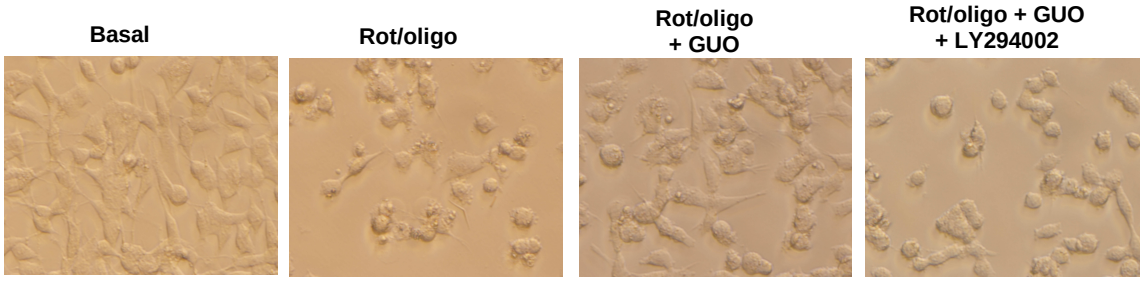

Figure 2. Signalling pathways involved in the neuroprotective effect of Guanosine against cell death induced by Rot/oligo in SHSY-5Y neuroblastoma cells. (A) SHSY-5Y neuroblastoma cells were incubated for 24 h with with 30 μM rotenone plus 10 μM oligomycin A (rot/oligo) with or without 1 mM guanosine (GUO). Cells were pre-incubated for 30 min with 10 μM PD98059 (MEK inhibitor), 0.1 μM cheleritrine (chel) (PKC inhibitor) or 10 μM LY294002 (PI3K inhibitor) and subsequently co-incubated 24 h with 1 mM guanosine and Rot/oligo. Cell death was normalized in each individual experiment, as percentage of the maximum cell death (Rot/oligo) which was considered as 100% (black column). Data are mean ± SEM of five different cell batches from five different cultures. ** $p< 0.01$ represents means significantly different from Rot/oligo; # $p < 0.05$ represents means significantly different from Rot/oligo+GUO. **(B)** Photomicrographs of control SHSY-5Y cells (Basal); cells exposed 24 h to Rot/oligo; cells co-incubated 24 h with 1mM guanosine and Rot/oligo (Rot/oligo+GUO) and cells pre-incubated for 30 minutes with 10μM LY294002 and subsequently co-incubated 24 h with 1mM guanosine and Rot/oligo (Rot/oligo+GUO+LY294002). Image magnification (40X).

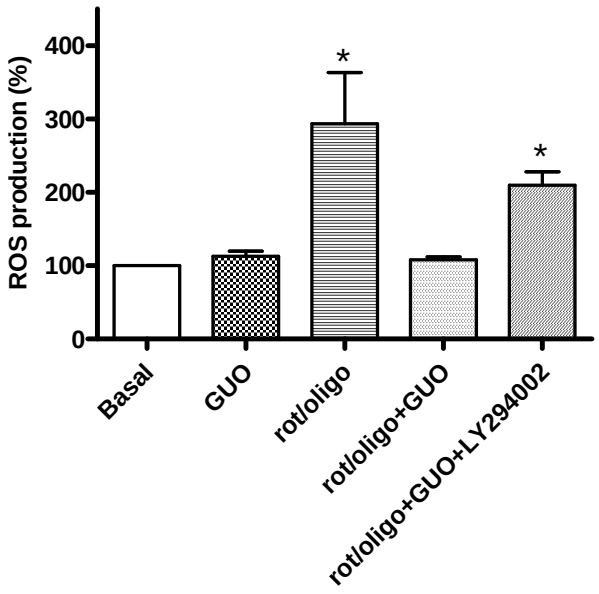

Figure 3. Guanosine inhibits mitochondrial ROS production elicited by Rot/oligo in SHSY-5Y neuroblastoma cells. SHSY-5Y neuroblastoma cells were incubated for 24 h with with 30μM rotenone plus 10μM oligomycin A (Rot/oligo) with or without 1mM guanosine (GUO). Cells were pre-incubated for 30 min with 10 μM LY294002 (PI3K inhibitor) and subsequently co-incubated 24 h with 1mM guanosine and Rot/oligo. Data are mean ± SEM of four different cell batches from four different cultures. * $p < 0.05$ represents means significantly different from all other groups.

CAPÍTULO 5

AVALIAÇÃO DO EFEITO NEUROPROTETOR DA GUANOSINA FRENTE À TOXICIDADE INDUZIDA PELO PEPTÍDIO β-AMILÓIDE EM CULTURAS DE CÉLULAS DE NEUROBLASTOMA HUMANO (SHSY-5Y)

Resultados preliminares

Estes resultados foram desenvolvidos no Departamento de Farmacologia da Universidade Autônoma de Madri, no laboratório 3 - Instituto Teófilo Hernando, sob coordenação da Prof[a]. Dra Manuela G. López

Introdução

A Doença de Alzheimer é uma enfermidade caracterizada por progressiva deterioração da memória e cognição apresentando mudanças neuropatológicas específicas, incluindo placas senis, emaranhados neurofibrilares e prejuízo nas sinapses (HARDLY & SELKOE, 2003). As placas senis são depósitos extracelulares de fibrilas e agregados amorfos de peptídeo β-amilóide (Aβ). Gu et al. (2004) demonstraram que o peptídeo $A\beta_{25–35}$ inibe fortemente a atividade de transportadores de glutamato e da Na^+-K^+-ATPase o que resulta em elevadas concentrações neurotóxicas de glutamato. Além disso, $A\beta_{25–35}$ causa estresse oxidativo, gerando radicais livres e produtos da peroxidação lípidica (ZENG et al., 2004; PIERMARTIRI, 2009) que são capazes de danificar a mitocôndria e inibir transportadores de glutamato (LAUDERBACK et al., 2001; PIERMARTIRI, 2009), correlacionando assim a cascata glutamatérgica de morte celular com o desenvolvimento da Doença de Alzheimer.

A morte celular induzida pelo peptídeo $A\beta_{25-35}$ já foi previamente demonstrada em culturas de células não diferenciadas de neuroblastoma humano (SHSY-5Y) (PETTIFER et al., 2004), porém, os efeitos deste peptídeo em células de neuroblastoma humano SHSY-5Y diferenciadas ainda não foi determinada. A diferenciação destas células para um fenótipo mais próximo de um fenótipo neuronal pode ser realizado através do tratamento das culturas de SH-SY5Y com ácido retinóico (10μM – 5 dias) seguido da incubação com BDNF (1ng/ml – 3 dias) (ENCINAS et al., 2000).

Os nucleotídeos e o nucleosídeo derivados da guanina desempenham um importante papel extracelular na modulação da transmissão glutamatérgica, apresentam efeitos sobre a memória e o comportamento e ainda efeitos tróficos em célula neurais (SCHMIDT et al., 2007). Assim como descrito anteriormente, estudos recentes também demonstraram um importante papel protetor da GUO em modelos de doenças neurodegenerativas (D'ALIMONTE et al., 2007; SU et al., 2009) via ativação da via PI3K/Akt (PETTIFER et al.,

2004; PETTIFER et al., 2007), porém, o efeito da GUO frente ao dano causado pelo peptídeo Aβ em culturas diferenciadas de células SH-SY5Y ainda não foi avaliado.

O objetivo desse trabalho foi analisar o efeito do peptídeo β-amilóide ($A\beta_{25-35}$) em culturas de células não-diferenciadas e diferenciadas de neuroblastoma SH-SY5Y e também avaliar o possível efeito protetor de GUO frente à toxicidade do $A\beta_{25-35}$ nestas culturas.

Materiais e Métodos

Reagentes:

Peptídeo $A\beta_{25-35}$ foi obtido da Sigma. Meio Dulbecco's modificado (DMEM), soro bovino fetal (SBF), penicilina e estreptomicina foram obtidos da GIBCO (Madrid, Spain).

Preparação das culturas de SHSY-5Y:

A linhagem de neuroblastoma SHSY5Y foi gentilmente fornecida pelo Centro de Biología Molecular Universidad Autonoma de Madrid/Consejo Superior de Investigaciones Cientificas (Madrid, Spain). As culturas de células SH-SY5Y foram mantidas em meio DMEM suplementado com 10% de soro bovino fetal (SBF), 2 mM glutamina, 50 unidades/ml penicilina, e 50g/ml estreptomicina e mantidas a 37°C em 5% CO_2, ar umidificado. As culturas estoques eram subcultivadas 1:3 duas vezes por semana; ou seja, um frasco era dividido em três outros frascos. Este procedimento foi realizado duas vezes por semana.

Cultivo e diferenciação das células SHSY-5Y:

Quando as células SHSY-5Y não foram submetidas à diferenciação, estas foram cultivadas em placas de 48 poços a uma densidade de 100.000 células/poço. Quando as células foram submetidas à diferenciação, a densidade celular foi de 30.000 células/poço (ENCINAS et al., 2000). Para a análise morfológica, as células foram cultivadas em placas de

6 poços a uma densidade de 8.000 células/poço para a análise morfológica. As células foram cultivadas por 24 horas em meio de cultura DMEM contendo 10% SBF e após este período, foram submetidas ou não á diferenciação.

A diferenciação das culturas de SH-SY5Y se deu pela incubação das células com de ácido retinóico (10μM) em meio de cultura DMEM contendo 15% SBF por 5 dias. Após este período, o meio era retirado e substituído por meio de cultura DMEM sem SBF contendo 1ng/ml de BDNF e as culturas foram incubadas por mais 3 dias (ENCINAS et al., 2000).

Tratamento das células SHSY-5Y:

As culturas de células SHSY-5Y diferenciadas e não-diferenciadas foram tratadas com concentrações crescentes do peptídeo $A\beta_{25-35}$ (3, 10 e 30μM) por um período de 24h. Antes de ser utilizado nas culturas, o peptídeo $A\beta_{25-35}$ foi solubilizado em água a uma concentração final de 1mM e posteriormente mantido a 37 ^{o}C por 48h para permitir a sua oligomerização (SCHUSTER et al., 2005). A avaliação do efeito da GUO se deu através da pré-incubação das culturas de células SHSY-5Y diferenciadas com concentrações crescentes de GUO (0,03, 0,1, 0,3, 3 e 1 mM) por um período de 24 horas antes da adição do peptídeo $A\beta_{25-35}$ e GUO permaneceu durante as 24 horas de exposição ao peptídeo $A\beta_{25-35}$.

Análise morfológica e imunofluorescência:

As células foram fixadas com paraformaldeído 4% por 10 minutos e cuidadosamente lavadas com tampão fosfato (PBS). Posteriormente, as células foram permeabilizadas com triton X-100 a 0,1% por 10 minutos. As células foram então bloqueadas com uma solução 3% albumina bovina, diluída em PBS e posteriormente incubadas por 12h com os anticorpos específicos diluídos em solução de bloqueio, a 4^{o}C. Foi utilizado anticorpo primário anti MAP-2 (1:500), seguido de 3 lavagens com PBS e incubação com anticorpo secundário (FITC-anti-mouse IgG, 1/100) .

Avaliação da viabilidade celular:

A viabilidade celular foi avaliada pelo método de redução do MTT (3-[4,5-Dimetiltiazol-2-il]-2,5-difenil-tetrazolium brometo = Thiazolyl blue). O MTT é um sal de tetrazolium solúvel em água, que é convertido em um formazam púrpura após clivagem do anel de tetrazólio por desidrogenases mitocondriais (MOSMANN, 1983). Após o período de 24 horas de exposição ao peptídeo $A\beta_{25-35}$, as células SHSY-5Y foram incubadas com MTT (0,5 mg/ml) à 37 ^{o}C por 3h e o formazam reduzido foi solubilizado pela adição de DMSO. A viabilidade celular foi proporcional à leitura da absorbância medida em leitora de Elisa (550 nm).

Análise estatística

Os resultados obtidos foram avaliados através da análise de variância de uma via (ANOVA), seguido do Teste de Duncan, quando necessário. Os resultados foram considerados significativos quando $p < 0,05$.

