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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

GUANOSINA: UMA NOVA ABORDAGEM DO SISTEMA PURINÉRGICO

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"Quem não traz na alma a imensidão do pampa se perde na pequenez de sua

própria existência."

"Isto nós sabemos.

Todas as coisas estão ligadas

Como o sangue

Que une uma família...

O Homem não tece a teia da vida;

Ele é apenas um fio.

Tudo o que faz à teia,

Ele faz a si mesmo"

Ted Perry – Inspirado no chefe Seattle

"Todo fazer é um conhecer e todo conhecer é um fazer."

Maturana & Varela

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Sumário

ABREVIATURAS

1S,3R-ACPD   1S,3R-aminociclopentano-1,3dicarboxilato

AMPA α-amino-3-hidróxi-5-metil-4-isoxazol-ácido propiônico

AMP_C Adenosina monofosfato cíclico

EAAT 1- 5   Transportador de aminoácidos excitatórios 1 até 5

GABA   Ácido gama-aminobutírico

GDP   Guanosina difosfato

GFAP   Proteína ácida fibrilar glial

GLAST   Transportador de glutamato e aspartato em ratos

GLT-1   Transportador de glutamato em cérebro de ratos

GMP   Guanosina monofosfato

GTP   Guanosina trifosfato

GTP-N   5´-Guanilimidodifosfato

iGluR   Receptores ionotrópicos para glutamato

KA   ácido caínico

L-AP4   L-2-amino-4-fosfonobutirato

mGluR   Receptores metabotrópicos para glutamato

MK-801   (+)-5-metil-10,11-diidro-5H-dibenzo[a,d]ciclohepteno-5,10-imina

NMDA   N-metil-D-aspartato

SNC   Sistema nervoso central

RESUMO

Durante as últimas décadas os estudos do sistema purinérgico concentraram seu foco de atenção nas ações dos derivados da adenina (como adenosina e o ATP). Seus efeitos, receptores, agonistas e antagonistas encontram-se muito bem estabelecidos dentro do sistema nervoso central. Os resultados obtidos com os diversos estudos dos derivados da guanina trazem uma nova perspectiva para o estudo do sistema purinérgico. Os nucleotídeos derivados da guanina são classicamente associados ao sistema de transmissão de sinal transmembrana via proteínas G. Além disto, suas ações extracelulares sobre o sistema nervoso central, especificamente sobre o sistema glutamatérgico, têm tornado essa classe de moléculas uma nova fronteira no estudo da neuroproteção. Estas moléculas também são capazes de promover processos trófico e mitóticos nas células do SNC, promover a liberação de fatores de crescimento e estimular o influxo de cálcio nos astrócitos.

Entretanto, suas ações sobre o sistema glutamatérgico são o alvo principal deste trabalho. Os derivados da guanina, especialmente a guanosina, são capazes de estimular a captação de glutamato em cultura de células astrocitárias e em fatias de tecido cerebral. Atuam como anticonvulsivantes pelas mais diversas vias de administração, e ainda possuem efeito amnésico. O aumento provocado na captação de glutamato parece ser realizado especificamente pela guanosina, uma vez que os nucleotídeos necessitam ser hidrolisados para exercerem tais efeitos. Assim a guanosina acaba assumindo um papel importante na neuroproteção contra os efeitos de concentrações extracelulares tóxicas de glutamato no SNC. Nosso trabalho demonstra que a guanosina também é a real efetora do

efeito anticonvulsivante apresentado pelo GMP, uma vez que o uso de inibidores da conversão de GMP para guanosina leva a uma diminuição do seu efeito. Ainda, a guanosina aumenta a V_{max} da captação de glutamato em fatias, o que indicaria um maior contingente de transportadores presentes na membrana da célula ou uma menor taxa de turnover dos mesmos. Esse efeito nos transportadores parece permanecer mesmo depois que a guanosina é retirada do meio de incubação. Os efeitos demonstrados pela guanosina *in vitro* foram confirmados em experimentos *ex vivo*. Além disso, a concentração de purinas no liquor dos ratos tratada não demonstrou aumentos significativos após a administração i.c.v. de guanosina ou GMP. Essa ação pode indicar que a guanosina dispara algum mecanismo que aumente o tônus glutamatérgico por um período maior do que o tempo de exposição a ela. Essas ações da guanosina devem ser desempenhadas através de seu receptor. Nossos resultados apontam para um novo receptor no sistema purinérgico, sensível à guanosina e adenosina, mas não antagonizado por cafeína e ATP. Esse receptor parece ter proteínas G acopladas, uma vez que o GTP-N foi capaz de inibir a união de guanosina ao receptor. Esse receptor deve responder de acordo com a purina que estiver ligada a ele, pois a adenosina e a guanosina têm ações diversas e muitas vezes contrárias no SNC. O sitio de união parece ser preferencialmente astrocitário, o que ajudaria a explicar as ações encontradas para a guanosina na captação de glutamato. Somando-se todas as ações já descobertas para a guanosina e as suas mais variadas formas de proteger o cérebro de excesso de glutamato extracelular, é impossível tratar a guanosina apenas como uma molécula coadjuvante no sistema purinérgico.

APRESENTAÇÃO

Essa tese traz uma breve introdução que trata do sistema glutamatérgico, enfocando o seu funcionamento geral, e ainda uma revisão sobre o papel da guanosina no sistema purinérgico, focando principalmente as suas ações sobre o sistema glutamatérgico.

Na seqüência, apresentamos os objetivos do nosso trabalho e os resultados obtidos. A apresentação dos resultados se dará na forma de artigos científicos. Em seguida faremos uma discussão geral dos resultados obtidos em conjunto com as conclusões do nosso trabalho. A seção das referências bibliográficas traz apenas os artigos utilizados na introdução, discussão e conclusões.

1 – INTRODUÇÃO

1.1 – O Sistema glutamatérgico

O aminoácido glutamato é o principal neurotransmissor excitatório do sistema nervoso central (SNC) de mamíferos, estando presente na maioria das sinapses centrais (Ozawa et al., 1998). O glutamato atua nas mais diferentes funções cerebrais, tais como aprendizado e memória, cognição e na formação de redes neurais durante o desenvolvimento e o envelhecimento (Collingridge & Lester, 1989; Izquierdo & Medina 1997; Ozawa et al., 1998; Castellano et al., 2001, Segovia et al., 2001). Além de desempenhar um papel fundamental em vários processos fisiológicos, em algumas situações patológicas existe uma hiperativação do sistema glutamatérgico que leva à toxicidade celular, chamada de excitotoxicidade (Lipton & Rosemberg, 1994).

O glutamato é sintetizado a partir de diversos precursores, sendo os mais comuns a glutamina, o oxalacetato e ainda através de outros aminoácidos por transaminação (Fonnum, 1984). Não há nenhuma comprovação de que alguma dessas vias seja específica para a formação de glutamato, que será usado como neurotransmissor ou para outras funções metabólicas. O armazenamento de glutamato no sistema nervoso central, que será utilizado como neurotransmissor, é realizado pelas vesículas que se encontram nos terminais pré-sinápticos. Essas vesículas podem concentrar 10 vezes mais glutamato do que os níveis encontrados normalmente no citosol dos terminais pré-sinápticos (Maycox et al.,

1990). Esse armazenamento é a forma como diferenciamos o glutamato quanto à sua utilização. Quando ocorre a despolarização dos terminais pré-sinápticos glutamatérgicos, o glutamato que se encontra nas vesículas é liberado para a fenda sináptica, por exocitose dependente da concentração de cálcio citosólico (Nicholls & Atwell, 1990, Vesce et al., 1999). O glutamato, uma vez liberado na fenda sináptica, vai interagir com seus receptores localizados nas membranas pré- e pós-sinápticas e também nas membranas gliais (Gallo & Ghiani, 2000; Scannevin & Huganir, 2000).

Para realizar as suas ações no SNC, o glutamato se une a uma multiplicidade de proteínas conhecidas como receptores. Esses receptores por sua vez estão divididos em duas classes: ionotrópicos e metabotrópicos (Conn & Pin 1997; Ozawa et al., 1998). Os receptores ionotrópicos (iGluR) são canais que permitem a passagem de íons específicos quando ativados (Ozawa et al., 1998) e foram subdivididos em N-metil-D-aspartato (NMDA), α-amino-3-hidroxi-5-metil-4-isoxazol-ácido propiônico (AMPA) e ácido caínico (KA), de acordo com a sensibilidade a agonistas. Os receptores NMDA são canais com grande permeabilidade a Ca^{2+}, baixa permeabilidade a Na^+ e K^+. Possui ainda uma cinética da abertura lenta. O receptor NMDA apresenta diversos sítios de modulação para a abertura do canal: um sítio para o glutamato ou NMDA, um sítio para o co-agonista glicina (insensível à estricnina), um sítio dentro do canal sensível à união de bloqueadores (MK-801, quetamina), um sítio para as poliaminas e sítios para o zinco e o magnésio (Ozawa et al., 1998). Quando a membrana neuronal está em repouso, o canal do receptor NMDA

encontra-se bloqueado por Mg^{2+}. A ativação do receptor NMDA e o influxo de íons só ocorrem se a membrana neuronal for previamente despolarizada, por exemplo através dos receptores AMPA, permitindo a saída do Mg^{2+} do canal. Alguns eventos neurotóxicos do glutamato são mediados por um grande influxo de Ca^{2+}, o que faz o receptor NMDA um alvo para alguns estudos referentes à excitotoxicidade (Ozawa et al., 1998). Os receptores AMPA são canais com alta permeabilidade ao Na^+ e K^+, com menor permeabilidade ao Ca^{2+}, enquanto os receptores do tipo KA são bastante permeáveis ao Ca^{2+} (Cotman et al., 1995). Os receptores AMPA estão amplamente distribuídos no SNC, mas com uma maior incidência nas regiões do hipocampo e na camada molecular do cerebelo (Ozawa et al., 1998). Os receptores iGluR do tipo AMPA e KA são responsáveis pela ativação rápida da neurotransmissão excitatória no SNC. Os receptores metabotrópicos (mGluR) pertencem a uma família de receptores que interagem com proteínas ligantes de nucleotídeos da guanina (proteínas G), que modulam a produção de efetores intracelulares, como através da adenilato ciclase e da fosfolipase C, responsáveis pela produção de segundos mensageiros (AMPc, diacilglicerol e inositol-3-fosfato), que por sua vez ativam e/ou inibem diversos eventos celulares (Conn & Pin, 1997; Ozawa et al., 1998). Os receptores mGluR podem ser ativados por ibotenato, quisqualato, 1S,3R-ACPD (1S,3R-aminociclopentano-1,3dicarboxilato) e L-AP4 (L-2-amino-4-fosfonobutirato), e estão subdivididos em subgrupos de acordo com a semelhança na sua seqüência de aminoácidos (Pin & Duvoisin, 1995) e sua sensibilidade a agonistas e respostas celulares envolvidas. Esses receptores

estão localizados em ambos os terminais pré- e pós-sinápticos, bem como em células gliais, e sua ativação pode promover efeitos inibitórios ou excitatórios (Ozawa et al., 1998).

Após a promoção de influxo iônico nas células pós-sinápticas e/ou a modulação da produção de segundos mensageiros, o glutamato precisa ser removido da fenda sináptica por sistemas de transporte dependentes de Na^+, localizados principalmente nas células astrocitárias (Robinson & Downd, 1997; Anderson & Swanson, 2000; Danbolt, 2001; Amara & Fontana, 2002). Devido à ausência de sistemas enzimáticos para metabolizarem o glutamato na fenda sináptica, os sistemas de captação de glutamato são responsáveis pela inativação da ação glutamatérgica. Os transportadores de glutamato são os seguintes: GLAST/EAAT1, GLT-1/EAAT2 (transportadores gliais), EAAC1/EAAT3 (transportador neuronal), EAAT4 (transportador predominante em células de Purkinje no cerebelo) e EAAT5 (transportador encontrado na retina) (Kanai & Hediger, 1992; Pines et al., 1992; Storck et al., 1992; Fairman et al., 1995; Arriza et al., 1997; Danbolt, 2001). Após a captação astrocitária, o glutamato pode ser metabolizado por duas diferentes vias: a formação da glutamina ou ser convertido a α-cetoglutarato. A transformação em glutamina é realizada pela ação da glutamina sintetase, enquanto que a formação do α-cetoglutarato é feita por desaminação através da glutamato desidrogenase ou ainda pela ação de transaminases. A glutamina e o α-cetoglutarato são liberados no fluído extracelular e podem ser captados pelos terminais pré-sinápticos neuronais. A glutamina é transportada para os neurônios, convertida pela glutaminase a glutamato, este por sua vez é captado

pelas vesículas sinápticas e liberado novamente, recomeçando o processo. Esse caminho do glutamato, passando pela sua transformação em glutamina, sua captação pelos neurônios e sua transformação novamente em glutamato, é conhecido como ciclo glutamato/glutamina (Anderson & Swanson, 2000; Danbolt, 2001; Amara & Fontana, 2002).

1.1.2 – A neurotoxicidade do glutamato

Olney e colaboradores (1970, 1978) observaram que altas doses de glutamato e seus agonistas provocavam dano e até mesmo a morte celular no SNC. Desta forma introduziram na literatura o termo excitotoxicidade para a morte neuronal provocada pela estimulação excessiva provocada pelo glutamato nos seus receptores. O papel do glutamato como mediador de excitotoxicidade parece estar relacionado ao aumento de Ca^{2+} intracelular causado pela estimulação excessiva de seus receptores e a subseqüente perda da homeostase do Ca^{2+} intracelular (Meldrum, 1994, Sattler & Tymianski, 2000). O aumento nos níveis de Ca^{2+} intracelular leva ao desencadeamento de uma série de eventos que incluem um aumento na produção de espécies reativas de oxigênio, maior influxo de cálcio e sódio e aumento do consumo de energia, culminando na morte neuronal (Dawson et al., 1991; Dubinsky & Rothman, 1991; Lei et al., 1992; Lafon-Cazal et al., 1993; Lipton & Rosenberg, 1994; Ozawa et al., 1998, Sattler & Tymianski, 2000). Entretanto, o comprometimento dos transportadores de glutamato gliais GLAST e GLT-1 tem merecido

destaque, desde quando experimentos com cultura de células neurais demonstraram que a morte neuronal por glutamato era atenuada quando neurônios eram co-cultivados com astrócitos (Rosenberg et al., 1992). Atualmente, está claro que os transportadores de glutamato astrocitários são os principais responsáveis pela manutenção da homeostase celular, uma vez que eles regulam os níveis extracelulares de glutamato na fenda sináptica (Anderson & Swanson, 2000; Tanaka, 2000; Gegelashvili et al., 2001; Danbolt, 2001, Amara & Fontana, 2002). Em estudos anteriores, em que os transportadores GLT-1 e GLAST não são expressos, foi observada uma maior susceptibilidade à epilepsia e neurodegeneração nos camundongos testados (Tanaka et al., 1997; Watase et al., 1998). A não expressão dos transportadores GLAST ou GLT-1 produz um aumento dos níveis extracelulares de glutamato e danos neuronais característicos de excitotoxicidade (Rothstein et al., 1996). Com todas essas evidências, tem-se estabelecido que os transportadores gliais são indiscutivelmente os responsáveis pela manutenção dos baixos níveis extracelulares de glutamato e por prevenir uma neurotoxicidade que possa ser causada por este aminoácido, sendo que aos transportadores neuronais caberia a função de carrear o glutamato para fins de modulação sináptica e metabolismo (Amara & Fontana, 2002).

As mortes celulares observadas em determinadas patologias no SNC como isquemia cerebral, esclerose lateral amiotrófica, Doença de Alzheimer, trauma cerebral, epilepsia, intoxicações por metais pesados, etc, foram relacionadas a comprometimentos

nos transportadores gliais GLAST e GLT-1 (Rothstein et al., 1992; 1995; Tanaka, 1997; Lipton, 1999; Aschner et al., 1995; 2000; Honig et al., 2000; Danbolt, 2001; Maragakis & Rothstein, 2001). Existem diversos protocolos onde podemos encontrar preparações passíveis de serem utilizadas para o estudo do transporte de glutamato, desde a cultura de astrócitos, passando pela utilização de fatias de cérebro de animais ou mesmo por preparações conhecidas como sinaptossomas (Frizzo et al., 2001, 2002; Leal et al., 2001). O fenômeno da modulação dos transportadores de glutamato torna-se um fator importante para o estudo do desenvolvimento dos danos neurotóxicos causados pelo excesso de glutamato na fenda sináptica. Dessa maneira, diversos trabalhos tratam do estudo da do transporte de glutamato no SNC, mostrando que a captação de glutamato pode ser afetada por fatores de crescimento, pelo próprio glutamato, derivados da guanina e também por algumas neurotoxinas (Swanson et al., 1997; Leal, et al., 2001; Danbolt, 2001; Frizzo et al., 2001, 2002; Gegelashvili et al., 2001; Tavares, et al., 2002; Emanuelli et al., 2003). Dessa forma o estudo de mecanismos de modulação parece ser a chave para prevenção dos danos neurotóxicos causados pelo glutamato em condições não fisiológicas.

1.2 – Os derivados da Guanina

Os nucleotídeos derivados da guanina (intracelulares) são classicamente associados ao sistema de transmissão de sinal transmembrana via proteínas G (Gudermann et al.,

1997). A união de neurotransmissores, ou agonistas aos seus receptores metabotrópicos, leva à formação de complexos ativos proteínas G/GTP. Quando ativadas por GTP, as proteínas G exercem dois efeitos simultâneos: modulam a atividade de efetores e diminuem a afinidade do agonista unido ao receptor (Morris & Malbon, 1999). A adenilato ciclase, fosfolipase C e os canais iônicos estão entre os efetores modulados por neurotransmissores, através de sistemas que operam com proteínas G (Gudermann et al., 1997). No SNC, esta forma de transdução do sinal celular está associada a subtipos de praticamente todos os receptores estudados: dopaminérgicos, glutamatérgicos, serotoninérgicos, purinérgicos, gabaérgicos, entre outros (Morris & Malbon, 1999). Estudos sobre a especificidade mostram que os nucleotídeos derivados da adenina não interagem com proteínas G (Gudermann et al., 1997).

Além da ação intracelular em proteínas G, existem evidências que indicam que os derivados da guanina podem atuar do lado externo da membrana plasmática celular, sem o envolvimento de proteínas G. Estudos sobre os efeitos dos derivados da guanina sobre a união do glutamato e agonistas foram feitos em preparações de membrana plasmática e demonstraram que GTP, GDP e GMP, e em alguns casos GMP cíclico, inibiram a união de glutamato, KA, L-AP4 e NMDA a seus receptores (Monahan et al., 1988; Baron et al., 1989; Yoneda et al., 1990; Souza & Ramirez, 1991; Gorodinsky et al., 1993; Migani et al., 1997; Ramos et al., 1997; Rubin et al., 1997A). Esses estudos foram realizados em preparações de membrana onde estavam presentes componentes pré- e pós-sinápticos, bem

como células gliais, não sendo possível determinar em quais componentes da sinapse os derivados da guanina estariam inibindo a união de glutamato e de seus agonistas. Os derivados da guanina também inibem a união de KA mesmo em condições onde a participação da proteína G pode ser minimizada (Souza & Ramirez, 1991; Barnes et al., 1993; Paz et al., 1994), e ainda inibem a união de antagonistas de glutamato, um efeito que não é modulado pelas proteínas G (Monahan et al., 1988; Baron et al., 1989; Barnes et al., 1993). Os derivados da guanina se mostraram capazes de inibir a união do glutamato e agonistas a seus receptores em preparações onde está presente apenas à porção pós-sináptica (densidade pós-sináptica) (Porciuncula et al., 2002). Além de atuarem sobre a união de glutamato e seus agonistas, os derivados da guanina atuam sobre a captação astrocitária do glutamato (Frizzo et al., 2003). Somados aos efeitos sobre sistema glutamatérgico, os derivados da guanina exercem um papel importante nos que diz respeito aos processos reparatórios mediados por astrócitos no SNC, e ainda possuem efeitos tróficos e mitóticos nos neurônios e em células da glia (Ciccarelli et al., 2000; Rathbone et al.,1999). Recentemente, foram preliminarmente descritos sítios de união específicos para guanosina em preparações de membranas de cérebro de ratos (Traversa et al., 2002). Como podemos observar, as mais diversas funções são desempenhadas pelos derivados da guanina, mas nessa tese abordaremos de uma maneira mais contundente os efeitos relacionados ao sistema glutamatérgico.