Resultados

Diferenciação das culturas de células SHSY-5Y:

A Figura 1 mostra a análise morfológica e a marcação com MAP-2 nas células SHSY-5Y não diferenciadas e diferenciadas. As células SHSY-5Y foram diferenciadas através da incubação com ácido retinóico e BDNF. As células SHSY-5Y não diferenciadas foram mantidas em meio de cultura D-MEM contendo 10% SBF durante todo o período. Pode-se verificar que a aplicação de ácido retinóico e BDNF às células SHSY-5Y promoveu a diferenciação destas células, uma vez que houve um aumento significativo da emissão de prolongamentos e formação de contatos entre as células (Fig. 1B) quando comparado com as células não tratadas com ácido retinóico e BDNF (Fig.1A). A imunomarcação com MAP-2

também demonstrou maior intensidade de marcação nas células SHSY-5Y diferenciadas, principalmente nos prolongamentos celulares (Fig. 1D) quando comparado às células SHSY-5Y não diferenciadas (Fig. 1C).

Avaliação da toxicidade do peptídeo $A\beta_{25-35}$ nas culturas de células SHSY-5Y não diferenciadas e diferenciadas:

Primeiramente avaliou-se a toxicidade do peptídeo $A\beta_{25-35}$ nas células SHSY-5Y não diferenciadas. Para tanto, vinte e quatro horas após o plaqueamento das células, o meio de cultura foi retirado e substituído por novo meio de cultura sem soro, contendo concentrações crescentes do peptídeo $A\beta_{25-35}$ (3, 10 e 30 μM). As culturas de SHSY-5Y não diferenciadas foram então incubadas por 24 horas e a viabilidade celular avaliada pelo método de redução do MTT. Pode-se verificar na figura 2 que nenhuma das concentrações testadas afetou a viabilidade celular das células SHSY-5Y.

A toxicidade do peptídeo $A\beta_{25-35}$ também foi avaliada nas células SHSY-5Y que foram diferenciadas com ácido retinóico e BDNF. Neste caso, após a diferenciação das culturas, o peptídeo $A\beta_{25-35}$ foi adicionado nas culturas e estas incubadas por 24 horas adicionais. Após este período, a viabilidade celular foi avaliada pelo método do MTT. Verificou-se que o peptídeo $A\beta_{25-35}$ nas concentrações de 10 e 30μM causa redução significativa da viabilidade celular (29% e 20%, respectivamente) (Fig. 3).

Avaliação do efeito neuroprotetor da GUO frente à toxicidade do peptídeo $A\beta_{25-35}$ em células SHSY-5Y diferenciadas:

Uma vez que a diferenciação das células SHSY-5Y para um fenótipo mais neuronal torna estas células mais susceptíveis ao dano com o peptídeo $A\beta_{25-35}$, e que não houve diferença significativa no dano celular produzido por 10 ou 30μM de $A\beta_{25-}$35, verificamos a capacidade da GUO em proteger estas células contra a perda de viabilidade celular induzida

pelo peptídeo $A\beta_{25-35}$ (10μM). Para tanto, após a diferenciação com ácido retinóico e BDNF, as culturas foram pré-incubadas por um período de 24 horas com concentrações crescentes de GUO (0,03, 0,1, 0,3, 3 e 1 mM) e posteriormente tratadas com o peptídeo $A\beta_{25-35}$. Pode-se observar que GUO, nas concentrações de 0,1 e 0,3 mM, protege parcialmente enquanto que GUO 1mM protege totalmente as células SHSY-5Y da toxicidade do peptídeo $A\beta_{25-35}$ (Fig. 4).

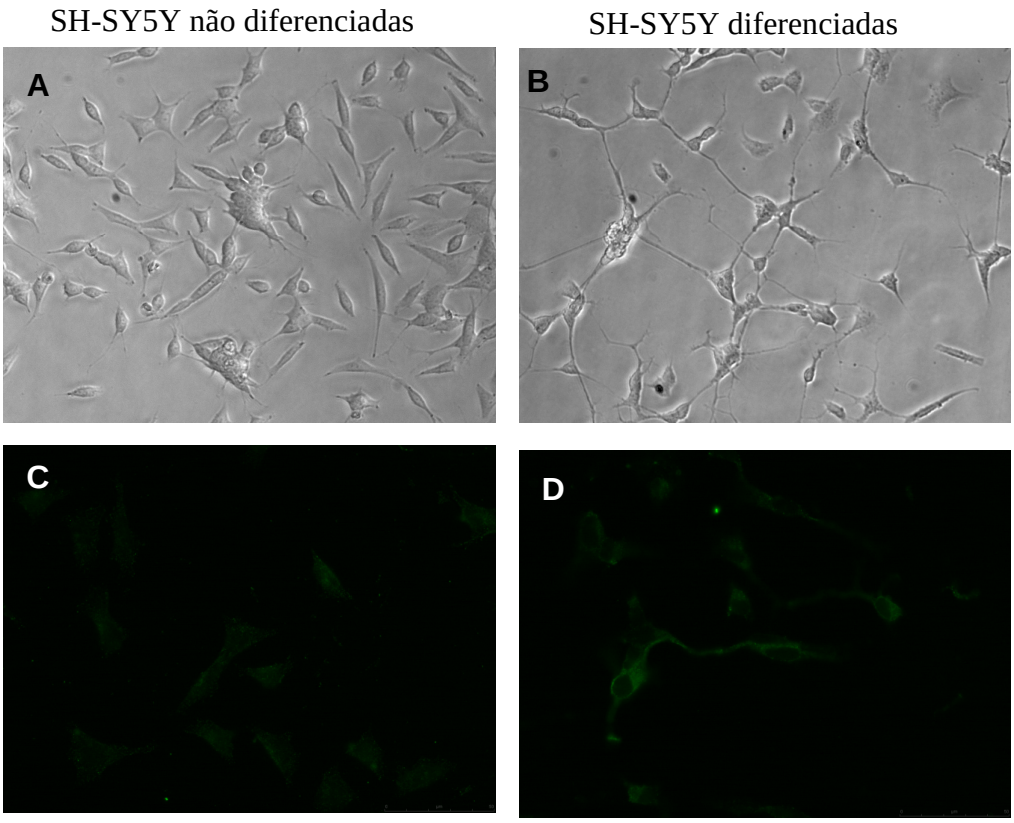

Figura 1. Avaliação morfológica das culturas de SHSY-5Y diferenciadas e não diferenciadas. As culturas de SHSY-5Y foram incubadas na presença ou não de ácido retinóico (10μM- 5 dias) e BDNF (1ng/ml – 3 dias). Posteriormente, as células SHSY-5Y não diferenciadas e diferenciadas foram submetidas à imunomarcação com MAP-2 e visualizadas em microscópio de fluorescência.

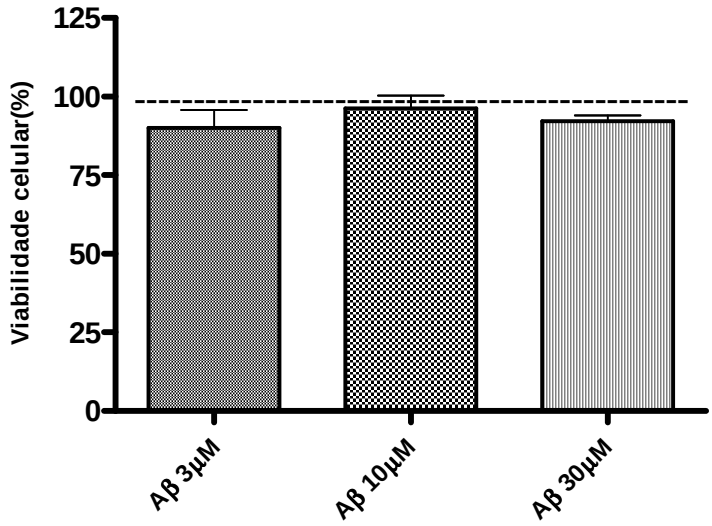

Figura 2. Avaliação da viabilidade celular de células SHSY-5Y não diferenciadas tratadas com o peptídeo $A\beta_{25-35}$. As células SHSY-5Y foram incubadas por 24 horas na presença de 3, 10 ou 30 μM de $A\beta_{25-35}$. Após este período, a viabilidade celular foi avaliada pelo método do MTT. O grupo controle foi considerando 100% e representa a viabilidade celular das células SHSY-5Y incubadas apenas em meio de cultura. Os valores representam a média $\pm$ erro padrão de 4 experimentos realizados em triplicatas.

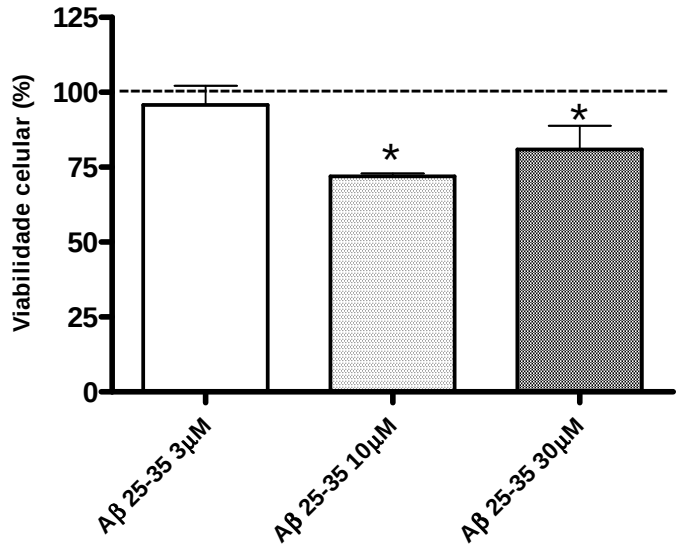

Figura 3. Avaliação da viabilidade celular de células SHSY-5Y diferenciadas tratadas com o peptídeo $A\beta_{25-35}$. As células SHSY-5Y diferenciadas foram incubadas por 24 horas na presença de 3, 10 ou 30 μM de $A\beta_{25-35}$. Após este período, a viabilidade celular foi avaliada pelo método do MTT. O grupo controle foi considerando 100% e representa a viabilidade celular das células SHSY-5Y diferenciadas incubadas apenas em meio de cultura. Os valores representam a média $\pm$ erro padrão de 3 experimentos realizados em triplicatas. * indica médias significativamente do controle; $p<0,05$.

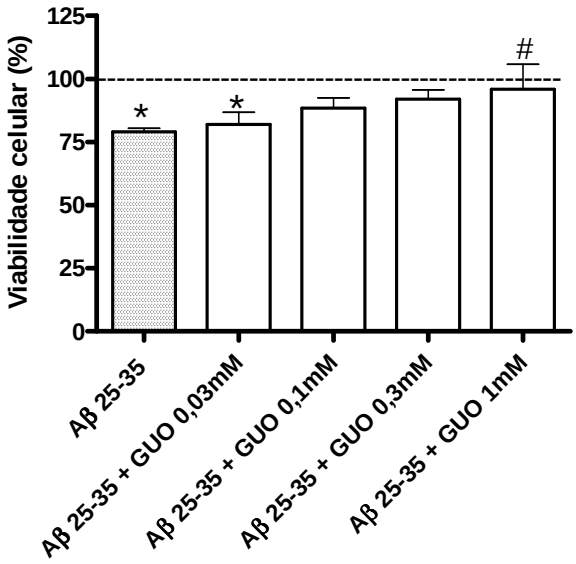

Figura 4. Avaliação do efeito neuroprotetor da guanosina (GUO) frente à toxicidade induzida pelo peptídeo $A\beta_{25-35}$. As células SHSY-5Y diferenciadas foram pré-incubadas com concentrações crescentes de GUO (0,03, 0,1, 0,3 3 1mM) por 24 horas e posteriormente submetidas a 24 horas de incubação com 10 μM de $A\beta_{25-35}$. GUO permaneceu no meio de cultura durante o período de exposição ao $A\beta_{25-35}$. Após este período, a viabilidade celular foi avaliada pelo método do MTT. O grupo controle foi considerando 100% e representa a viabilidade celular das células SHSY-5Y diferenciadas e incubadas por 48 horas em meio de cultura. Os valores representam a média $\pm$ erro padrão de 5 experimentos realizados em triplicatas. * indica médias significativamente do controle; # indica médias significativamente diferente de $A\beta_{25-35}$; $p<0,05$.