1.2.1 – As ações dos derivados da Guanina no SNC

Alguns trabalhos importantes vêm sendo realizados no sentido de elucidar o papel exercido pela guanosina no SNC. Em culturas de astrócitos, a exposição a condições de hipóxia associada à hipoglicemia (isquemia *in vitro*) eleva a concentração extracelular da guanosina em aproximadamente quatro vezes, sendo esta elevação maior e mais prolongada do que a elevação nos níveis de adenosina (Ciccarelli et al., 1999). *In vivo*, a isquemia cerebral produz um aumento dos níveis de guanosina de cerca de 140% por mais de uma semana após a injúria (Uemura et al., 1991). Além disso, um estudo de microdiálise no tálamo de ratos demonstrou que a despolarização in vivo por K^+, cainato e ouabaína elevam a concentração de guanosina e adenosina (Dobolyi et al., 2000). Com relação a efeitos fisiológicos, a guanosina exerce efeitos tróficos e mitóticos em células neurais, na faixa de concentração de 30 a 300 μM (Rathbone et al., 1999). Esses efeitos parecem ser mediados, em parte pela adenosina já que são atenuados por antagonistas de receptores de adenosina do tipo P1 e pela enzima adenosina deaminase (Ciccarelli et al., 2000). Além disso, foi demonstrado que a guanosina promove a liberação de adenosina de astrócitos (Ciccarelli et al., 2000). Por outro lado, a própria adenosina não é capaz de mimetizar o efeito da guanosina na sua totalidade, e parte do efeito da guanosina não é inibido por antagonistas P1 e pela adenosina deaminase (Rathbone et al., 1999; Ciccarelli et al., 2000). A guanosina parece ainda possuir uma ação antiapoptótica (Di Iorio et al., 2004), diferente da ação

encontrada para a adenosina (Di Iorio et al., 2002). O efeito antiapoptótico da guanosina está relacionado com a ativação de vias de comunicação intracelulares, como a da PI3-quinase/AKT/PKB em culturas de astrócitos. A guanosina ainda é capaz de proteger células da linhagem SH-SY5Y contra a apoptose provocada pela proteína β-amilóide (Pettifer et al., 2004). Ciccarelli e colaboradores (2001) descreveram o papel importante dos astrócitos nos processos reparadores que ocorrem no cérebro e mais recentemente descreveu-se um efeito remielinizante da guanosina na medula espinhal de ratos e levando ainda à recuperação da função perdida com a desmielinização (Jiang et al., 2003). O GTP atua sinergisticamente com o EGF para estimular o crescimento de neuritos em células da linhagem PC12 (Gysbers & Rathbone, 1996) e esse efeito envolve a via da ERK e ainda a mobilização de Ca^{2+} intracelular (Guarnieri et al., 2004). A guanosina também estimula o crescimento de neuritos em células PC12 através da ativação da heme oxigenase e aumento do GMPc intracelular (Bau et al., 2005). A guanosina aumenta os níveis intracelulares de Ca^{2+} livre em astrócitos de maneira similar à adenosina, a diferença entre os efeitos está na velocidade de desensibilização à droga (Chen et al., 2001). Esse aumento independe dos níveis de cálcio extracelulares e poderia ser exercido via receptores A3 (Chen et al.,2001).

1.3 – Interações entre o sistema glutamatérgico e os derivados da Guanina

Uma outra visão dos derivados da guanina diz respeito às suas ações sobre o sistema glutamatérgico. Os derivados da guanina bloqueiam respostas celulares à ação de glutamato ou seus agonistas tais como: inibem a quimioluminescência induzida por glutamato (Regner et al., 1998), bloqueiam o influxo de cálcio induzido por NMDA em retinas de pintos (Burgos et al., 2000), diminuem a fosforilação de GFAP e o aumento de AMP_C induzido por glutamato em preparações em que os nucleotídeos da guanina não penetram no espaço intracelular (Tasca et al., 1995; Tasca & Souza, 2000). No entanto, os efeitos dos nucleotídeos da guanina sobre os receptores glutamatérgicos não são particularmente potentes, ocorrendo geralmente na faixa de 100 μM a 1 mM (Monahan et al., 1988; Baron et al., 1989; Tasca et al., 1995; Regner et al., 1998; Burgos et al., 1998). Em estudos comportamentais o GMP é capaz de reverter o efeito facilitador do glutamato na tarefa de esquiva inibitória (Rubin et al., 1996; 1997B).

Nosso grupo de pesquisa tem apontado, a partir de vários trabalhos, para um possível papel neuroprotetor dos derivados da guanina contra as desordens do sistema glutamatérgico (excitotoxicidade) e ainda a processos fisiológicos que envolvam esse sistema. Trabalhos demonstram que o GMP e a guanosina são capazes de prevenir convulsões provocadas pelo ácido quinolínico, administrado no ventrículo lateral de cérebro de camundongos e ratos, (Schmidt et al., 2000). Também quando administrada por

via oral crônica ou agudamente administrada, (Lara et al., 2001; de Oliveira et al., 2004; Vinadé et al., 2003, 2004) a guanosina é capaz de atuar como anticonvulsivante. O GMP ainda protegeu as células de lesão induzida por ácido quinolínico em estriado de ratos (Malcon et al., 1997). Os nucleotídeos da guanina (GMP, GDP e GTP) são capazes de proteger culturas de neurônios hipocampais e corticais dos efeitos excitotóxicos do NMDA e KA, efeito que a guanosina não consegue repetir (Morciano et al., 2003). Ainda o GMP é capaz de proteger fatias de hipocampo de ratos submetidas à privação de glicose e a modelos de neurotoxicidade induzidos por glutamato e outros agonistas glutamatérgicos (ionotrópicos e metabotrópicos), diminuindo a liberação de lactato desidrogenase nestas fatias (Molz et al., 2005). Os derivados da guanina ainda são capazes de afetar a captação vesicular de glutamato, entretanto o efeito inibitório encontrado parece não depender do potencial eletroquímico vesicular, exceção feita ao GTP (Tasca et al., 2004).

Recentes trabalhos demonstraram que a guanosina quando testada na tarefa de esquiva inibitória apresentou efeitos amnésicos semelhantes aos antagonistas glutamatérgicos (Roesler et al., 2000; Vinadé et al., 2003, 2004, 2005), bem como protegeu as fatias corticais dos efeitos deletérios da privação de glicose e oxigênio, e estimulou a captação de glutamato em culturas de astrócitos (Frizzo et al., 2001; 2002). Estudos complementares demonstraram que a guanosina parece ser a principal responsável pelos efeitos estimulatórios na captação de glutamato exercido pelos derivados da guanina (Frizzo et al., 2003). Os efeitos da guanosina, via oral crônica, parecem não estar

relacionados com os receptores purinérgicos tradicionais, uma vez que a cafeína não foi capaz de reverter o efeito amnésico da guanosina (Vinade et al, 2004). Essa ausência de ação da cafeína está de acordo com outros estudos que mostram que ela também não consegue reverter os efeitos anticonvulsivantes da guanosina, quando esta é administrada oralmente (Lara et al., 2001). O efeito sobre a captação de glutamato parece ser especifico da guanosina, uma vez que os nucleotídeos precisam ser hidrolisados para exercer esse efeito, e que não ocorre efeito aditivo quando testados em conjunto com a guanosina (Frizzo et al., 2003). Ainda, os derivados da adenina não apresentam nenhum efeito significativo sobre o parâmetro de captação do glutamato em astrócitos (Frizzo et al., 2003). O efeito sobre a captação se repete em fatias de cérebro de ratos em condições normais e ainda a guanosina protege as fatias quando submetidas a condições de privação de glicose e oxigênio, provavelmente por aumentar a captação de glutamato (Frizzo et al., 2002). A administração crônica de guanosina via oral também é capaz de aumentar a captação de glutamato em fatias corticais de cérebro de ratos, e ainda o nível de guanosina no fluido cérebro-espinhal encontra-se aumentado no grupo tratado, quando comparado com o grupo controle (Vinade et al., 2005). A ação sobre a captação ainda parece ser específica sobre o sistema de captação do glutamato, uma vez que a guanosina não consegue aumentar a captação astrocitária do GABA (Frizzo et al., 2003). Recentes estudos demonstraram que, como na captação, os efeitos anticonvulsivantes dos nucleotídeos derivados da guanina são exercidos a partir da sua hidrólise, uma vez que compostos pouco

hidrolisáveis como o GTP e o GDP rígidos não são capazes de prevenir convulsões, efeito não encontrado quando se utilizam seus análogos, o GTP e o GDP (Schmidt et al., 2005). O aumento provocado pela guanosina na captação de glutamato parece depender da idade, uma vez que os efeitos da guanosina foram observados em culturas de astrócitos com 10 dias *in vitro* e não em 40 dias (Gottifried C., et al., 2002). Esse efeito parece se repetir em modelos mais complexos, uma vez que em fatias de ratos de diferentes idades, a guanosina só estimulou a captação de glutamato em ratos de 10 dias de vida e ainda seu efeito apresentou-se como dependente da estrutura cerebral utilizada (Thomazi et al., 2004).

Essa especificidade em relação à captação do glutamato, e ainda a valorização por ser um efeito exclusivo dos derivados da guanina, parece confluir para um novo *status* para os derivados da guanina. Já que se lembrarmos que em momentos de desajustes do SNC (hipóxia/isquemia), as purinas são liberadas, veremos que a guanosina é liberada em maior quantidade e a sua concentração permanece maior por mais tempo (Ciccarelli et al., 1999; Uemura et al., 1991). Além disso, a hidrólise dos nucleotídeos purinérgicos no fluído cérebro-espinhal, tem uma V_{max} e um K_m maiores para os derivados da Guanina, quando comparamos as velocidades de hidrólise do GDP e ADP, condição essa associada a um sabido aumento na liberação de purinas em condições de insulto neuronais, indica a formação de uma quantidade maior de guanosina e GMP (Portela et al., 2002). Somando-se a esses aspectos, os efeitos exercidos exclusivamente pela guanosina e não pela adenosina, como efeito antiapoptótico (Di Orio et al., 2002, 2004), e os efeitos sobre o sistema

glutamatérgico (Frizzo et al., 2003). Podemos inferir um papel diferenciado para a guanosina no campo do sistema purinérgico, à parte dos efeitos classicamente associados à adenosina. Além disso, essas novas funções parecem ter papéis importantes dentro das funções fisiológicas clássicas associadas aos derivados da guanina. Prova disso é o recente trabalho que apresenta uma hipótese sobre o papel de que alguma deficiência no metabolismo dos derivados da guanina, especificamente da guanosina, pode estar associada com anormalidades no neurodesenvolvimento, na neuromodulação e neurotransmissão associados com a síndrome de Lesch-Nyhan (Deutsch et al., 2005). Essa hipótese tem sua base teórica nos nossos trabalhos já citados, onde a guanosina é um modulador do sistema glutamatérgico, fisiologicamente, e em situações de insulto.

2 – OBJETIVOS

Essa tese tem como objetivo geral o estudo da modulação do sistema glutamatérgico exercida pelas purinas derivadas da guanina, principalmente sobre o transporte de glutamato. Esse estudo se deu com objetivos específicos que foram traduzidos nos artigos que fazem parte dos resultados dessa tese. Os objetivos específicos dessa tese são:

a) Determinar qual das purinas derivadas da guanina é responsável pela ação anticonvulsivante encontrada em um modelo de convulsão induzido pela administração i.c.v. do ácido quinolínico.

b) Realizar um estudo cinético dos efeitos da guanosina sobre a captação de glutamato em fatias de cérebro de ratos.

c) Determinar os efeitos da guanosina *ex vivo* e comparar com os efeitos *in vitro*.

d) Verificar a existência de um sitio de união para guanosina em preparações de membraans plasmáticas no SNC.

3 – RESULTADOS

Os resultados dessa tese serão apresentados na forma de artigos científicos enviados para publicação em revistas científicas internacionais. Os artigos serão organizados de acordo com os objetivos específicos apresentados para essa tese.

3.1 – ARTIGO I

Artigo publicado no Brain Research 1005:182-186 (2004)

Research report

Anticonvulsant effect of GMP depends on its conversion to guanosine

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Abstract

Studies on the purinergic system normally deal with adenine-based purines, namely, adenine nucleotides and adenosine. However, a guanine-based purinergic system may also have important neuromodulatory roles. Guanine-based purines exert trophic effects on neural cells, protect brain slices in a model of hypoxia and stimulate glutamate uptake. In vivo, both guanosine 5′-monophosphate (GMP) and guanosine (GUO) protected against seizures. In this study, we investigated if the anticonvulsant effect of GMP is mediated by guanosine and if guanosine or GMP treatments were able to increase adenosine levels. Intraperitoneal (i.p.) treatments with 7.5 mg/kg GMP or guanosine prevented 50% of seizures by quinolinic acid (QA) and increased guanosine cerebrospinal fluid (CSF) levels around twofold and threefold, respectively; GMP and adenosine levels remained unchanged. Intracerebroventricular treatment with 960 nmol GMP prevented 80% of seizures and the 5′-nucleotidase inhibitor α-β-methyleneadenosine 5′-diphosphate (AOPCP), when injected 3 min before, reduced this anticonvulsant effect to 30% protection as well as significantly decreased the conversion of GMP into guanosine measured in the CSF. This study shows that the previously reported effect of GMP as an anticonvulsant seems to be related to its ability to generate guanosine through the action of ecto-5′-nucleotidase.
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1. Introduction

Studies on the purinergic system normally deal with adenine-based purines, namely adenine nucleotides (e.g., ATP) and adenosine [20]. Others and we have suggested that a guanine-based purinergic system also has important neuromodulatory roles, which, similarly to the adenine-based purinergic system, are exerted by guanine nucleotides and the nucleoside guanosine (GUO). Of particular importance for the modulation of the purinergic system is the conversion of extracellular nucleotides to nucleosides, which regulates the relative concentrations of purines in the synaptic cleft and therefore their physiological roles. The enzymes responsible for the breakdown of GTP and GDP include one of the isoenzymes of the ecto-NTDPase family, whereas the conversion of guanosine 5′-monophosphate (GMP) into GUO by the action of ecto-5′-nucleotidases, which are also the enzymatic chain responsible for breakdown of adenine nucleotides [19].

In particular, in vitro studies have shown that GMP and GUO exert trophic effects on neural cells [3,13], protect brain slices in a model of hypoxia [5] and stimulate the removal of extracellular glutamate by astrocytes [4–6]. In vivo, both GMP and GUO protected against seizures induced by the glutamatergic agents, quinolinic acid (QA) or α-dendrotoxin (α-DTX) in rodents [1,10,15,17]. In these studies, the relative contribution of the conversion of GMP for GUO has not been addressed. Recently, our group demonstrated that glutamate

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uptake by astrocyte cultures was increased by GUO and by guanine nucleotides, but the inhibition of the breakdown of GMP into GUO, through addition of the ecto-5′-nucleotidase inhibitor α-β-methyleneadenosine 5′-diphosphate (AOPCP), prevented the effect of guanine nucleotides. Also, a poorly hydrolysable guanine nucleotide (GMP-PNP) failed to stimulate glutamate uptake, reinforcing the hypothesis that GUO is the final mediator of such effects of guanine nucleotides [6]. Another issue that has been addressed is the relative contribution of adenosine to the effects of GUO. Whereas adenosine may participate in the trophic effects of GUO [3,13], nonselective adenosine receptor antagonists, such as caffeine and theophylline, did not affect the effects of GUO on glutamate uptake [4] and on seizures [10].

In this study, we investigated if the anticonvulsant effect of GMP [10,15,17] is mediated by GUO and if in vivo GUO or GMP treatments were able to increase adenosine levels. To this end, we measured purine levels in the cerebrospinal fluid (CSF) of rats injected intraperitoneally (i.p.) with GMP and GUO as well as evaluated the anticonvulsant effect of GMP injected i.c.v. with or without pretreatment with the ecto-5′-nucleotidase inhibitor AOPCP.

2. Materials and methods

2.1. Materials

Guanosine (GUO), guanosine 5′-monophosphate (GMP), α, β-methyleneadenosine 5′-diphosphate (AOPCP), MK-801 (MK) and quinolinic acid (QA) were obtained from Sigma (St Louis, MO, USA). The anesthetic sodium thiopental was obtained from Cristália (Itapira, SP, Brazil). All solutions were dissolved in saline (NaCl 0.9%) and buffered with 0.1 N NaOH or 0.1 N HCl to pH 7.4 when necessary. All other chemicals were of analytical reagent grade and purchased from local suppliers.

2.2. Animals

Male adult Wistar rats (250–350 g) were kept on a 12 h light/dark cycle (light on at 7:00 am) at a constant temperature of 22±1 °C. They were housed in plastic cages (five per cage) with commercial food and tap water ad libitum. Our institutional protocols for experiments with animals, designed to minimize suffering and limit the number of animals sacrificed, were followed throughout.

2.3. Surgical procedure

Animals were anesthetized with sodium thiopental (40 mg/kg, 1 ml/kg, i.p.). In a stereotaxic apparatus, the skin of the skull was removed and a 27-gauge 9-mm guide cannula was unilaterally placed at 0.9 mm posterior to bregma, 1.5 mm right from the midline and 1.0 mm above the right lateral brain ventricle. Through a 2-mm hole made at the cranial bone, the cannula was implanted 2.6 mm ventral to the superior surface of the skull, and fixed with acrylic cement. In the experiments, performed 72 h after surgery, we used for i.c.v. infusion a 30-gauge cannula that was fitted into the implanted guide cannula and connected by a polyethylene tube to a microsyringe.

2.4. Treatments

2.4.1. Treatment 1

Rats were pretreated with an i.p. administration of vehicle (saline 0.9%), MK-801 (0.5 mg/kg), a noncompetitive NMDA receptors antagonist used as anticonvulsant in seizure models induced by over stimulation of the glutamatergic system [10,15], or three different doses (0.75, 2.0, 7.5 mg/kg) of GUO or GMP. After 30 min, an i.c.v. infusion of QA 39.2 mM (4 μl, 156.8 nmol) was performed, which produced tonic–clonic seizures within approximately 1 min in all rats. Animals were observed for 10 min in Plexiglas chambers for the occurrence of tonic–clonic seizures lasting more than 5 s [9]. Animals not displaying seizures during these 10 min were considered protected. Methylene blue (4 μl) was injected through the cannula and animals without contrast in the lateral brain ventricle were discarded.

2.4.2. Treatment 2

Another group of rats was treated similarly with i.p. administration of GUO and GMP; however, after 30 min, rats were anesthetized with sodium thiopental (40 mg/kg, 1 ml/kg, i.p.) and placed in a stereotaxic apparatus, where the CSF was drawn (40–60 μl per rat), for measuring GUO, GMP and adenosine, by direct puncture of the cisterna magna with an insulin syringe (27 gauge×1/2 in length), as described elsewhere [12]. All samples were centrifuged 12,000×g in an Eppendorf centrifuge 5402 during 5 min to obtain cell-free supernatants and stored in separate tubes in −70 °C until the quantification of purines.

2.4.3. Treatment 3

Rats were treated with an i.c.v. infusion of vehicle (saline 0.9%) or AOPCP 50 mM (5 μl, 250 nmol). After 3 min, an i.c.v. infusion (4 μl) of vehicle or 120 or 240 mM GMP (480 and 960 nmol, respectively) was made, followed 5 min latter by an i.c.v. infusion of either 4 μl of vehicle or 39.2 mM QA (156.8 nmol), the lowest dose causing seizures in all control animals. The observation procedures for seizures and the protection parameters were the same as described above.

2.4.4. Treatment 4

In other set of experiments, rats were treated with an i.c.v. infusion of 5 μl of vehicle or 50 mM AOPCP. After 3 min, an i.c.v. injection of 4 μl of vehicle or 240 mM GMP was made and the animals were observed for 5 min. Then, CSF was collected for measuring guanosine and GMP and treated as described before.
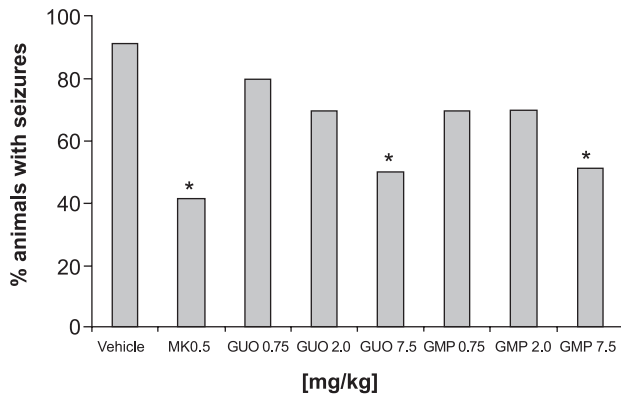
Fig. 1. Anticonvulsant effect of GUO and GMP i.p. against quinolinic acid in rats. All the animal groups were treated with QA (156.8 nmol in 4 μl) i.c.v. Either vehicle, MK801 or various doses of GUO and GMP were administered i.p. 30 min before i.c.v. injection of QA. The animals were then observed for 10 min for the occurrence of tonic–clonic seizures. $n=10-12$ animals per group. $*p<0.05$ (Fischer's exact test), as compared to vehicle group.

2.5. HPLC procedure

HPLC analyses of purines were performed with aliquots obtained of the cell-free supernatants from CSF. The measurement of purines was done according to Frizzo et al. [6]. Briefly, separation was carried out with a reverse phase column (Supelcosil LC-18, 25 cm×4.6 mm, Supelco) in a Shimadzu Instruments liquid chromatograph (100 μl loop valve injection). The elution was carried out applying a linear gradient from 100% of solvent A (60 mM KH_2PO_4 and 5 mM of tetrabutylammonium phosphate, pH 6.0) to 100% of solvent B (70% 100 mM KH_2PO_4 and 5 mM of tetrabutylammonium phosphate, pH 6.0, plus 30% acetonitrile) over a 40-min period (flow rate at 1.2 ml/min). The amount of purines was measured based on the absorption at 254 nm. The retention time of standards was used as identification and quantification parameters.