DISCUSSÃO

O aminoácido glutamato é considerado o principal neurotransmissor excitatório do SNC de mamíferos. Desempenha importante papel na manutenção de aspectos funcionais do cérebro, tais como: cognição, aprendizagem, memória, indução sináptica, migração e diferenciação. Entretanto, quando ocorre algum desequilíbrio da transmissão glutamatérgica, a concentração de glutamato pode se elevar na fenda sináptica e causar a super-estimulação dos receptores de glutamato, culminando com a morte de células do SNC. Existe uma correlação direta entre a disfunção dos transportadores de glutamato, aumento da concentração do neurotransmissor na fenda sináptica e desenvolvimento de dano celular excitotóxico tanto em modelos de estudo *in vivo* e *in vitro* (MARAGAKIS & ROTHSTEIN, 2004). A disfunção do transporte de glutamato pode ser observada em situações em que ocorre desequilíbrio iônico, diminuição nos níveis de ATP intracelular e estresse oxidativo. Nestes casos, a atividade dos transportadores pode estar diminuída, comprometendo assim a retirada do glutamato da fenda sináptica, ou o transportador pode estar funcionando de maneira reversa, ou seja, contribuir para a liberação de glutamato (ROSSI ET AL., 2000; BONDE ET AL., 2003).

O acúmulo de glutamato na fenda sináptica decorrente desses processos pode então ativar de maneira excessiva os receptores de glutamato, culminando na morte celular excitotóxica. O hipocampo é uma das estruturas cerebrais mais susceptíveis à excitotoxicidade, e a perda de neurônios hipocampais está relacionada à patogênese de doenças degenerativas agudas e crônicas. Dessa forma, um dos focos de interesse deste trabalho foi estudar os mecanismos envolvidos na morte celular excitotóxica utilizando-se fatias de hipocampo de ratos. Além disso, verificamos a interação dos derivados da guanina (GMP e GUO) na morte celular excitotóxica, pois investigamos o efeito protetor destes compostos frente à excitotoxicidade, como também o possível efeito deletério destes

compostos e a sua correlação com a excitotoxicidade. Outro aspecto abordado neste estudo foi o efeito da GUO em outras situações envolvidas em processos neurodegenerativos, como por exemplo, o estresse oxidativo mitocondrial e a morte neuronal induzida pelo peptídeo Aβ.

No primeiro capítulo deste estudo, demonstramos que glutamato e NMDA induzem morte celular com característica de apoptose em fatias de hipocampo de ratos, pois a redução da viabilidade celular induzida por estes compostos foi acompanhada de fragmentação do DNA (Figs. 1, 2, 3 e 4, capítulo 1). Neste trabalho, também estamos demonstrando que concentrações tóxicas de glutamato, mas não NMDA, pode ocasionar a reversão dos transportadores de glutamato e que este pode ser um mecanismo adicional de toxicidade glutamatérgica em fatias de hipocampo de ratos (Figs. 5 e 6, capítulo 1). Portanto, a toxicidade induzida por NMDA ocorre pela ativação de seus receptores e não envolve a reversão dos transportadores de glutamato. Os transportadores de glutamato são vulneráveis ao aumento das espécies reativas de oxigênio que ocorrem após um insulto excitotóxico (TROTTI et al., 1998), causando uma disfunção do transporte de glutamato que pode ser parcialmente revertida pela utilização de agentes redutores (BRONGHOLI et al., 2006).

A redução da viabilidade celular e a fragmentação de DNA induzida por NMDA foram completamente bloqueadas na presença de GMP 1mM (Figs. 2 e 4, capítulo 1) um derivado da guanina endógeno que age como antagonista de receptores NMDA (BARON et al., 1989). Entretanto, GMP 1mM não foi capaz de prevenir a perda de viabilidade celular induzida por glutamato (que também agiu via estimulação do transporte reverso) (Figs. 1 e 3, capítulo 1), sugerindo assim que o efeito protetor do GMP só é evidente quando o dano celular é induzido pela estimulação de receptores NMDA.

Estudos prévios demonstram a capacidade neuroprotetora do GMP (1mM) frente à privação de glicose e oxigênio, porém neste caso, o transporte de glutamato não foi avaliado (OLIVEIRA et al., 2002) ou não foi alterado (MOLZ et al., 2005). Considerando que GMP

pode ser hidrolisado pela ação da enzima 5´-ectonucleotidase (ZIMMERMANN, 1996), não se pode excluir completamente a participação da GUO como mediadora dos efeitos protetores do GMP frente à estimulação de receptores NMDA. Entretanto, estudos realizados em nosso laboratório demonstraram que o efeito protetor da GUO frente à privação de glicose e oxigênio não é bloqueado com antagonistas ionotrópicos de receptores de glutamato (OLESKOVICZ et al., 2008) e Morciano e colaboradores (2004) demonstraram que a neurotoxicidade induzida por NMDA em culturas de neurônios hipocampais é revertida na presença dos derivados fosfatados da guanina (GTP, GDP e GTP), porém este efeito não é observado com a utilização de GUO. Adicionalmente, GUO tem pouca capacidade de deslocar a união de glutamato à receptores NMDA quando comparado ao GMP (MONAHAN et al., 1988) e evidências sugerem que o efeito neuroprotetor da GUO não está relacionada ao bloqueio de receptores de glutamato, mas sim a sua capacidade de modular o transporte de glutamato (FRIZZO et al., 2002; 2003). Diante destas informações, a segunda parte do nosso estudo enfocou a avaliação do papel neuroprotetor da GUO frente à toxicidade do glutamato em fatias de hipocampo de ratos e o mecanismo envolvido nos efeitos da GUO.

Os efeitos neuroprotetores da GUO são desencadeados em uma faixa de concentração bastante ampla (FRIZZO et al., 2001; DI IORIO et al., 2004), entretanto, a maioria dos estudos utilizando fatias de hipocampo de ratos demonstram que o efeito neuroprotetor da GUO ocorre na faixa de 100-300μM (OLESCOVICZ et al., 2008; THOMAZI et al., 2008; DAL-CIM, 2008). Portanto, avaliamos o efeito de diferentes concentrações de GUO (30, 100 e 300 μM) frente à toxicidade do glutamato em fatias de hipocampo de ratos e verificamos que apenas a concentração de 100 μM foi capaz de reduzir significativamente a perda de viabilidade celular induzida por glutamato. O padrão em “U” invertido da curva dose-resposta para GUO (Fig. 1, capítulo 1) sugere que dependendo do agente indutor de dano celular, e, possivelmente também, do mecanismo envolvido neste dano, pode haver diferenças

entre as concentrações efetivas de GUO. Portanto, em nosso estudo, verificamos que, ao contrário do GMP, a GUO foi capaz de prevenir a morte celular induzida por glutamato.

Como mencionado anteriormente, o principal mecanismo pelo qual a GUO protege frente à excitotoxicidade envolve a sua capacidade de modular o transporte de glutamato e contribuir assim para a diminuição da concentração de glutamato na fenda sináptica. Em nosso estudo, também verificamos que o efeito protetor de GUO (Fig. 2, capítulo 2) envolve a modulação do transporte de glutamato, pois GUO diminui a liberação de glutamato induzida por glutamato de maneira semelhante ao DL-TBOA, o que indica que provavelmente o efeito da GUO se deve à diminuição do transporte reverso do glutamato. Contudo, não podemos excluir a possibilidade de que GUO também esteja aumentando a atividade de transportadores receptores funcionais de glutamato (FRIZZO et al., 2001), ou atuando sobre os transportadores que estão inibidos (mas não revertidos), aumentando a captação de glutamato e contribuindo assim para a maior retirada do glutamato da fenda sináptica. Alternativamente, GUO também pode estar modulando a expressão ou o tráfego dos transportadores de glutamato para a membrana celular. Dessa maneira, estamos demonstrando que além de estimular a captação de glutamato (OLIVEIRA et al., 2004; DAL-CIM, 2008), diminuir a liberação sinaptossomal de glutamato (TAVARES et al., 2005), diminuir a captação vesicular de glutamato (TASCA et al., 2004), a GUO pode diminuir a liberação de glutamato decorrente da reversão de seus transportadores de membrana celular. Portanto, as informações contidas no capítulo 2 dessa Tese, corroboram estudos prévios e reforça a participação da GUO na modulação do transporte de glutamato.

O efeito neuroprotetor da GUO e o seu efeito na inibição da liberação de glutamato foram abolidos na presença de LY204002 e GUO foi capaz de aumentar os níveis de p-Akt, indicando que o efeito neuroprotetor da GUO é decorrente da diminuição da liberação de glutamato e é depende a ativação da via PI3K/Akt (Figs. 2, 3 e 4, capítulo 2). A ativação da enzima GSKβ tem sido correlacionada com a morte neuronal que ocorre devido a diversos

estímulos neurotóxicos, incluindo glutamato (NISHIMOTO et al., 2008). Um dos alvos da Akt é a enzima GSK3β. A Akt fosforila a serina 9 da GSK3β, inibindo-a. Trinta minutos de exposição à GUO aumentou a fosforilação da Akt e da GSK3β^{Ser9} (Fig. 5, capítulo 2) demonstrando assim que a via de sinalização envolvida no efeito neuroprotetor da GUO e possivelmente sua ação sobre o transporte de glutamato envolve a ativação da PI3K/Akt e a conseqüente inibição da GSK3β. Um estudo realizado em nosso laboratório também demonstrou que GUO (100μM) é capaz de reverter a diminuição da captação de glutamato induzida pela privação de glicose e oxigênio, e que este efeito da GUO está correlacionado com a sua capacidade de estimular a via PI3K/Akt (DAL-CIM, 2008). Alguns dados na literatura têm demonstrado que a via da PI3K/Akt aumenta a expressão dos transportadores de glutamato (SIMS et al., 2000) e também o tráfego dos transportadores de glutamato do citoplasma para a membrana, o que resulta em aumento da captação de glutamato (KRIZMAN-GENDA et al., 2005). Em estudo realizado por Guillet e colaboradores (2005) foi demonstrado que a inibição da via da PI3K diminui a captação de glutamato e que essa via está envolvida com a regulação da expressão dos transportadores de glutamato, GLAST e GLT-1 na superfície celular. Assim, a diminuição da liberação de glutamato promovida pela GUO, envolve a ativação da via da PI3K/Akt, que por sua vez estaria modulando a atividade dos transportadores de glutamato ou aumentando a expressão desses transportadores na membrana celular. A disfunção dos transportadores de glutamato está envolvida em uma série de doenças neurodegenerativas (MARAGAKIS & ROTHSTEIN, 2004), logo, compostos que normalizem a atividade ou expressão destes transportadores apresentam potencial terapêutico frente á patologias que acometem o sistema nervoso central.

GUO reduziu a expressão de iNOS induzida por glutamato (Fig.6, capítulo 2). Já está demonstrado na literatura que a ativação de receptores de glutamato induz a produção de mediadores pró-inflamatórios (citocinas), as quais ativam a transcrição do NF-κB. Conjuntamente, estes mediadores ativam a transcrição do gene iNOS (DE BOCK et al., 1996;

MORO et al., 2004). Outros estudos correlacionam a ativação da via da $p38^{MAPK}$ como um importante mediador da ativação da expressão de iNOS (SAHA & PAHAN, 2006). De fato, neste mesmo modelo de estudo, demonstramos que a morte celular induzida por glutamato depende da ativação da via $p38^{MAPK}$ (MOLZ et al., 2008). O tratamento de células de glioma C6 e culturas primárias de astrócitos com inibidores da via PI3K (por exemplo, o LY294002) aumentam a expressão de iNOS em resposta ao LPS ou citocinas, demonstrando que a manutenção da atividade da via PI3K é importante para bloquear o aumento da expressão de iNOS (PAHAN et al., 1999).

Em resumo, diante dos dados que foram apresentados nos capítulos 1 e 2 desta Tese, podemos sugerir que em situações de excitotoxicidade, a liberação de derivados fosfatados da guanina em doses de baixo milimolar de vesículas sinápticas (SANTOS et al., 2006) ou dos astrócitos (CICCARELLI et al., 1999) leva ao aumento dos níveis de GMP que seria responsável pelo bloqueio da ativação de receptores NMDA. Ou, após sua hidrólise, GMP formaria GUO, a qual diminui a liberação de glutamato através da ativação da via da PI3K, inibição da enzima GSK3β e diminuição da expressão de iNOS induzida por glutamato, resultando em neuroproteção.