2.6. Statistical analysis

Statistical analysis between groups was performed by the Fisher exact test for the occurrence of seizures. Others analysis performed were ANOVA followed by Duncan's test when necessary and Student's t-test for comparison
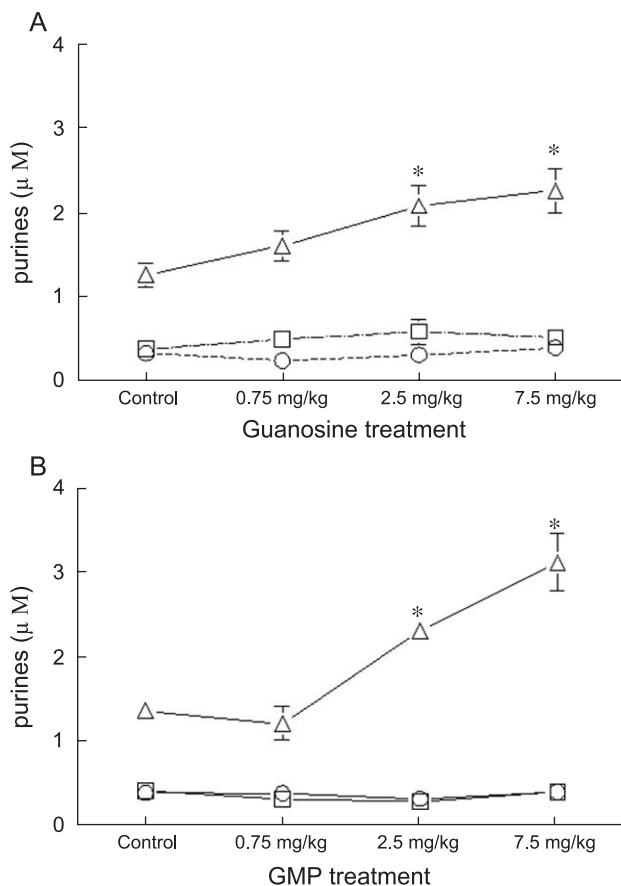

Fig. 2. I.p. administration of GUO and GMP increase GUO levels in CSF of rats. Animals received different doses of GUO (A) and GMP (B) and the levels of purines in the CSF were measured ($n=5$ for both treatments) 30 min later. △s represent GUO levels, Os are GMP levels, and □s are adenosine levels. (*) indicates difference from control, $p<0.05$.
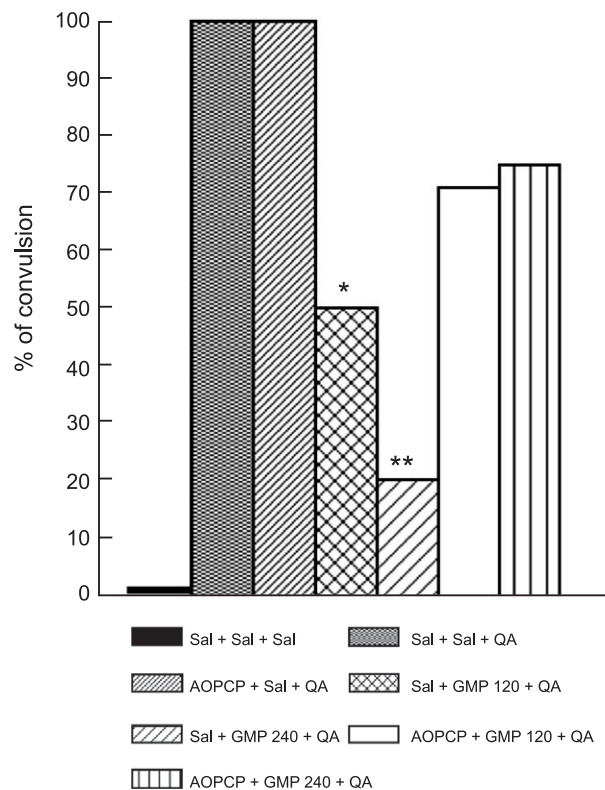

Fig. 3. AOPCP reduces the protection of GMP against quinolinic acid induced seizure. For procedure details, see Materials and methods section. For each group, $n\geq10$. (**) indicates a difference from control (Sal+Sal+AQ) at $p<0.01$, and (*) indicates a difference from same control at $p<0.05$.

between groups with and without AOPCP. All results with $p < 0.05$ were considered significant.

3. Results

In accordance with previous reports, i.p. administration of GMP or GUO dose-dependently prevented the induction of seizures by quinolinic acid, reaching a 50% protection at 7.5 mg/kg (Fig. 1). Interestingly, when we measured purine levels in the CSF of rats 30 min after the i.p. treatment with 7.5 mg/kg GUO or GMP (Fig. 2A and B, respectively), GUO levels increased around twofold and threefold for GUO and GMP, respectively, but GMP and adenosine levels remained stable.

In order to further evaluate the contribution of guanosine on the anticonvulsant effect of GMP, we established the protocol of i.c.v. route of GMP administration. Using this route, GMP exerted a significant and dose-dependent protection (up to 80%) against seizures induced by quinolinic acid (Fig. 3). I.c.v. treatment with the 5′-nucleotidase inhibitor AOPCP had no influence on seizures induced by quinolinic acid, but when injected 3 min before i.c.v. GMP, it inhibited the anticonvulsant effect of GMP (Fig. 3) to around 30% of protection. In order to confirm the involvement of rapid in vivo conversion of GMP to GUO, we measured purine levels 5 min after i.c.v. GMP administration with and without pretreatment with AOPCP. As expected, compared to the control group, the relative proportion of GMP over GUO in the CSF was significantly increased by the administration of AOPCP (Fig. 4). When purines were i.c.v. administered, their absolute levels varied considerably among rats: GUO and GMP levels for AOPCP group were 67.7 ± 47.2 and 203.2 ± 119.0 μM, respectively;

and for the group without AOPCP were 233.3 ± 106.7 and 13.23 ± 2.38 μM for GUO and GMP respectively. Thus, the results were expressed as the ratio of GMP/GMP+GUO.

4. Discussion

Recent studies on the extracellular effects of guanine nucleotides and GUO, along with evidence of specific binding sites [16], have strengthened the proposal of an independent guanine-based purinergic system in addition to the adenine-based purinergic system. In particular, GUO has been shown to exert in vitro trophic [13] and neuroprotective activity [5] as well as to stimulate glutamate uptake [4–6]; in vivo GUO has been shown to present anticonvulsant and amnesic effects in rodents [10,14,15,17]. The fact that both GMP and GUO were similarly effective in some of these actions, along with the presence of the converting enzyme ecto-5′-nucleotidase, prompted the hypothesis that GUO could be the mediator of GMP actions, which was confirmed in vitro for stimulation of glutamate uptake [6]. Because transport of nucleosides has been evidenced in intestinal cells [11], and they are transported across the blood brain barrier via a carrier-mediated process in cerebral microvessels [8], it is reasonable that guanosine can be active orally. Additionally, in previous works of our group, using guanine based purines in similar protocols, we demonstrated that neither guanosine nor GMP affects the body temperature [10,17]. The results of the present study suggest that also the in vivo anticonvulsant effect of GMP requires its hydrolysis to GUO, as shown by the increase of GUO after i.p. GMP administration and the significant inhibition of both GMP hydrolysis and its anticonvulsant effect by the ecto-5′-nucleotidase inhibitor AOPCP.

The protocol used in this study has the limitation that the assessment of seizures and purine levels in the CSF had to be performed separately. However, it is interesting to note that AOPCP was not fully effective in either parameter because after AOPCP administration, 20–30% of rats showed both lack of seizures after i.c.v. treatment with GMP and considerable GUO levels after i.c.v. treatment with GMP. This may be due to an uneven distribution of AOPCP and/or GMP after i.c.v. administration. Additionally, we could postulate that GMP itself may present less anticonvulsant activity, or that AOPCP could not optimally inhibit ecto-5′-nucleotidases using such protocol, allowing significant levels of GUO to be formed. Zhang et al. [18] showed that AOPCP injected into the rat prepiriform cortex produce generalized seizures, in contrast to our observations with i.c.v. injections of AOPCP, making the site of injection a likely explanation for this difference. In addition, to our knowledge, this is the first time that AOPCP treatment was used to affect in vivo behavioral responses to guanine-based purines, which reinforces the relevance of AOPCP in studies involving purine nucleotides and nucleosides in vivo. Another limitation of our study is that GUO receptor antago-
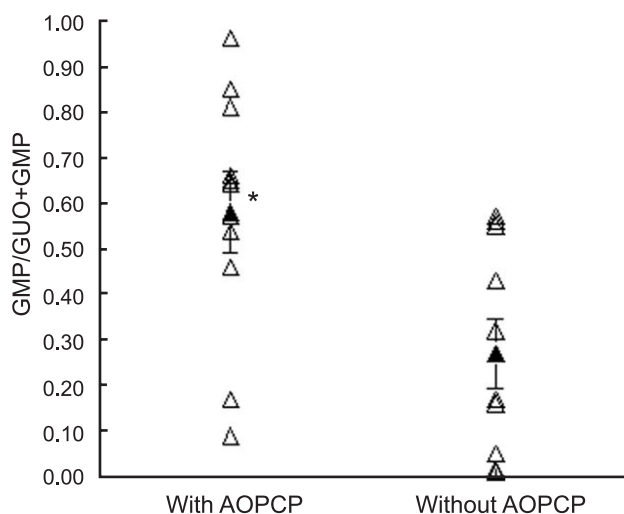
Fig. 4. AOPCP treatment alters the proportion of GUO and GMP levels in the CSF of rats treated with GMP. Animals are treated with ($n=11$) or without ($n=10$) AOPCP and received GMP 240 mM (980 nmol). (*) indicates a difference from groups with $p < 0.05$.

nists are not available, which would more directly assess the requirement of GUO production for GMP to exert its anticonvulsant effect.

As regards the involvement of adenosine on the effects of GUO, different experimental approaches have yielded conflicting results. GUO treatment has been shown to increase extracellular adenosine levels in astrocyte cultures and to induce proliferative effects that were partially inhibited by treatment with adenosine deaminase and adenosine receptor antagonists [2], pointing to some involvement of adenosine on GUO effects. In contrast, other guanosine effects as enhancement of neurite outgrowth in PC12 cells were not affected by adenosine receptor antagonists [7], which is also the case for the effect of guanosine on glutamate uptake [4], seizures induced by quinolinic acid [10] and impairment in inhibitory avoidance task (unpublished observations). In the present study, i.p. administered GUO and GMP, which latter produced GUO, failed to increase adenosine levels in the CSF.

Reinforcing the demonstration that the conversion of GMP to GUO seems to play a crucial role on its effects on glutamate uptake by astrocyte cultures [6], our results demonstrate that this conversion is also necessary for the in vivo model of convulsions because AOPCP diminished the protective effect of GMP. Importantly, the specificity of GUO an in vivo paradigm is relevant due to complexity of model used and is in line with results obtained with cell cultures.

In conclusion, this study shows that the previously reported effect of GMP treatment as an anticonvulsant seems to be related to its ability to generate GUO through the action of ecto-5′-nucleotidase. Therefore, administration of GMP may also be an effective strategy to increase GUO levels in future studies of possible therapeutic actions of GUO. In addition, most of the effects of GMP and GUO seem to be independent of adenosine, reinforcing the notion of a specific guanine-based purinergic system.

Acknowledgements

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3.2 – ARTIGO II

Artigo aceito para publicação no Cellular and Molecular Neurobiology 25: 911-919

Guanosine Enhances Glutamate Transport Capacity in Brain Cortical Slices

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SUMMARY

1. The effect of guanosine on L-[^{3}H] glutamate uptake was investigated in brain cortical slices within physio-pathological range of glutamate (1–1000 μM). In these conditions, glutamate uptake was significantly enhanced in slices treated with 100 μM guanosine only at 100 and 300 μM glutamate (44 and 52%, respectively).

2. Evaluation of kinetic parameters showed that guanosine affected significantly only uptake V_{max} (23%).

3. The guanosine withdrawal did not abolish its significant effect on glutamate uptake when 100 or 300 μM glutamate were used (an increase of 66 and 35%, respectively).

4. These results support the hypothesis of a protective role for guanosine during excitotoxic conditions when glutamate levels are enhanced (e.g. brain ischemia and seizures), possibly by activating glutamate uptake. Moreover, our results may contribute to understand the antiexcitotoxic mechanism of guanosine on glutamate transport, giving new information concerning its mechanism of action.

INTRODUCTION

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS), acting on ionotropic and metabotropic receptors to play several essential physiological roles, such as learning and memory, neural development and aging (Ozawa *et al.*, 1998; Izquierdo *et al.*, 1999; Meldrum, 2000; Segovia *et al.*, 2001). Considering that the extent of receptor stimulation depends on glutamate concentration in the surrounding extracellular fluid, the extracellular glutamate concentrations must be kept under physiological levels. In fact, a kind of toxicity called excitotoxicity,

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which is involved in the pathogenesis of various acute and chronic brain diseases, can be triggered if glutamate receptors are overstimulated (Greene and Greenamyre, 1996; Ozawa *et al.*, 1998; Lee *et al.*, 1999; Meldrum, 2000). Therefore, glutamate is responsible, e.g., for the "paroxysmal depolarizing shifts" characteristically recorded intracellularly in association with epileptic discharges (Meldrum *et al.*, 1999), and has long been recognized to play a key role in the pathology of ischemia (Nishizawa, 2001).

There is a general consensus concerning the role of adenosine in the CNS as an inhibitor of excitatory neurotransmission and as a neuroprotective agent during ischemic- and seizure-induced neuronal injury (Brundege and Dunwiddie, 1997). Nevertheless, compared to adenosine's well-established endogenous neuroprotective actions, the role of guanosine in the CNS has been less well characterized. However, it is noteworthy that extracellular concentrations of both nucleosides increase after depolarization (Dobolyi *et al.*, 2000) as well as after *in vivo* (Uemura *et al.*, 1991) and *in vitro* ischemia (Ciccarelli *et al.*, 1999), conditions where glutamate is also released (Meldrum *et al.*, 1999; Nishizawa, 2001; Phillis and O'Regan, 2003). Moreover, several studies showed a protection by guanosine against glutamate-receptors overstimulation (Schmidt *et al.*, 2000, 2004; Lara *et al.*, 2001; Oliveira *et al.*, 2004; Vinadé *et al.*, 2003, 2004; Soares *et al.*, 2004) probably via its activation of glutamate uptake (Frizzo *et al.*, 2001, 2002, 2003). However, the mechanisms involved with its neuroprotective and stimulatory effects are unknown. Therefore, the purpose of this study was to investigate if the nucleoside guanosine could affect kinetic parameters of glutamate transport. Additionally, we also investigated if guanosine effect would be maintained after its withdrawal.

METHODS

Animals and Reagents

Ten-day-old Wistar rats of both genders were used. The animals were maintained under controlled light and environmental conditions (12:12 h light-dark cycle, $22 \pm 1°C$) and had free access to food and water. The experimental protocols were approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, and followed the "Principles of Laboratory Animal Care" (NIH publication 85–23, revised 1985). The animals were decapitated, their brains immediately removed and submerged into Hank's balanced salt solution (HBSS). Cortices were dissected and coronal slices (0.4 mm) were obtained from the parietal area using a McIlwain tissue chopper, being separated with the help of a magnifying glass. All reagents were of P.A. purity and unless otherwise stated were purchased from Sigma (St Louis, MO).

Guanosine Treatment

The effect of guanosine was assessed in two different conditions: (a) when guanosine was present during pre-incubation (60 min) and glutamate uptake (7 min)

periods and, (b) only during pre-incubation time. In the last situation, after 60 min of exposure, guanosine was removed from the incubation medium by one wash with 1 mL of HBSS, before uptake assay. During the pre-incubation period the plates were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 35°C.

Glutamate Uptake

Uptake was assessed by adding 0.33 μCi mL^{-1} L-[2,3-^{3}H] glutamate (Amersham) with 1–1000 μM unlabeled glutamate in HBSS at 35°C. Incubation was stopped after 7 min by two ice-cold washes with 1 mL HBSS immediately followed by addition of 0.5N NaOH, which was kept overnight. Aliquots of lysates were taken for determination of intracellular content of L-[2,3-^{3}H] glutamate through scintillation counting. In order to determine the actual glutamate uptake, parallel experiments were done under ice and using *N*-methyl-D-glucamine instead of sodium chloride in the incubation medium, being subtracted from the uptake at 35°C. All experiments were performed in triplicate. Determination of protein was assessed using the method described by Peterson (1977).

Statistical Analysis

Data were analyzed using one-way ANOVA for multiple group comparison. Post-hoc analysis was carried out by Duncan multiple range test. Guanosine effect was analyzed using Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Stimulation of Glutamate Uptake by Guanosine

Considering our previous results showing that guanosine stimulates glutamate uptake *in vitro* (Frizzo *et al.*, 2001, 2002, 2003), we investigated the profile of guanosine effect on a broad range of glutamate concentration (1–1000 μM) in rat cortical slices. Figure 1 shows that the basal specific uptake of glutamate was significantly stimulated by 100 μM guanosine at 100 and 300 μM glutamate concentrations. At 100 μM glutamate, the basal uptake (0.123 ± 0.012) was enhanced up to 44% (0.177 ± 0.005) and at 300 μM (0.232 ± 0.012) it was increased up to 52% (0.353 ± 0.015). In lower and higher concentrations no stimulatory effect was observed.

Effect of Guanosine on Glutamate Uptake Kinetic Parameters

In order to determine whether this stimulation mediated by guanosine could modify kinetics parameters of the glutamate transport, we analyzed the results using Eadie–Hofstee plot. As shown in Fig. 2, treatment with guanosine caused a significant increase in glutamate transport capacity ($V_{\max}$) (up to 23%) but had no effect on the
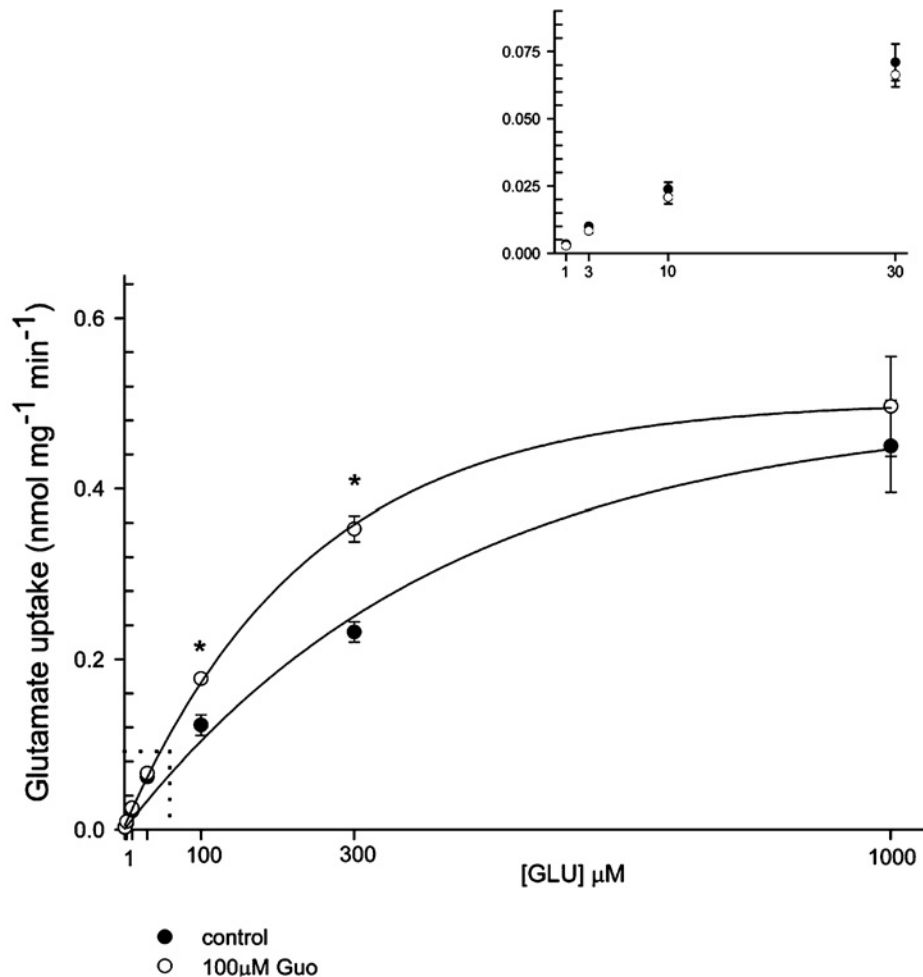
Fig. 1. Glutamate uptake in cortical slices with and without treatment with $100 \, \mu$M guanosine (Guo). Inset shows values among 1–30 μM glutamate (GLU). Data are expressed by mean $\pm$ SEM of 10 independent experiments. $*P < 0.01$, by unpaired Student's t-test when compared with respective slices untreated.

apparent glutamate K_m. Values of the calculated kinetics parameters are depicted in Table I.

Effects on Glutamate Uptake After Guanosine Withdrawal

In attempt to verify whether the guanosine-stimulatory effect observed persisted after removal of the nucleoside, slices exposed to 100 μM guanosine by 60 min were washed before glutamate uptake assay. In these set of experiments we used only glutamate concentrations that presented significant effects (100 and 300 μM).