Apesar de seus comprovados efeitos neuroprotetores, as purinas (adenosina e ATP) em altas concentrações podem ser importantes toxinas (FRANKE et al., 2006). GMP apresenta efeito neuroprotetor quando usado em uma faixa de concentração de baixo milimolar (MOLZ et al., 2005). Entretanto, também demonstramos (MOLZ, 2003) que o GMP potencializa a fragmentação de DNA induzida por glutamato em um modelo de neurotoxicidade em fatias de hipocampo de ratos. Diante destas observações, o capítulo 3 deste trabalho avaliou o possível efeito neurotóxico do GMP em fatias de hipocampo de ratos. Demonstramos que o GMP, na dose de 5mM, diminui a viabilidade celular em fatias de hipocampo de ratos (Figs 1 e 2, capítulo 3). O aumento da incorporação de iodeto de propídeo observada indica que GMP 5mM pode estar induzindo um padrão necrótico de morte celular. Tal observação foi

reforçada pela ausência de fragmentação de DNA na presença de 5mM de GMP (dados não mostrados). De maneira similar, 5 mM de GTP ou Gpp(NH)p (um análogo não hidrolizável do GMP) aumenta o dano oxidativo induzido por glutamato em fatias de hipocampo de ratos (REGNER et al., 1998). A toxicidade celular do ATP (5mM) já foi descrita em culturas organotípicas de hipocampo (FRIZZO et al., 2007) e até este momento não há dados na literatura com relação à toxicidade de um derivado da guanina. Portanto, estamos demonstrando pela primeira vez que assim como já foi demonstrado para os derivados da adenina, a disfunção da neurotransmissão purinérgica devido a altas concentrações de um derivado da guanina pode levar á morte de células em fatias de hipocampo de ratos.

O tratamento das fatias de hipocampo com GUO não causou diminuição da viabilidade celular em nenhuma das concentrações testadas (Fig. 1, inset, capítulo 3) demonstrando que a toxicidade promovida pelo GMP não envolve a sua conversão até GUO e que a concentração de GMP avaliada neste estudo (5mM) se sobrepõe a qualquer efeito protetor que a GUO poderia apresentar. Em um estudo realizado por Di Iorio et al. (2002), GUO induziu apoptose em cultura de astrócitos, porém os autores correlacionam este efeito devido ao fato de que GUO induz a liberação de ADO, a qual, através da estimulação de receptores A_3 de ADO, desencadeia a morte celular apoptótica nestas células.

O pré-tratamento das fatias de hipocampo com dipiridamol não preveniu a perda de viabilidade celular induzida por GMP (Fig. 3A, capítulo 3), o que demonstra que a toxicidade induzida pelo GMP ocorre através da sua interação com sítios extracelulares de ligação ao GMP. Os receptores para os DG ainda não estão totalmente caracterizados e algumas evidências demonstraram que DG podem se unir com baixa afinidade aos receptores de adenosina (MULER & SCIOR, 1993). Entretanto, em nosso estudo, a utilização de antagonistas de receptores A_1 ou A_{2A} de adenosina não preveniu a perda de viabilidade celular induzida por GMP (Fig. 3B, capítulo 3). O fato de que o inibidor do transporte de nucleosídeos (dipiridamol) ou o antagonista de receptores A_1 de ADO (DPCPX) foram *per se*

capazes de reduzir a viabilidade celular na mesma proporção do GMP, indica que o transporte de nucleosídeos e a ativação basal de receptores A_1 de ADO é necessária para manter a integridade das fatias de hipocampo. Porém, a ativação de receptores A_1 ou A_{2A} de ADO na presença de 5mM de GMP não bloquearam o efeito neurotóxico do GMP (Fig. 3C, capítulo 3). Estes resultados são consistentes com o papel clássico da adenosina como agente neuroprotetor através da ativação de seus receptores A_1 (CUNHA, 2001), e exclui o sistema adenosinérgico no mecanismo de toxicidade do GMP.

GMP desloca a união de glutamato a receptores ionotrópicos de glutamato (SOUZA & RAMÍREZ, 1991; PORCIÚNCULA et al., 2002) sugerindo que GMP possa se unir a estes receptores. A utilização de antagonistas de receptores NMDA ou AMPA de glutamato (MK-801 ou DNQX, respectivamente) preveniu a morte celular induzida por GMP (Fig. 4, capítulo 3). Concentrações neurotóxicas de GMP diminuíram a captação de glutamato e este efeito foi prevenido pela utilização de DL-TBOA (Fig. 5, capítulo 3). Este conjunto de resultados indica que o mecanismo envolvido no efeito neurotóxico do GMP envolve, portanto, a diminuição da captação de glutamato, aumento da concentração extracelular de glutamato e super-estimulação de receptores NMDA e AMPA de glutamato.

Nessa parte do estudo (capítulos 1, e 3), foi possível demonstrar em fatias de hipocampo de ratos, que assim como acontece com outros neurotransmissores e neuromoduladores (glutamato, ATP e adenosina), dependendo da concentração, o GMP pode ser neuroprotetor (1mM) ou neurotóxico (5mM).

Muitos estudos utilizam culturas primárias de neurônios para investigar os mecanismos envolvidos na neurodegeneração. Entretanto, a quantidade reduzida de células obtidas a partir de culturas primárias, aliado a problemas éticos devido à utilização de embriões humanos para estudos patofisiológicos associados á doenças neurodegenerativas, torna a utilização de células de neuroblastoma humano (SHSY-5Y) uma alternativa para estudos de neurodegeneração e neuroproteção. A linhagem SHSY-5Y constitui um sistema

amplamente utilizado para estudar processos neuronais, incluindo vias de sinalização e mecanismo de morte neuronal. As células SHSY-5Y são derivadas de neurônios simpáticos, que sintetizam e armazenam noradrenalina (BIEDLER et al., 1973). Nos dois últimos capítulos desta Tese, estudamos os efeitos do estresse oxidativo e do peptídeo Aβ em células de neuroblastoma humano SHSY-5Y e o papel da GUO frente a estes processos.

O estresse oxidativo mitocondrial associado à superprodução de ROS está envolvido na perda neuronal que ocorre em diversas doenças neurodegenerativas e pode ser evidenciado em diversas situações relacionadas à neurotoxicidade, como no dano induzido por peróxido de hidrogênio, MPP^+, Aβ e também devido ao glutamato. No quarto capítulo desta Tese, utilizamos um modelo de estresse oxidativo mitocondrial induzido pela combinação de dois inibidores da cadeia respiratória (rotenona e oligomicina A, rot/oligo) em células de neuroblastoma humano (SHSY-5Y) e estudamos o efeito neuroprotetor da GUO. Verificamos que GUO nas concentrações de 0,3 e 1 mM, quando co-incubada com rot/oligo, protege significativamente as células SHSY-5Y da toxicidade neuronal induzida por rot/oligo (Fig. 1A, capítulo 4), sendo 1mM de GUO a concentração mais efetiva (Fig. 1B, capítulo 4). Recentemente, Guarnieri e colaboradores (2009) demonstraram que GUO induz proliferação celular em culturas de SHSY-5Y somente após 4 dias de incubação com GUO, o que sugere que o efeito protetor da GUO observado em nosso estudo não se deve a um efeito da GUO na proliferação celular.

A morte celular induzida por rot/oligo envolve a super-produção de ROS, perda do potencial de membrana mitocondrial e ativação de caspase-3 (EGEA et al., 2007). Em nosso estudo, a produção de ROS induzida por rot/oligo foi totalmente abolida na presença de GUO (Fig. 3, capítulo 4), e esse efeito da GUO não envolveu as vias de sinalização MEK/ERK1/2 ou PKC, mas foi dependente da ativação da via PI3K (Fig 2A e 2B, capítulo 4). Além disso, a inibição da via PI3K também aboliu o efeito neuroprotetor da GUO na morte celular induzida por rot/oligo. Outros estudos já demonstraram o envolvimento das vias de

sinalização PI3K/Akt e MEK/ERK1/2 no efeito neuroprotetor da GUO em modelos de toxicidade quando GUO era pré-incubada, ou seja, adicionada antes do agente neurotóxico (DI IORIO et al., 2004; PETTIFER et al., 2004), porém, assim como evidenciado em nosso estudo, quando a GUO era co-incubada com o agente tóxico, o efeito neuroprotetor ocorreu somente através da ativação da via PI3K/Akt (PETTIFER et al., 2007), indicando que a via da PI3K pode ser a principal via de sinalização ativada pela GUO. A GUO também protege células SHSY-5Y da morte neuronal induzida pelo MPP^+ (PETTIFER et al., 2007), uma agente que causa toxicidade principalmente por induzir estresse oxidativo, porém, nesse estudo, os autores não avaliaram o efeito da GUO na produção de ROS.

Alguns estudos sugerem que o efeito protetor da GUO frente à inibição mitocondrial se deve a uma ação intracelular (JURKOWITZ et al., 1998; LITZKY et al., 1999). Entretanto, estudos posteriores demonstraram que a utilização de dipiridamol (um inibidor do transporte de nucleosídeo) não afeta o efeito neuroprotetor da GUO, indicando que seu efeito é extracelular (DI IORIO et al., 2004; DECKER et al., 2007).

No capítulo 5, também avaliamos o efeito protetor de GUO frente à toxicidade do peptídeo $A\beta_{25-35}$ em culturas de células de neuroblastoma humano SH-SY5Y. Nesta parte do estudo, verificamos que as culturas não-diferenciadas de células SH-SY5Y são resistentes à toxicidade do peptídeo $A\beta_{(25-35)}$ (Fig. 2, capítulo 5), porém, a diferenciação destas células para um fenótipo mais neuronal (Fig. 1) as torna susceptíveis ao peptídeo $A\beta_{25-35}$ (Fig. 3, capítulo 5). A diferenciação das culturas de SH-SY5Y se deu pela adição de ácido retinóico (10μM – 5 dias) seguido da incubação com BDNF (1ng/ml – 3 dias). A adição de ácido retinóico aumenta a expressão de receptores TrKB nas células SHSY-5Y, tornando-as responsivas ao BDNF, mantendo-as na fase G1 do ciclo celular, permitindo a sua diferenciação e aumento da projeção de neuritos (ENCINAS et al., 2000). Adicionalmente, as células SHSY-5Y diferenciadas com ácido retinóico e BDNF apresentam aumento da expressão e do conteúdo da tau fosforilada, sugerindo que este seja um modelo de Doença de Alzheimer associado à

hiperfosforilação de tau (JÄMSÄ et al., 2004). Como os efeitos do Aβ ocorrem predominantemente sobre os neuritos (VAISID et al., 2008) e que existe uma forte correlação entre o peptídeo Aβ e a hiperfosforilação da tau, estes fatores podem explicar a susceptibilidade destas células à ação do $A\beta_{25-35}$. Pettifer e colaboradores (2004) demonstraram que o peptídeo $A\beta_{25-35}$ causa morte celular por apoptose em culturas não diferenciadas de SHSY-5Y. Neste estudo, a apoptose foi avaliada através da fragmentação de DNA induzida por 5μM de $A\beta_{25-35}$, portanto, o fato de não termos observado morte celular com a aplicação de $A\beta_{25-35}$ nas culturas de SHSY-5Y não diferenciadas pode ser devido a metodologia utilizada em nosso estudo para a avaliação da viabilidade celular.

A pré-incubação das culturas diferenciadas de SH-SY5Y por 24h com GUO diminuiu a morte neuronal induzida pelo $A\beta_{25-35}$ de maneira dependente de concentração, sendo a concentração de 1mM a mais efetiva (Fig. 4). Desta forma GUO também apresenta potencial efeito protetor no tratamento da doença de Alzheimer. Os resultados neuroprotetores obtidos com GUO frente ao modelo de toxicidade mediada pelo $A\beta_{25-35}$ em células SHSY-5Y diferenciadas ainda são preliminares e, portanto, necessitam de maior investigação pelo nosso grupo. Por exemplo, a investigação do efeito da GUO sobre parâmetros apoptóticos induzidos pelo $A\beta_{25-35}$. Ainda, como existem evidências de que a proteína tau está hiperfosforilada neste modelo, e que esta hiperfosforilação da proteína tau depende da ativação da GSK3β, os níveis fosforilação da tau na presença do $A\beta_{25-35}$ e sua relação com a atividade da GSK3β poderia ser determinados nas culturas de SHSY-5Y diferenciadas. Adicionalmente, o efeito da GUO nestes parâmetros poderia ser avaliado.

A GUO protege as células SHSY-5Y do estresse oxidativo mitocondrial induzido por rot/oligo e da toxicidade do peptídeo $A\beta_{25-35}$ de maneira dependente de concentração, sendo a concentração de 1 mM a mais efetiva. Em contrapartida, nas fatias submetidas à excitotoxicidade, a concentração efetiva de GUO foi 100μM. Esta diferença de concentração

de GUO para se evidenciar a neuroproteção pode ser devido á diferenças no modelo de estudo (cultura de células e fatias de hipocampo) assim como ao tipo de agente neurotóxico (rot/oligo, $A\beta_{25-35}$ ou glutamato) e também devido ao mecanismo envolvido no dano celular induzido pelos diferentes agentes neurotóxicos.

De maneira geral, os resultados apresentados nos capítulos 4 e 5 dessa Tese indicam que a GUO apresenta potencial efeito neuroprotetor frente à mecanismos relacionadas ao estresse oxidativo mitocondrial e à Doença de Alzheimer.

Finalmente, podemos propor através dos dados apresentados nessa Tese de Doutorado, que altas concentrações de GMP podem ser neurotóxicas via diminuição da captação de glutamato e ativação de receptores ionotrópicos de glutamato (Figura 3) indicando que dependendo da concentração, GMP pode ser neuroprotetor (Figura 4) ou neurotóxico. Em situações de excitotoxicidade, a liberação de derivados da guanina de vesículas sinápticas ou de astrócitos leva ao aumento dos níveis destes compostos, que agem bloqueando a ativação de receptores NMDA (GMP) ou modulando o transporte de glutamato (GUO), resultando em neuroproteção. GUO também apresenta efeito neuroprotetor em situações que podem estar envolvidas na patogênese de doenças neurodegenerativas, como a produção de ROS e a toxicidade do peptídeo $A\beta_{25-35}$ (Figura 4), demonstrando assim que GUO apresenta potencial efeito terapêutico em diferentes modelos de neurodegeneração.

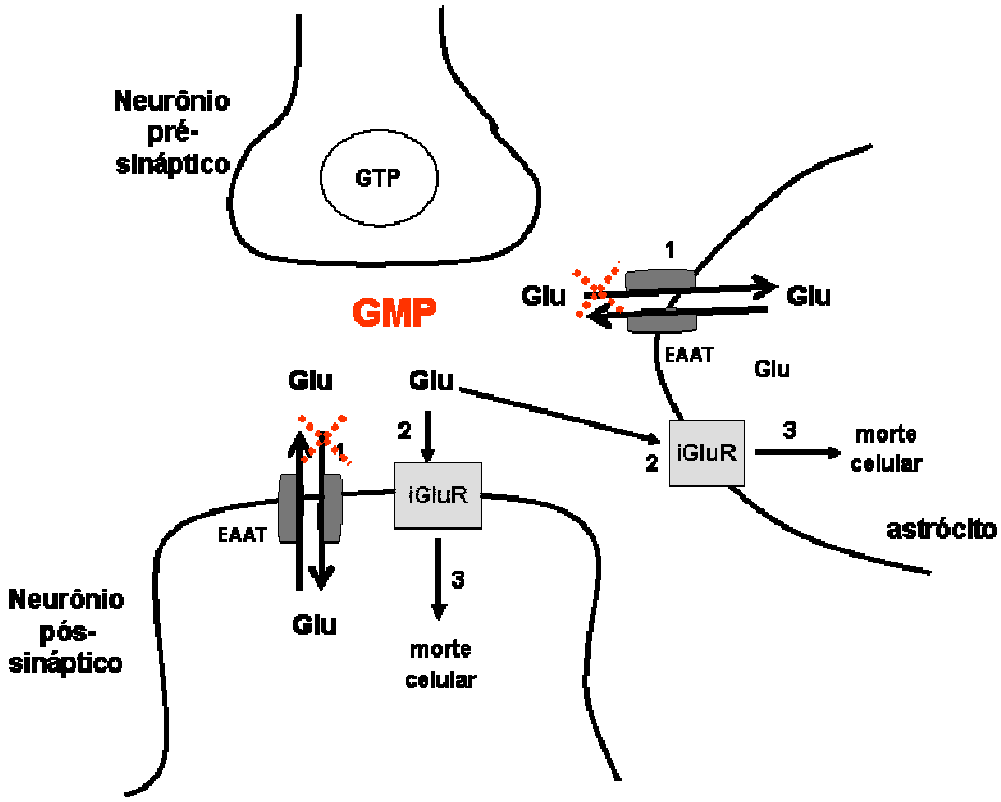

Figura 3: Efeito neurotóxico do GMP em fatias de hipocampo de ratos. Concentrações tóxicas de GMP (5mM) diminuem a captação de glutamato **(1)** aumentando a concentração de glutamato na fenda sináptica. Glutamato estimula receptores ionotrópicos de glutamato (NMDA e AMPA) **(2)** levando à morte celular **(3)** em fatias de hipocampo de ratos.

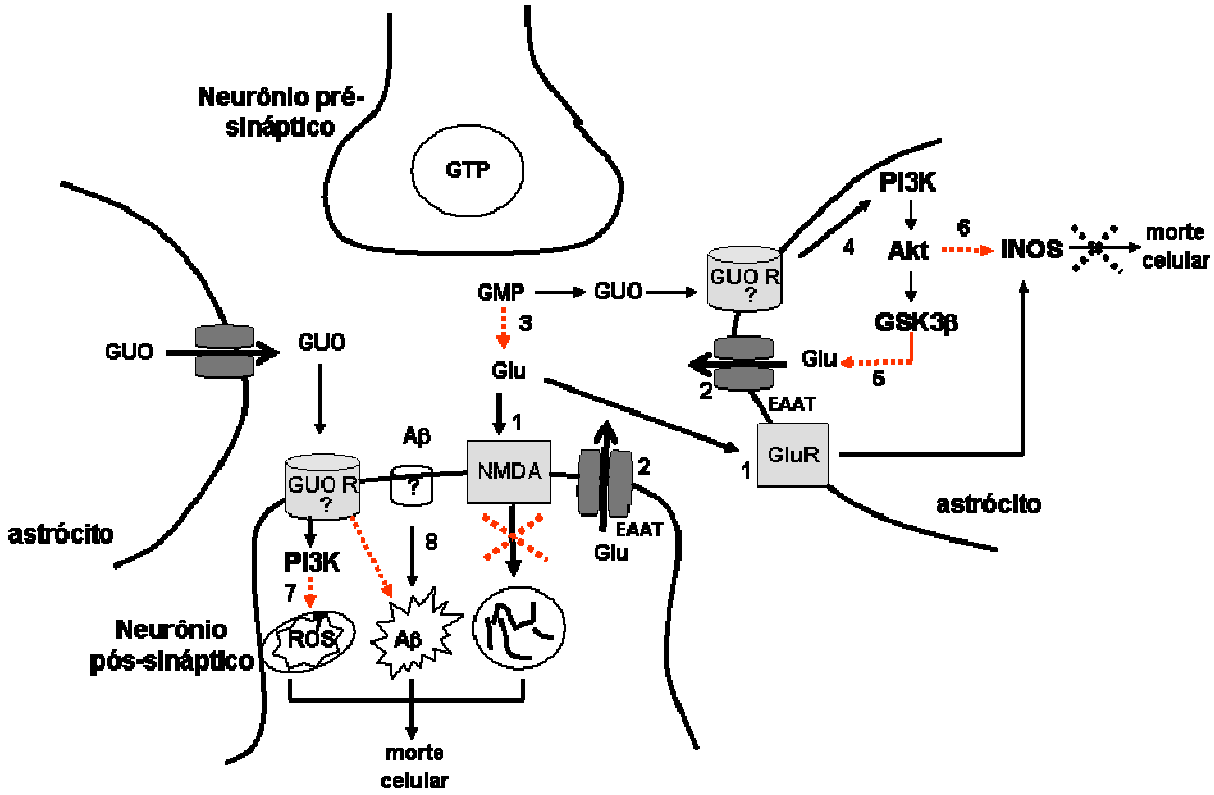

Figura 4: Efeito neuroprotetor dos derivados da guanina (GMP e GUO) em diferentes situações de neurotoxicidade: A redução da viabilidade celular induzida por glutamato em fatias de hipocampo de ratos ocorre devido à estimulação de seus receptores **(1)** e através da atividade reversa de seus transportadores **(2).** GMP 1mM apresentou efeito neuroprotetor frente à apoptose induzida pela estimulação de receptores NMDA **(3)**. A neuroproteção promovida por GUO (100μM) frente ao dano celular induzido por glutamato envolve: ativação da via da PI3K/Akt, inibição da enzima GSK3β **(4)** e diminuição da liberação de glutamato **(5),** assim como a redução da expressão de iNOS induzida por glutamato **(6)**. GUO (1mM) protege células de neuroblastoma humano SHSY-5Y da neurotoxicidade induzida pela super-produção de ROS através da ativação da via de sinalização celular PI3K **(7)** e também diminuiu significativamente a morte celular induzida pelo peptídeo $A_{\beta 25-35}$ em culturas diferenciadas de células de neuroblastoma SHSY-5Y **(8)**.

CONCLUSÕES

- A redução da viabilidade celular induzida por glutamato em fatias de hipocampo de ratos pode ocorrer devido à estimulação de seus receptores e através da atividade reversa de seus transportadores.
- GMP 1mM apresentou efeito neuroprotetor frente à apoptose induzida pela estimulação de receptores NMDA e não frente frente à apoptose induzida por glutamato nas fatias de hipocampo de ratos.
- A neuroproteção promovida por GUO (100μM) frente ao dano celular induzido por glutamato envolve: diminuição da liberação de glutamato através da ativação da via da PI3K/Akt; inibição indireta da enzima GSK3β e redução da expressão de iNOS em fatias de hipocampo de ratos.
- GUO (1mM) protege células de neuroblastoma humano SHSY-5Y da neurotoxicidade induzida pela super-produção de ROS e envolve a ativação da via de sinalização celular PI3K.
- As culturas de células SHSY-5Y não diferenciadas são resistentes à toxicidade do peptídeo $A\beta_{25-35}$, porém, a diferenciação destas células para um fenótipo mais neuronal as torna susceptíveis ao peptídeo $A\beta_{25-35}$. GUO (1mM) diminuiu significativamente a morte celular induzida pelo peptídeo $A_{\beta 25-35}$ em culturas de neuroblastoma humano diferenciadas.
- A diminuição da viabilidade celular induzida por concentrações tóxicas de GMP (5mM) em fatias de hipocampo de ratos ocorre devido à diminuição da captação de glutamato e conseqüente estimulação de receptores ionotrópicos de glutamato (NMDA e AMPA).

REFERÊNCIAS BIBLIOGRÁFICAS

ABBRACCHIO M.P., CERUTI S., BARBIERI D., FRANCESCHI C., MALORNI W., BIONDO L., BURNSTOCK G., CATTABENI F. **Biochem** Biophys **Res Com** v. 213, p. 908-915, 1995.

AMADIO S., D'AMBROSI N., CAVALIERE F., MURRA B., SANCESARIO G., BERNARDI G., BURNSTOCK G., VOLONTÉ C. P2 receptor modulation and cytotoxic function in cultured CNS neurons. **Neuropharmacol.** v. 42, p. 489-501, 2002.

ANDERSON C.M., SWANSON R.A. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. **Glia**. v. 32, p. 1-14, 2000.

ANDERSON C.M., BERGHER J.P., SWANSON R.A. ATP-induced ATP release from astrocytes. **J Neurochem**., v. 88, n. 1, p. 246-56, 2004.

ANKARCRONA M., DYPBURKT J.M., BONFOCO E., ZHYVOTOVSKY B., ORRENIUS S. LIPTON, S.A. NICOTERA P. Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. **Neuron**. v. 15, p. 961-973, 1995.

BARON B.M., DUDLEY M.W., MCCARTY D.R., MILLER F.P., REYNOLDS I.J., SCHMIDT C.J. Guanine nucleotides are competitive inhibitors of N-methyl-D-aspartate at its receptor site both in vitro and in vivo. Journal of Pharmacology and **Exp Ther**. v. 250, p. 162-169, 1989.

BHAT R.V., BUDD HAEBERLEIN S.L., AVILA J. Glycogen synthase kinase 3: a drug target for CNS therapies**. J Neurochem.** v. 89, p. 1313-7, 2004.

BIEDLER J.L., HELSON L., SPENGLER B.A. Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. **Cancer Res.**, v. 33, p. 2643-52, 1973.