Fig. 2. Eadie-Hofstee plot of glutamate uptake in cortical slices. Filled circles represent control groups; $100\,\mu$M guanosine corresponds to open circles. Lines were fitted by linear regression analysis. Data are represented by mean $\pm$ SEM of five independent experiments; V is expressed by nmol mg^{-1}min^{-1}.
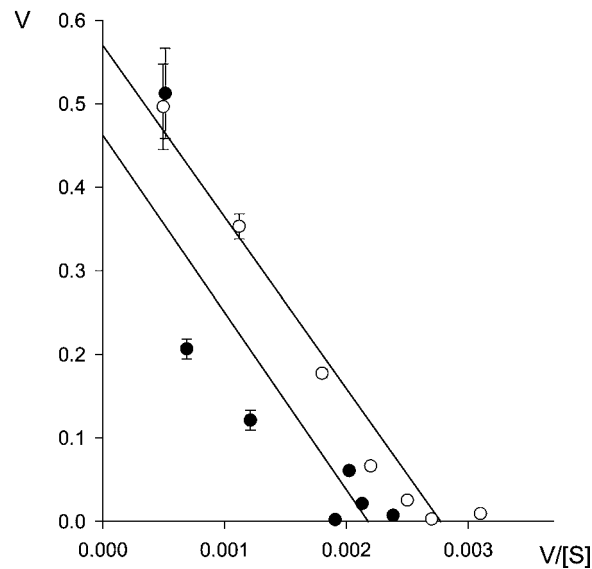
Results depicted in Fig. 3 show that the increase evoked by guanosine on glutamate uptake remained after its withdraw, by 66 and 35% respectively.

DISCUSSION

Cerebral tissue contains huge amounts of glutamate, but only a tiny fraction of it is normally present extracellularly. Despite its several brain functions in CNS (Ozawa *et al.*, 1998; Izquierdo *et al.*, 1999; Meldrum, 2000; Segovia *et al.*, 2001), glutamate at extracellular excessive concentrations can function as an excitotoxin, inducing neuronal injury and death (Ozawa et al., 1998; Lee *et al.*, 1999; Meldrum, 2000; Maragakis and Rothstein, 2004). In this context, astrocytic glutamate transporters play an important role in maintaining extracellular glutamate concentrations

Table I. Kinetics of L-[^{3}H] Glutamate Uptake into Cortical Slices

Treatment	V_{max} (nmol mg^{-1} min^{-1})	K_m (μM)
Control	0.469 ± 0.04	214 ± 8
$100\,\mu$M guanosine	$0.576 \pm 0.05^*$	206 ± 19

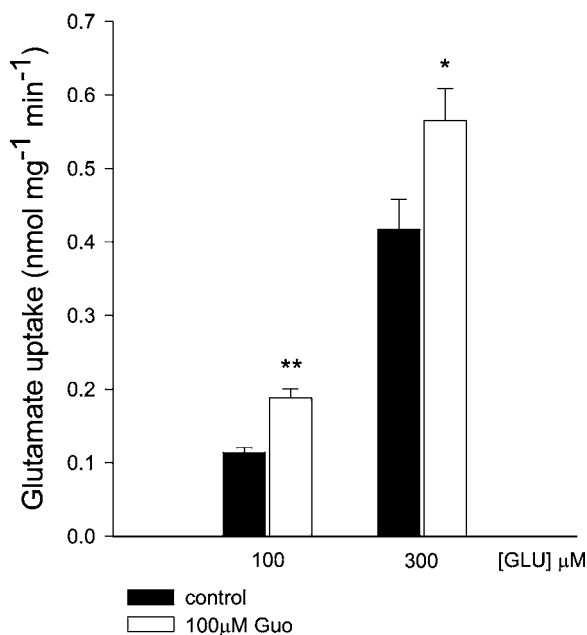$^*P < 0.05$, by unpaired Student's *t*-test when compared treated with untreated groups.

Fig. 3. Effect of guanosine pre-treatment on glutamate uptake. Slices treated with 100 μM guanosine (Guo) by 60 min were washed before addition of L-[^{3}H] glutamate (GLU) and compared with respective controls. Data are expressed by mean $\pm$ SEM of six independent experiments. *$P < 0.05$, **$P < 0.001$, by unpaired Student's t-test when compared with respective group.

below neurotoxic levels (Anderson and Swanson, 2000; Danbolt, 2001). Thus, the balance between physiological and pathological conditions is provided by a sophisticated modulatory system. Indeed, it has been shown that glutamate uptake can be stimulated by guanosine (Frizzo *et al.*, 2001, 2002, 2003), by glutamate and analogs (Gegelashvili *et al.*, 1996; Duan *et al.*, 1999; Munir *et al.*, 2000) as well as by synthetic neuroprotective compounds (Asai *et al.*, 1999; Shimada *et al.*, 1999).

Even though there are several studies showing neuroprotective actions of guanosine against glutamatergic overstimulation (Schmidt *et al.*, 2000, 2004; Lara *et al.*, 2001; Oliveira *et al.*, 2004; Vinadé *et al.*, 2003, 2004; Soares *et al.*, 2004), the mechanism involved is still unknown. One possible explanation could involve its stimulatory effect on glutamate uptake, shown by different *in vitro* models (Frizzo *et al.*, 2001, 2002, 2003). Here the guanosine effect on glutamate uptake was investigated on a broad range of glutamate concentration (1–1000 μM). The fact that guanosine presented stimulatory effect only at higher concentrations (100 and 300 μM) is relevant to its neuroprotective actions, and corroborates our previous results (Frizzo *et al.*, 2002). So it is reasonable to suppose that guanosine exerts neuroprotective roles only when glutamate concentrations are getting higher, being ineffective at physiological glutamate levels.

Considering our results, guanosine-stimulatory role on glutamate uptake at physio-pathological concentrations could be explained by its capacity of increasing the uptake V_{max}, without affecting the affinity of glutamatergic receptors by glutamate (K_m). Other works using glutamate and analogs during a pre-treatment period showed a stimulatory effect on glutamate uptake (Gegelashvili *et al.*, 1996; Duan *et al.*, 1999; Munir *et al.*, 2000), some of them by enhancement of uptake V_{max} and involvement of transporter translocation to (or decreased removal from) the cell membrane (Duan *et al.*, 1999; Munir *et al.*, 2000). Therefore, one might hypothesize that the mediated increase in V_{max} by guanosine could also be due to the increase in the number of glutamate transporters in the cell surface (or in their catalytic efficiency). Importantly, in the works that studied up-regulation of glutamate transport substrate-induced, no significant alteration on K_m values was observed, similar to our results.

Here, we also observed that guanosine stimulatory effect remained after its withdrawal, which is similar to results where glutamate pre-incubation was used to up-regulate substrate-transport even after washing procedures (Duan *et al.*, 1999). Although we are sure that guanosine was removed from the incubation medium, and that we are reproducing previous observations from the literature (Duan *et al.*, 1999), we cannot rule out the possibility that guanosine had not been completely removed from the neurophil. Of note, the stimulatory effect on glutamate uptake presented similar magnitude in the absence or presence of the nucleoside. Our results obtained using slices strengthen previous findings with astrocyte cultures (Frizzo *et al.*, 2001, 2003), given that it is a brain preparation where interactions among neural cells are mostly preserved.

Thus, the results presented here provide new important information over previous works of our group: (i) indicate that the stimulatory effect of guanosine on glutamate uptake was exerted on the transporter capacity (number of transporters?) and (ii) indicate that the stimulatory effect remains (at least for short time) even after the guanosine withdrawal (which reinforce that the guanosine effect could be due to the recruitment of glutamate transporters to cells surface).

The ability to stimulate the uptake confers to guanosine a putative therapeutic relevance by its anti-excitotoxic role.

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3.3 – ARTIGO III

Artigo submetido para publicação no Neurochemical Research

Guanosine reinforces the glutamatergic tonus by increasing the uptake and release of glutamate

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Running head: Guanosine reinforces the glutamatergic tonus

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Abstract

The aim of this study was to determine the effect of guanosine on the glutamatergic tonus in *ex vivo* and *in vivo* experiments. Thus, we studied if guanosine *in vitro* could affect L-[^{3}H]glutamate release, uptake and accumulation by synaptosomes, as well as vesicular glutamate uptake. I.c.v. guanosine effects on the same parameters in *ex vivo* preparations were also studied. Our results show an increase in synaptosomal release, synaptosomal uptake, and vesicular uptake both *in vitro* and *ex vivo* assays. *In vitro* synaptosomal accumulation was also increased. Our results indicated that effects *in vitro* of guanosine could be reproduced in a more complex *ex vivo* study. We could suggest that guanosine may reinforce the glutamatergic tonus.

Keywords: guanosine, glutamate uptake, glutamate release, synaptosomes, synaptic vesicles, glutamatergic tonus.

Introduction

Glutamate, the main excitatory neurotransmitter in the mammalian central nervous system (CNS), plays important functions in several brain physiological and pathological events. Glutamate participates in various plastic processes, being involved in ontogeny and ageing, memory and learning, and formation of neuronal networks (1,2). However, overstimulation of the glutamatergic system may lead to excitotoxicity, a phenomenon involved in physiopathogeny of various acute and chronic disorders of the CNS (1-3). Thus, the maintenance of extracellular glutamate concentrations below toxic levels is essential for normal brain development and function.

The removal of glutamate from the synaptic cleft, which occurs through high affinity of sodium-dependent membrane transporters located mainly in astrocytes, is a key mechanism for modulation of glutamate actions, and also for maintaining its extracellular concentrations under control (1). Inside astrocytes, glutamate is converted to glutamine, which is transferred to presynaptic terminals (glutamate/glutamine cycle), where it is converted again to glutamate and a low affinity Na^+-independent carrier finally takes up glutamate into synaptic vesicles. This mechanism counts to maintain the glutamate/glutamine cycle activity, which is crucial for the glutamatergic synapses activity and is responsible by most of energy used by these synapses (4).

Extracellular guanine based-purines (GBPs) modulate the glutamatergic system in physiological and pathological conditions. GBPs inhibit cell responses to glutamatergic ligands (5,6); GMP and guanosine (GUO) prevent seizures induced by quinolinic acid (QA), kainate and α-dendrotoxin (7-10). Furthermore, the inhibitory effect of GBPs on the binding of glutamate and analogs in different preparations from chick, rat and goldfish brain was described (11-13). GBPs have been demonstrated to enhance glutamate uptake in astrocyte cultures (14,15) and brain cortical slices (16). In addition, extracellular GBPs

exert additional important functions such as inductors of trophic effects on neural cells and astrocytes growth and proliferation (17).

The presence of nucleotides, nucleosides and their metabolites in cerebrospinal fluid has been earlier described (18), and it has been shown that their concentrations are elevated during ischemic events (19,20). *In vivo*, the extracellular concentration of purines depends on a balance between the cell release and uptake as well as on extracellular metabolism (17).

Recent works (9,15) established that the GBPs effects on glutamate uptake and as anticonvulsants were exerted specifically by GUO, being the observed effects attributed to other GBPs due to their conversion to GUO. As GUO effects persist in the presence of classic purine receptor antagonists or nucleoside uptake inhibitors (14,17), it is reasonable to presume that GUO effects involve specific membrane receptors in the brain (21).

Moreover, GUO has been shown to exert trophic effects on neural cells (17), to preserve viability in mouse spinal cord cultures during chemical hypoxia (22), to promote myelination and functional recovery in chronic spinal injury (23), and to stimulate neurite outgrowth in PC2 cells (24).