BONDE C., SARUP A., SCHOUSBOE A, GEGELASHVILI G., ZIMMER J., NORABERG J. Neurotoxic and neuroprotective effects of the glutamate transporter inhibitor DL-threo-beta-benzyloxyaspartate (DL-TBOA) during physiological and ischemia-like conditions. **Neurochem Int.** v. 43, p. 371-380, 2003.

BRONGHOLI K., SOUZA D.G., BAINY A.C., DAFRE A.L., TASCA C.I. Oxygen-glucose deprivation decreases glutathione levels and glutamate uptake in rat hippocampal slices. **Brain Res**. v. 1083, p. 211-218, 2006.

BURGOS J.S., BARAT A., RAMÍREZ G. Guanine nucleotides block agonist-driven $^{45}Ca^{2+}$ influx in chick embryo retinal explants**. Neuroreport** v. 11, p. 2303-2305, 2000.

BURNSTOCK G. Purinergic signalling. British J Pharmacol. v. 147, p. S172-181, 2006.

CAMACHO A., MASSIEU L. Role of glutamate transporters in the clearance and release of glutamate during ischemia and its relation to neuronal death. **Arch Med. Res**. v. 37, 11-18, 2006.

CAÑAS N., VALERO T., VILLARROYA M., MONTIELL E., VERGÉS J., GARCÍA A.G., LÓPEZ M.G. Chondroitin Sulfate Protects SH-SY5Y Cells from Oxidative Stress by Inducing Heme Oxygenase-1 via Phosphatidylinositol 3-Kinase/Akt. J. **Pharmacol .Exp. Ther**. v. 323, p. 946-953, 2007.

CARDENAS A., MORO M.A., HURTADO O., LEZA J.C., LORENZO P., CASTILLO A., BODELON O.G., BOSCA L., LIZASOAIN I. Implication of glutamate in the expression of inducible nitric oxide synthase after oxygen and glucose deprivation in rat forebrain slices**. J. Neurochem**. v. 74, p. 2041-2048, 2000.

CHEN J.F., HUANG Z., MA, J. ZHU J., MORATALLA R., STANDAERT D., MOSKOWITZ, M.A., FINK J.S., SCHWARZSCHILD, M.A. A_{2A} adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. **J Neurosci**., v. 19, p. 9192-200, 1999.

CHEN J.F., SONSALLA P.K, PEDATA F., MELANI A., DOMENICI M.R., POPOLI P., GEIGER J., LOPES L.V., DE MENDONÇA A. Adenosine A_{2A} receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and "fine tuning" modulation. **Prog Neurobiol**., v. 83, n. 5, p. 310-31, 2007.

CICCARELLI R., BALLERINI P., SABATINO G., RATHBONE M.P., D'ONOFRIO M., CACIAGLI F., DI IORIO P. Involvement of astrocytes in purine-mediated reparative processes in the brain. **Int. J Dev Neurosci**. v. 19, p. 395-414, 2001.

CICCARELLI R., DI IORIO P., GIULIANI P., D'ALIMONTE I., BALLERINI P., CACIAGLI F., RATHBONE M.P. Rat cultured astrocytes release guanine-based purines in basal conditions and after hypoxia/hypoglycemia. **Glia.** v. 25, p. 93-98, 1999.

CROSS D.A., ALESSI D.R., COHEN P., ANDJELKOVICH M., HEMMINGS B.A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. **Nature.** v. 378, p. 785–789, 1995.

CUNHA R.A Neuroprotection by adenosine in the brain: From A1 receptor activation to A2A receptor blockade. **Purinergic Sign.** v. 1, p. 111–134, 2005.

CUNHA R.A. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. **Neurochem Int.** v. 38, p. 107-125, 2001.

DAL-CIM T.A.. Mecanismos de neuroproteção da guanosina em fatias de hipocampo de ratos submetidas à privação de glicose e oxigênio. Dissertação de mestrado, UFSC, Florianópolis, 2008.

D'ALIMONTE I., FLATI V, D'AURO M, TONIATO E, MARTINOTTI S, RATHBONE MP, JIANG S, BALLERINI P, DI IORIO P, CACIAGLI F, CICCARELLI R. Guanosine inhibits CD40 receptor expression and function induced by cytokines and beta amyloid in mouse microglia cells. **J Immunol**. 178, 720-31.

DANBOLT, N.C., 2001. Glutamate uptake. **Prog Neurobiol**. v. 65, p. 1-105, 2007.

DE BOCK F., DORMAND J., RONDOUIN, G. Release of TNFα in the rat hippocampus following epileptic seizures and excitotoxic neuronal damage. **NeuroReport**, v. 7, p. 1125-1129, 1996.

DECKER H., FRANCISCO S.S., MENDES-DE-AGUIAR C.B., ROMÃO L.F., BOECK C.R., TRENTIN A.G., MOURA-NETO V., TASCA C.I. Guanine derivatives modulate extracellular matrix proteins organization and improve neuron-astrocyte co-culture. J **Neurosci** Res. v. 85, p. 1943-1951, 2007.

DI IORIO P., BALLERINI P., TRAVERSA U., NICOLETTI F., D'ALIMONTE I., KLEYWEGT S., WERSTIUK E.S., RATHBONE M.P., CACIAGLI F., CICCARELLI R. The antiapoptotic effect of guanosine is mediated by the activation of the PI 3-kinase/AKT/PKB pathway in cultured rat astrocytes. **Glia.** v. 46, p. 356-368, 2004.

DI IORIO, P., CICCARELLI, R., TRAVERSA, U., ANDREW, C.M., CROCKER, C.E., WERSTIUK, E.S., RATHBONE, M.P. Mechanism of purine nucleosides induced apoptosis in astrocytes. **Glia.** v. 38, p. 179-190, 2002.

DI VIRGILIO F., CERUTI S., BRAMANTI, P., ABBRACCHIO, M.P. Purinergic signalling in inflammation of the central nervous system. **Trends Neurosci.**, v. 32, n 2, p. 79-87, 2009.

DUAN S., ANDERSON, C.M., STEIN, B.A., SWANSON, R.A. Glutamate induces rapid upregulation of astrocyte glutamate transport and cell-surface expression of GLAST. **J Neurosci.**, v. 19, p. 10193-200, 1999.

DUAN, S., ANDERSON, C.M., KEUNG, E.C., CHEN, Y., CHEN, Y., SWANSON RA. P2X7 receptor-mediated release of excitatory amino acids from astrocytes. **J Neurosci.**, v. 23, n. 4, p. 1320-8, 2003.

EGEA, J., ROSA, A.O., CUADRADO, A., GARCÍA, A.G., LÓPEZ, M.G. Nicotinic receptor activation by epibatidine induces heme oxygenase-1 and protects chromaffin cells against oxidative stress. **J. Neurochem.** v. 02, p. 1842-52, 2007.

ENCINAS, M., IGLESIAS, M., LIU, Y., WANG, H., MUHAISEN, A., CEÑA, V., GALLEGO, C., COMELLA JX. Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. **J Neurochem**., v. 75, p. 991-1003, 2000.

FELLIN, T., POZZAN, T., CARMIGNOTO, G. Purinergic receptors mediate two distinct glutamate release pathways in hippocampal astrocytes. **J Biol Chem**., v. 281, n. 7, p. 4274-84, 2006.

FERREIRA, J.M., PAES-DE-CARVALHO, R. Long-term activation of adenosine A(2a) receptors blocks glutamate excitotoxicity in cultures of avian retinal neurons. **Brain Res.**, v. 900, n.2, p. 169-76, 2001

FIELDS, R.D., BURNSTOCK, G. Purinergic signalling in neuron-glia interactions. **Nat Rev Neurosci.** v. 7, p. 423-436, 2006.

FRANKE, H., ILLES, P. Involvement of P2 receptors in the growth and survival of neurons in the CNS. **Pharmacol** & Ther. v. 109, p. 297-324, 2006.

FREDHOLM, B.B. Adenosine, an endogenous distress signal, modulates tissue damage and repair. **Cell Death Differ.,** v. 14, n. 7, p. 1315-23, 2007.

FRIZZO ME, LARA DR, PROKOPIUK AS, VARGAS CR, SALBEGO CG, WAJNER M, SOUZA D.O. Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. **Cell. Mol. Neurobiol.** v. 22, p. 353-363, 2002.

FRIZZO, M.E., SOARES, F.A., DALL'ONDER, L.P., LARA, D.R., SWANSON, R.A., SOUZA, D.O. Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake. **Brain Res.** v. 972, p. 84-89, 2003.

FRIZZO, M.E, FRIZZO, J.K., AMADIO, S., RODRIGUES, J.M., PERRY, M.L., BERNARDI, G., VOLONTÉ, C. Extracellular Adenosine Triphosphate Induces Glutamate Transporter-1 Expression in Hippocampus. **Hippocampus.** v. 17, p. 305–315, 2007.

FURUTA, A., ROTHSTEIN, J.D., MARTIN, L.J. Glutamate transporter protein subtypes are expressed differentially during rat CNS development. **J Neurosci.**, v. 17, p. 8363-75, 1997

GÄHWILER, B.H., CAPOGNA, M., DEBANNE, D., McKINNEY, R.A., THOMPSON, S M. Organotypic slices cultures: A technique has come of age. **Trends Neurosci**., v.20, p.471-477, 1997.

GONZÁLEZ, M.I., ROBINSON, M.B. Protein KINASE C–Dependent Remodeling of Glutamate Transporter Function. **Mol. Interv**., v.4, p. 48-58, 2004.

GREENE, R.W., HAAS, H.L. The electrophysiology of adenosine in the mammalian central nervous system. **Prog Neurobiol**, v. 36, p. 329Y41, 1991.

GRIMES CA, JOPE RS. The multifaceted roles of glycogen synthase kinase in cellular signalling. **Prog Neurobiol**. v. 65, p. 391-426, 2001.

GU Q.B., ZHAO J.X., FEI, J., SCHWARZ, W. Modulation of Na(+),K(+) pumping and neurotransmitter uptake by beta-amyloid**. Neuroscience**, v. 126, p. 61-7, 2004.

GUILLET BA, VELLY LJ, CANOLLE B, MASMEJEAN FM, NIEOULLON AL, PISANO P. Differential regulation by protein kinases of activity and cell surface expression of glutamate transporters in neuron-enriched cultures**. Neurochem Int.** v. 46, p. 337-46, 2005.

GYSBERS, J.W., RATHBONE, M.P. Neurite outgrowth from PC12 cells is enhanced by guanosine through both cAMP-dependent and –independent mechanisms. **Int J Dev Neurosci.** v. 41, p. 19-34, 2000.

HARA, M.R., SNIDER, S.H. Cell signaling and neuronal death. **Annu. Rev. Pharmacol. Toxicol**., v. 47, p. 117–41, 2007.

HARDY, J., SELKOE, D.J. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. **Science**, v. 297, p.353-6, 2003.

HERNÁNDEZ F, GÓMEZ DE BARREDA E, FUSTER-MATANZO A, GOÑI-OLIVER P, LUCAS JL, AVILA J. The role of GSK3 in Alzheimer disease. **Brain Res Bul** v. 80, p. 248–250, 2009.

HETMAN M, CAVANAUGH JE, KIMELMAN D, XIA Z. Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal. **J Neurosci.** v. 20, p. 2567–2574, 2000.

HOOPER, C., KILLICK, R., LOVESTONE S. The GSK3 hypothesis of Alzheimer's disease. **J Neurochem**., v. 104, n. 6, p. 1433-9, 2008.

HUETTLER, J.E. Kainate receptors and synaptic transmission. **Prog Neurobiol.** v. 70, p. 387-407, 2003.

IZQUIERDO, I, MEDINA, J.H. Memory Formation: The Sequence of Biochemical Events in the Hippocampus and Its Connection to Activity in Other Brain Structures **Neurobiol Learn Mem.**, v. 68, p. 285-316, 1997.

JÄMSÄ A., HASSLUND K., COWBURN R.F., BÄCKSTRÖM A., VASÄNGE, M. The retinoic acid and brain-derived neurotrophic factor differentiated SH-SY5Y cell line as a model for Alzheimer's disease-like tau phosphorylation. **Biochem Biophys Res Commun**., v. 319, p. 993-1000, 2004.

JURKOWITZ M. S., LITSKY M. L., BROWNING M. , HOHL, C. M. Adenosine, inosine, and guanosine protect glial cells during glucose deprivation and mitochondrial inhibition: correlation between protection and ATP preservation. **J. Neurochem**. v. 71, p. 535-548, 1998.

KENNY, P.J., MARKOU, A. “The ups and downs of addiction: role of metabotropic glutamate receptors”. **Trends Pharmacol. Sci**., v 25, p. 265-272, 2004.

KNOTT, A.B, BOSSY-WETZEL, E. Nitric oxide in health and disease of the nervous system. **Antioxidants and Redox sign.** v. 11, p. 541-553, 2009.

KRIZMAN-GENDA E, GONZÁLEZ MI, ZELENAIA O, ROBINSON MB. Evidence that Akt mediates platelet-derived growth factor-dependent increases in activity and surface

expression of the neuronal glutamate transporter, EAAC1. **Neuropharmacol.** v. 49, p. 872-882, 2005.

LARA D. R., SCHMIDT A. P., FRIZZO M. E. S., BURGOS J. S.; RAMIREZ G.; SOUZA D. O. Effect of orally administered guanosine on seizures and death induced by glutamatergic agents. **Brain Res.**, v. 912, p. 176−180, 2001.

LARA D.R., SCHMIDT A.P., FRIZZO M.E.S., BURGOS J.S., RAMIREZ G., SOUZA D.O. Effect of orally administered guanosine on seizures and death induced by glutamatergic agents. **Brain Res.** v. 912, p. 176−180, 2001.

LAUDERBACK C.M., HACKETT J.M., HUANG F.F., KELLER J.N., SZWEDA L.I., MARKESBERY W.R., BUTTERFIELD D.A. The glial glutamate transporter, GLT-1, is oxidatively modified by 4-hydroxy-2-nonenal in the Alzheimer's disease brain: the role of Abeta1-42. **J Neurochem**., v. 78, p. 413-6, 2001.

LINSEMAN D.A., BUTTS B.D., PRECHT T.A., PHELPS R.A., LE S.S., LAESSIG T.A. BOUCHARD R.J., FLOREZ-MCCLURE M.L., HEIDENREICH KA. Glycogen synthase kinase-3beta phosphorylates Bax and promotes its mitochondrial localization during neuronal apoptosis. **J Neurosci**., v 24, n. 44, p. 9993-10002, 2004.

LITSKY M. L., HOHL C. M., LUCAS J. H., JURKOWITZ M. S. Inosine and guanosine preserve neuronal and glial viability in mouse spinal cord cultures during chemical hypoxia. **Brain Res.** v. 821, p. 426-432, 1999.

MADDEN, D.R. The structure and function of glutamate receptor ion channels. **Nat Rev Neurosci**, v. 3, p. 91-101, 2002.

MALARKEY MB, PARPURA V. Mechanisms of glutamate release from astrocytes. **Neurochem Int**., v. 52, p. 142–154, 2008.

MALCON C, ACHAVAL M, KOMLOS F., PARTATA W., SAURESSING M., RAMÍREZ G, SOUZA D.O. GMP protects against quinolinic acid-induced loss of NADPH-diaphorase-positive cells in the rat striatum. **Neurosci Lett.** v. 225, p. 145-148, 1997.

MARAGAKIS N.J., ROTHSTEIN J.D. Glutamate transporters: animal models to neurologic disease. **Neurobio Dis.** v. 15, p. 461-473, 2004.

MATTSON, M.P. Calcium and neurodegeneration. **Aging Cell.**, v. 6, n. 3, p. 337-50, 2007.

MATTSON, M.P. Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. **Neuromolecular Med**. v. 3, p. 65-94, 2003.

MATTSON, M.P., MAGNUS, T. Ageing and neuronal vulnerability. **Nat. Neurosci**. v. 7, p. 278-294, 2006.

MATUTE, C. Interaction between glutamate signalling and immune attack in damaging oligodendrocytes. **Neuron Glia Biol**., v. 3, p. 281-285, 2007.

MELDRUM, B.S. Glutamate as a neurotransmitter in the brain: review of phisiology and pathology. **J Nutr**. v. 130, p. 1007S-1015S, 2000.

MENDIETA J., GAGO F., RAMÍREZ G. Binding of 5'-GMP to the GluR2 AMPA receptor: insight from targeted molecular dynamics simulations. **Biochemistry** v. 44, p. 14470-14476. 2005.

MOLZ S, DECKER H., DAL-CIM T., CREMONEZ C., CORDOVA F.M. LEAL R.B., TASCA C.I., Glutamate-induced Toxicity in Hippocampal Slices Involves Apoptotic Features and $p38^{MAPK}$ Signaling. **Neurochem Res.** v. 33, p. 27-36, 2008.

MOLZ, S. "Estudo da morte celular induzida por glutamato e sua modulação por guanosina-5´-monofosfato em fatias de hipocampo de ratos". Dissertação de mestrado, UFSC, Florianópolis, 2003.

MOLZ S., OLIVEIRA I.J.L., DECKER H., SOUZA D.O., TASCA C.I. Neurotoxicity induced by glutamate in glucose-deprived rat hippocampal slices is prevented by GMP. **Neurochem Res**. v. 30, p. 83-89, 2005.

MONAHAN J.B., HOOD W.F., MICHEL J., COMPTON R.P. Effects of guanine nucleotides on N-methyl-D-aspartate receptor-ligand interactions. **Mol Pharmacol**., v. 34, n 2, p. 111-6, 1988.

MORCIANO M., ORTINAU S., ZIMMERMANN H. Guanine nucleotides inhibit NMDA and kainate-induced neurotoxicity in cultured rat hippocampal and neocortical neurons. **Neurochem Int.** v. 45, p. 95-101, 2004.

MORETTO MB, ARTENI NS, LAVISKI D, NETTO CA, ROCHA JBT, SOUZA DO, WOLCHUK S. Hipoxic-ischemic insult decreases glutamate uptake by hippocampal slices from neonatal rats: Prevention by guanosine. **Exper. Neurob**. v. 195, p. 400-406, 2005.

MORO MA, CÁRDENAS A, HURTADO O, LEZA JC, LIZASOAIN I. Role of nitric oxide after brain ischemia. **Cell calcium** v. 36, p. 265-275, 2004.

MULLER, C.E., SCIOR, T. Adenosine receptors and their modulators. **Pharmaceutica Acta Helvetiae.** v. 68, p. 77-111, 1993.

MURPHY, S. Production of nitric oxide by glial cells: regulation and potential roles in the CNS. **Glia.** v. 29, n. 1, p. 1-13, 2000.

NAITO S., UEDA T. Characterization of glutamate uptake into synaptic vesicles. J**. Neurochem**., v.44, p.99-109, 1985.

NICHOLLS D.G. Oxidative Stress and Energy Crises in Neuronal Dysfunction. **Ann N Y Acad Sci**., v. 1147, p. 53-60, 2008.

NISHIMOTO T, KIHARA T, AKAIKE A, NIIDOME T, SUGIMOTO H. α-amino-3-hydroxy-5-methyl-4-isoxazole propionate attenuates glutamate-induced caspase-3 cleavage via regulation of glycogen synthase kinase 3β. **J. Neurosci Res.** v. 86, p. 1096–1105, 2008.

NOSHITA N., LEWÉN A., SUGAWARA, T., CHAN, P.H. Evidence of phosphorylation of Akt and neuronal survival after transient focal cerebral ischemia in mice. **J Cereb Blood Flow Metab.** v. 21, p. 1442-50, 2001.

OLESKOVICZ S.P., MARTINS M.C., LEAL R.B., TASCA C.I. Mechanism of guanosine-induced neuroprotection in rat hippocampal slices submitted to oxygen-glucose deprivation. **Neurochem Int.** v. 52, p. 411-418, 2008.

OLIVEIRA DL, HORN JF, RODRIGUES JM, FRIZZO ME, MORIGUCHI E, SOUZA DO. Quinolinic acid promotes seizures and decreases glutamate uptake in young rats: reversal by orally administered guanosine. **Brain Res**. v. 1018, p. 48−54, 2004.

OLIVEIRA I.J.L., MOLZ S., SOUZA D.O., TASCA C.I. Neuroprotective effect of GMP in hippocampal slices submitted to an in vitro model of ischemia. **Cel Mol Neurobiol**. v. 22, p. 335-344, 2002.

OSES J.P., VIOLA G.G, DE PAULA COGNATO G., JÚNIOR V.H., HANSEL, G., BÖHMER A.E., LEKE R., BRUNO A.N., BONAN C.D., BOGO, M.R., PORTELA, L.V., SOUZA D.O., SARKIS J.J. Pentylenetetrazol kindling alters adenine and guanine nucleotide catabolism in rat hippocampal slices and cerebrospinal fluid. **Epilepsy Res.**, v. 75, p. 104-11, 2007.

OUYANG Y.B., TAN Y., COMB, M., LIU, C.L., MARTONE, M.E., SIEAJÖ, B.K., HU, B.R. Survival- and death-promoting events after transient cerebral ischemia: phosphorylation of Akt, release of cytochrome C and Activation of caspase-like proteases. **J Cereb Blood Flow Metab**., v. 19, p. 1126-35, 1999.

OZAWA S., KAMIYA H., TSUZUKI, K. Glutamate receptors in the mammalian central nervous system. **Progr. Neurobiol**. v.55, p.581-618, 1998.

PAAS Y., DEVILLERS-THIERY A., CHANGEUX J.-P., MEDEVIELLE F., TEICHBERG V.I. Identification of an extracellular motif involved in the binding of guanine nucleotides by a glutamate receptor. **Europ Mol Biol Organ J**. v. 15, p. 1548-1556, 1996.

PAZ, M.M., RAMOS, M., RAMÍREZ, G., SOUZA, D.O. Differential effects of guanine nucleotides on kainic acid binding and on adenylate cyclase activity in chick optic tectum **FEBS Letters**. v. 355, p. 205-208, 1994.

PETTIFER, K.M., JIANG, S., BAU, C., BALLERINI, P., D'ALIMONTE, I., WERSTIUK, E.S., RATHBONE, M.P. MPP^+-induced cytotoxicity in neuroblastoma cells: Antagonism and reversal by guanosine. **Purinergic Signal**. v. 3, p. 399-409, 2007.

PETTIFER, K.M., KLEYWEGT, S., BAU, C.J., RAMSBOTTOM, J.D., VERTES, E., CICCARELLI, R., CACIAGLI F, WERSTIUK ES, RATHBONE MP. Guanosine protects SH-SYSY cells against beta-amyloid-induced apoptosis. **Neuroreport.** v. 15, p. 833-836, 2004.

PIERMARTIRI, T.C.B. Estudo bioquímico e comportamental em camundongos submetidos à infusão intracerebroventricular dos peptídeos beta-amilóide AB1-40 e AB25-35 e o papel neuroprotetor da atorvastatina. Dissertação de mestrado, UFSC, Florianópolis, 2009.

PIN J. P., DUVOISIN, R. Neurotransmitter receptors I. The metabotropic glutamate receptors: structure and functions. **Neuropharmacol**., v.34, p.1-26, 1995.

PORCIÚNCULA L.O., VINADÉ L., WOLFCHUK S., SOUZA D.O. Guanine based purines inhibit [^{3}H]glutamate and [^{3}H]AMPA binding at postsynaptic densities from cerebral cortex of rats. **Brain Res**. v. 928, p. 106-112, 2002.

PORTELA L.V., OSES J.P., SILVEIRA A.L., SCHMIDT A.P., LARA D.R., OLIVEIRA BATTASTINI A.M., RAMIREZ G., VINADÉ L., FREITAS SARKIS, J.J. SOUZA D.O. Guanine and adenine nucleotidase activities in rat cerebrospinal fluid. **Brain Res**., v. 950, p. 74−78, 2002.

RATHBONE MP, PILUTTI L, CACIAGLI F, JIANG S. Neurotrophic effects of extracellular guanosine**. Nucleosides, Nucleotides, and Nucleic Acids.** v. 27, p. 666–672, 2008.

RATHBONE M.P., MIDDLEMISS P.J., GYSBERS J.W., ANDREW C., HERMAN M.A., REED J.K., CICCARELLI R., DI IORIO P., CACIAGLI F. Trophic effects of purines in neurons and glial cells. **Prog Neurobiol.** v. 59, p. 663-690, 1999.

REGNER A., CRESTANA R.E., SILVEIRA F.J.P., FRIEDMAN G., CHEMALE I., SOUZA D. Guanine nucleotides are present in human CSF. **Neuroreport**, v. 8, n. 17, p. 3771-4, 1997.

REGO A.C., OLIVEIRA C.R. Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. **Neurochem Res**., v. 28, p. 1563-74, 2003.

ROSSI DJ, OSHIMA T, ATTWELL D. Glutamate release in severe brain ischaemia is mainly by reversed uptake. **Nature.** v. 403, p. 316-321, 2000.

RUPINDER S.K., GURPRET A.K., MANJEET S. Cell suicide and caspases. **Vascular Pharmacol**, v. 46, p. 383-93, 2007.

RYTER S.W., KIM H.P., HOETZEL A., PARK J.W., NAKAHIRA K., WANG X., CHOI AM. Mechanisms of cell death in oxidative stress. 2007. **Antioxid Redox Signal.** v. 9, n. 1, p. 49-89, 2007.

SAHA R.N., PAHAN K. Signals for the induction of nitric oxide syntase in astrocytes. **Neurochem Int**., v. 49, p. 154-163, 2006.

SAI K., YANG D., YAMAMOTO H., FUGIKAWA H., YAMAMOTO S., NAGATA T., SAITO M., YAMAMURA T., NISHIZAKI T. A1 adenosine receptor signal and AMPK involving caspase 9/3 activation are responsible for adenosine induced RCR-1 astrocytoma cell death. **Neurotoxicol.** v. 27, p. 458-467, 2006.

SANTOS T.G., SOUZA D.O., TASCA C.I. GTP uptake into rat brain synaptic vesicles. **Brain Res.** v. 1070, p. 71-76, 2006.

SAPOLSKY R. M. Cellular defences against excitotoxic insults. **J. Neurochem**. v. 76, p.601-1611, 2001.

SCHLAG B.D., VONDRASEK J.R., MUNIR M., KALANDADZE A., ZELENAIA O.A., ROTHSTEIN J.D., ROBINSON M.B. Regulation of the glial Na+-dependent glutamate transporters by cyclic AMP analogs and neurons. **Mol Pharmacol.**, v. 53, n. 3, p. 355-69, 1998.

SCHMIDT AP, LARA DR, SOUZA DO. Proposal of a guanine-based purinergic system in the mammalian central nervous system. **Pharmacol & Ther**. v. 116, p. 401-416, 2007.

SCHMIDT A.P., LARA D.R., DE FARIA MARASCHIN J., DA SILVEIRA PERLA A., ONOFRE SOUZA, D.Guanosine and GMP prevent seizures induced by quinolinic acid in mice. **Brain Res.** v. 864, p. 40-43, 2000.

SCHOUSBOE A., WAAGEPETERSEN H.S. Role of astrocytes in glutamate homeostasis: implications for excitotoxicity. **Exp. Brain Res**., v. 103, p. 51-58, 2005.

SCHUH R.A., MATTHEWS C,C., FISHMAN, P.S. Interaction of mitochondrial respiratory inhibitors and excitotoxins potentiates cell death in hippocampal slice cultures. **J Neurosci Res**., v. 86, p. 3306-13, 2008.

SCHUSTER D., RAJENDRAN A., HUI S.W., NICOTERA T., SRIKRISHNAN T., KRUZEL M.L. Protective effect of colostrinin on neuroblastoma cell survival is due to reduced aggregation of beta-amyloid. **Neuropeptides**, v. 4, p.419-26, 2005.

SELENICA ML, JENSEN HS, LARSEN AK, PEDERSEN ML, HELBOE L, LEIST M, LOTHARIUS J. Efficacy of small-molecule glycogen synthase kinase-3 inhibitors in the postnatal rat model of tau hyperphosphorylation. **Brit J Pharmacol**, v. 152, p. 959–979, 2007.

SEROU M.J., DECOSTER M.A., BAZAN N.G. Interleukin-1 beta activates expression of cyclooxygenase-2 and inducible nitric oxide synthase in primary hippocampal neuronal culture: platelet-activating factor as a preferential mediator of cyclooxygenase-2 expression. **J Neurosci Res**., v. 58, n 4, p. 593-8, 1999.

SHELDON A.L., ROBINSON M.B. The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. **Neurochem Int.**, v 51, p. 333-55, 2007.

SIMS KD, STRAFF DJ, ROBINSON MB. Platelet-derived growth factor rapidly increases activity and cell surface expression of the EAAC1 subtype of glutamate transporter through activation of phosphatidylinositol 3-kinase. **J. Biol Chem.** v. 275, p. 5228-5237, 2000.

SIMS K.D., ROBINSON, M.B. Expression patterns and regulation of glutamate transporters in the developing and adult nervous system. **Crit Rev Neurobiol**., v. 13, n. 2, p. 169-97, 1999.

SMAILI S.S, HSU I.-T, CARVALHO A.C.P., ROSENTOCK T.R., SHARPE J.C., YOULE R.J. Mitochondria, calcium and pro-apoptotic proteins as mediators in cell death signaling. **Braz J Med Biol Res.**, v. 36, p. 183-190, 2003.

SOARES F.A., SCHMIDT A.P., FARINA M., FRIZZO M.E., TAVARES R.G., PORTELA L.V., LARA D.R., SOUZA D.O. Anticonvulsant effect of GMP depends on its conversion to guanosine. **Brain Res**., v. 1005, p. 182-186, 2004.

SOUZA D.O., RAMÍREZ G. Effects of guanine nucleotides on kainic acid binding and on adenylate cyclase in chick optic tectum and cerebellum. **J Mol Neurosci**. v. 3, p. 39-45, 1991.

STONE T.W., ADDAE, J.I. The pharmacological manipulation of glutamate receptors and neuroprotection. **Eur J Pharmacol**. v. 447, p. 285-296, 2002.

SU C., ELFEKI N., BALLERINI P., D'ALIMONTE I., BAU C., CICCARELLI R., CACIAGLI F., GABRIELE J., JIANG S. Guanosine improves motor behavior, reduces apoptosis, and stimulates neurogenesis in rats with parkinsonism. **J Neurosci Res**., v. 87, p. 617-25, 2009.

TASCA C. I., SOUZA D. O. Interaction of adenosine and guanine derivatives in the rat hippocampus: effects on cyclic AMP levels and on the binding of adenosine analogues and GMP. **Neurochem Res.,** v, 25, p. 181−188, 2000.

TASCA C.I., BURGOS J.S., BARAT A., SOUZA D.O., RAMIREZ G. Chick kainate binding protein lacks GTPase activity**. Neuroreport**. v. 10, p. 1981-1983, 1999.

TASCA C.I., WOFCHUK S.T., SOUZA D.O., RAMÍREZ G., RODNIGHT R. Guanine nucleotides inhibit the stimulation of GFAP phosphorylation by glutamate. **Neuroreport.** v. 6, p. 249-252, 1995.

TAVARES R.G., TASCA C.I., SANTOS C.E., ALVES L.B., PORCIÚNCULA L.O., EMANUELLI T., SOUZA D.O. Quinolinic acid stimulates synaptosomal glutamate release and inhibits glutamate uptake into astrocytes. **Neurochem. Int.** v. 40, p. 621-627, 2002.

TAVARES R. G., SCHMIDT A. P., ABUD J., TASCA C. I., SOUZA D. O. In vivo quinolinic acid increases synaptosomal glutamate release in rats: reversal by guanosine. **Neurochem Res**., v. 30, p. 439−444, 2005.

TAVARES R. G., TASCA C. I., SANTOS C. E. S., WAJNER M., SOUZA D. O., DUTRA-FILHO C. S. Quinolinic acid inhibits glutamate uptake into synaptic vesicles from rat brain. **NeuroReport**, v. 27, p. 249−253, 2000.

TAVARES R.G., SCHMIDT A.P., TASCA C.I., SOUZA D.O. Quinolinic acid-induced seizures stimulate glutamate uptake into synaptic vesicles from rat brain: effects prevented by guanine-based purines, **Neurochem Res**., v. 33, n. 1, p. 97-102, 2008.

THOMAZI AP, BOFF B, PIRES TD, GODINHO G, BATTÚ CE, GOTTFRIED C, SOUZA DO, SALBEGO C, WOFCHUK ST. Profile of glutamate uptake and cellular viability in hippocampal slices exposed to oxygen and glucose deprivation: Developmental aspects and protection by guanosine. **Brain Res.** v. 1188, p. 233–240, 2008.

THOMAZI A.P., GODINHO G.F., RODRIGUES J.M., SCHWALM F.D., FRIZZO, M.E., MORIGUCHI E., SOUZA D.O.. WOFCHUK, S.T. Ontogenetic profile of glutamate uptake in brain structures slices from rats: sensitivity to guanosine. **Mech Ageing Dev**., v. 125, p. 475−481, 2004.

THORN J.A., JARVIS, S.M. Adenosine transporters. **Gen Pharmacol.**, v. 27(4), p. 613-20, 1996.

TRAVERSA U., BOMBI G., DI IORIO P., CICCARELLI R., WERSTIUK E.S., RATHBONE M.P. Specific [^{3}H]-guanosine binding sites in rat brain membranes. **British J Pharmacol.** v. 135, p. 969-976, 2002.

TROTTI D., DANBOLT N.C., VOLTERRA A. Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? **Trends Pharmacol Sci**., v.19, p. 328-334, 1998.

UEMURA Y., MILLER J. M., MATSON W. R. & BEAL, M. F. Neurochemical analysis of focal ischemia in rats. **Stroke ,** 22, p. 1548-1453, 1991.

VAISID T., KOSOWER N.S., ELKIND E., BARNOY S. Amyloid beta peptide toxicity in differentiated PC12 cells: calpain-calpastatin, caspase, and membrane damage. **J Neurosci Res.** v. 86, n. 10, p. 2314-25, 2008.

VESCE S., ROSSI D., BRAMBILLA L., VOLTERRA A. Glutamate release from astrocytes in physiological conditions and in neurodegenerative disorders characterized by neuroinflammation. **Int Rev Neurobiol.**, v. 82, p. 57-71, 2007.

VINADÉ E. R., SCHMIDT A. P., FRIZZO M. E. S., IZQUIERDO I., ELIZABETSKY E., SOUZA D. O. Chronically administered guanosine is anticonvulsant, amnesic and anxiolytic in mice. **Brain Res**., v. 977, p. 97−102, 2003.

VINADÉ E.R., SCHMIDT A.P., FRIZZO M.E., PORTELA L.V., SOARES F.A., SCHWALM F.D., ELISABETSKY E., IZQUIERDO I., SOUZA DO. Effects of chronic

administered guanosine on behavioral parameters and brain glutamate uptake in rats. **J Neurosci Res**., v. 79, p. 248−253, 2005.

WANG X., ARCUINO G., TAKANO T., LIN, J., PENG W.G., WAN P., LI P., XU Q., LIU Q.S., GOLDMAN S,A., NEDERGAARD M. P2X7 receptor inhibition improves recovery after spinal cord injury. **Nat Med**., v. 10, n 8, p. 821-7, 2004.

WATKINS J.C., JANE D.E. The glutamate story. **Br J Pharmacol.** v. 147, Suppl 1, S100-8, 2006.

WHITE B.C., SULLIVAN J.M., DEGRACIA D.J., O'NEIL B.J., NEUMAR R.W., GROSSMAN L.I., RAFOLS J.A., KRAUSE G.S. Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. **J. Neurol. Sci**., v. 179, p. 1-33, 2000.

WIERASZKO A., SEYFRIED, T.N. Increased amount of extracellular ATP in stimulated hippocampal slices of seizure prone mice. **Neurosci Lett.**, v. 106, n. 3, p. 287-93, 1989.

YAMAGUCHI A., TAMATANI M., NAMIKAWA K., KIYAMA H., VITEK M.P., MITSUDA N., TOHYAMA M. Akt activation protects hippocampal neurons from apoptosis by inhibiting transcriptional activity of p53. **J.Biol. Chem**, v. 276, n. 7, p. 256-64, 2001.

ZENG H., CHEN Q., ZHAO B. Genistein ameliorates beta-amyloid peptide (25-35)-induced hippocampal neuronal apoptosis. **Free Radic Biol Med**., v. 36, p. 180-8, 2004.

ZHAO H., SAPOLSKY R.M., STEINBERG G.K. Phosphoinositide-3-kinase/akt survival signal pathways are implicated in neuronal survival after stroke. **Mol Neurobiol**. v. 34, n. 3, p. 249-70, 2006.

ZIMMERMANN H., Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. **Progress in Neurobiology**. v. 49, p. 589-618, 1996.

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