The main objective of this study was to determine the effect of GUO on various parameters of the glutamatergic system through *in vitro* studies and if the effect of guanosine could be also observed in an *ex vivo* protocol. Accordingly, we investigated in rats if: (i) GUO *in vitro* affects L-[^{3}H]glutamate release from synaptosomes; (ii) GUO *in vitro* affects the synaptosomal and vesicular L-[^{3}H]glutamate uptake; (iii) *in vivo* acute unilateral intracerebroventricular (i.c.v.) administration of GUO (4uL/660uM) affects synaptosomal L-[^{3}H]glutamate release and uptake.

Experimental Procedures

Animals and reagents

Two month old Wistar rats (180-250g) were maintained at 25°C on a 12:12h light/dark cycle (lights on at 06:00 h), with tap water and standard lab chow (Guabi, Santa Maria, RS, Brasil) *ad libitum*. They were housed in plastic cages (five per cage). Animals were killed by decapitation, the brains rapidly removed and the cerebral cortices dissected. Our institutional protocols for experiments with animals, designed to minimize suffering and limit the number of animals sacrificed, were followed throughout the experiments.

L-[^{3}H]glutamate (56 Ci/mmol) was from Amersham International, (UK), and other chemicals were of analytical reagent grade. The anesthetic sodium thiopental was obtained from Cristália (Itapira, SP, Brasil).

Surgical procedure and i.c.v. infusion techniques

Animals were anesthetized with sodium thiopental (25 mg/kg, 2 mL/kg, i.p.). In a stereotaxic apparatus, the skin of the skull was removed and a 27-gauge guide cannula was placed 0.9 mm posterior to bregma, 1.5 mm right from the midline and 1 mm above the lateral brain ventricle. The cannula was implanted through a 2 mm hole made at the cranial bone, and fixed with jeweler acrylic cement. Experiments were performed 48 h after surgery. A 30-gauge cannula was fitted into the guide cannula and connected by a polyethylene tube to a 10 uL Hamilton syringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula aiming the lateral brain ventricle. We performed an i.c.v. infusion of 4 uL of saline or GUO (660 uM) and rats were observed for 30 min in Plexiglas chambers before being employed in synaptosomal or synaptic vesicle experiments.

Purines measurement (HPLC procedures)

Animals received an i.c.v. infusion of either 4uL of saline (control), GUO or GMP (both 660 uM, 2.64 nmoles). After 30 min, these animals were anesthetized and placed in a stereotaxic apparatus and the CSF was drawn (40-60 uL per rat) by direct puncture of the cisterna magna with an insulin syringe (27 gauge×1/2" length), as described elsewhere (25).

All samples were centrifuged to obtain cell-free supernatants and stored in separate tubes in -70ºC until the quantification of purines.

The quantification of purines in the CSF was performed in accordingly to Frizzo (15). Briefly, HPLC analyses were carried out with a reverse phase column (Supelcosil LC-18, 25 cm x 4.6 mm, Supelco) in a Shimadzu Instruments liquid chromatograph (50 uL loop valve injection). The elution was carried out applying a linear gradient from 100% of solvent A (60 mM KH_2PO_4 and 5 mM of tetrabutylammonium phosphate, pH 6.0) to 100% of solvent B (70% 100 mM KH_2PO_4 and 5 mM of tetrabutylammonium phosphate, pH 6.0, plus 30% acetonitrile) over a 40 min period (flow rate at 1.2 mL/min). The amounts of purines were measured on the basis of the absorption at 254 nm. The retention time of standards was used as an identification and quantification parameter.

Synaptosomal preparations

Animals were decapitated and the forebrain was used to prepare synaptosomes on a discontinuous Percoll gradient according to Dunkley and colleagues (26). Synaptosomes were used in the same day of preparations. Synaptosomal preparations were suspended in oxygenated Krebs-Ringer buffer (KRB) containing 150 mM NaCl, 2.4 mM KCl, 1.2 mM Na_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM MgSO4, 5 mM Hepes-Tris, pH 7.4, and 10 mM glucose. These preparations contain 5% contamination with inner and outer mitochondrial membranes fragments, microsomes, myelin, as well as neural and glial plasma membranes (27).

Measurement of lactate dehydrogenase (LDH) activity

The synaptosomal preparation integrity was evaluated by lactate dehydrogenase (LDH; E1.11.27) release. LDH release was monitored by incubation synaptosomes with GUO (10nM-1mM) or saline (control) for 15 min. The LDH activity in the incubation medium and the total LDH content, which was determined by disruption of the

synaptosomal preparation using 1.5% Triton X-100, were evaluated spectrophotometrically using an assay kit (Labtest reagents, Brasil), which measure the amount of a colored complex derived from the NADH formed by the enzymatic reaction using a spectrophotometric method (510 nm).

L-[³H]glutamate accumulation by synaptosomal preparations previously depleted from endogenous glutamate and non-previous depleted

The glutamate accumulation was performed accordingly to Bole (28), with a few modifications. Briefly, synaptosomes in 1-ml aliquots (0.9–1.6 mg/mL) were pelleted (16,000 x g, 3 min), suspended in HBSS (composition in mM: HEPES 24, NaCl 119, KCl 2.1, $MgSO_4$ 1.08, KH_2PO_4 1.08, glucose 10.8, $CaCl_2$ 0.9), pH 7.4 (adjusted with HCl). In the synaptosome fraction that depleted from endogenous glutamate the KCl concentration used was 56 mM. The aliquots are incubated at 37º C for 20 min. After this procedure, synaptosomes were washed twice and suspended in ice-cold nondepolarizing HBSS medium. Pretreated synaptosomes were dispensed in 0.1-mL aliquots for glutamate uptake assay (500 nM of glutamate and 45 nM of [³H]-glutamate) for 1 min at 37º C in the presence or absence of GUO. Synaptosomal uptake was terminated by adding 0.9 mL ice-cold HBSS. Aliquots (0.1 mL) were immediately removed and filtered on glass fiber filters (Whatman GF/C) to determine the total amount of glutamate taken up.

L-[³H]glutamate uptake by synaptosomes

Synaptosomal preparations were washed twice in 3 vol 0.3 M sucrose, in 15 mM Tris/acetate buffer (pH 7.4) and centrifuged at 35,000 x g for 15 min. The final pellet was resuspended in 0.3 M sucrose, 15 mM Tris/acetate buffer (pH 7.4), and incubated in HBSS, pH 7.4, in the presence of 100 nM L-[³H]glutamate, for 1 min at 37° C. The reaction was stopped by filtration through GF/B filters (Whatman). The filters were washed three times with 3 mL ice-cold 15 mM Tris/acetate buffer (pH 7.4) in 155 mM ammonium acetate. The

radioactivity retained on the filters was measured in Wallac model 1409 liquid scintillation counter. Na^+-dependent L-[^{3}H]glutamate uptake was calculated as the difference between the uptake obtained in the incubation medium described above, and the uptake obtained with a similar incubation medium in which choline chloride was substituted for NaCl.

L-[^{3}H]glutamate release from synaptosomal preparations

L-[^{3}H]glutamate release was measured according to Migues (29). Briefly, synaptosomal preparations were incubated in HBSS, pH 7.4, for 15 min at 37° C in the presence of 500 nM L-[^{3}H]glutamate. Aliquots of labeled synaptosomes (1.3 mg protein) were centrifuged at 13,000 x g for 1 min at 4°C. Supernatants were discarded, and the pellets were washed four times in HBSS by centrifugation at 13,000 x g for 1 min at 4° C. In order to measure the basal release of L-[^{3}H]glutamate the final pellet was resuspended in HBSS and incubated for 1 min at 37° C in the presence or absence of GUO. Incubation was terminated by immediate centrifugation (16.000 x g for 1 min at 4°C). K^+-stimulated L-[^{3}H]glutamate release was assessed as described for basal release, except that the incubation medium contained 40mM KCl to induce synaptosomal depolarization. The total amount of glutamate loaded into synaptosomes under these conditions was 9.9 pmol/mg of protein.

Radioactivity present in supernatants and pellets was separately determined in a Wallac scintillation counter. The released L-[^{3}H]glutamate was calculated as percentage of the total amount of radiolabel in the synaptosomal preparation at the start of the incubation period (preloaded synaptosomes).

Synaptic vesicles preparation

Synaptic vesicles were isolated from rat brains as described by Fykse and Fonnum (30). Two whole brains were homogenized (10% wt/vol) in a buffer containing 0.32 M sucrose, 10 mM Mops/Tris, pH 7.4, and 1 mM EDTA and centrifuged twice for 10 min at

1000 x g. Both supernatants were pooled and centrifuged for 30 min at 20,000 x g to obtain the crude synaptosomal fraction (P2). This fraction was osmotically shocked by resuspension in 10 mM Mops/Tris, pH 7.4, containing 0.1 mM EGTA (~0.8 mL/g of fresh tissue) and centrifuged at 17,000 x g for 30 min. The supernatant containing synaptic vesicles was subjected to 0.4 M and 0.6 M sucrose density gradient centrifugation at 65,000 x g for 2 h. The synaptic vesicle fraction was isolated from the 0.4 M sucrose band and stored at -70 °C.

L-[^{3}H]glutamate uptake by synaptic vesicles

Uptake experiments were performed in a standard medium (final volume of 200 uL) composed of 10 mM Mops/Tris, pH 7.4, 4 mM KCl, 140 mM potassium gluconate, 0.12 M sucrose, 2 mM MgCl$_2$, 2 mM ATP, as previously described (31), with 50 uM L-[^{3}H]glutamate (3 uCi/mL). Incubation was carried out for 10 min at 35° C, and stopped by rapid filtration of the suspension through 0.45 um Millipore filters. The filters were quickly flushed three times with 4 mL of 10 mM Mops/Tris, pH 7.4, at room temperature. Specific uptake was calculated by discounting the uptake measured in the absence of ATP. Radioactivity was measured with a Wallac scintillation counter.

Measurement of protein content

Protein content was determined by the method of Lowry (32), using serum bovine albumin as standard.

Statistics

All experiments were performed at least in triplicates and the mean was used for the calculations. Data were evaluated by one-way or two-way ANOVA, followed by the Duncan multiple range when F was significant. Linear regression was used to detect dose-

dependent effects. Student's *t*-test was used in the *ex vivo* assay. Significance occurred when P<0.05.

Results

Synaptosomal preparations did not show any significant leakage of the cytosolic marker LDH after incubations with 100 nM - 1 mM GUO (data not shown).

Synaptosomes previously depleted from endogenous glutamate were able to take up 1,5 times more glutamate (4.19 ± 0.07 pmol/min/mg of protein, Fig.1) than synaptosomes not previously depleted (2.67 ± 0.12 pmol/min/mg of protein, Fig. 1). Guanosine effect over glutamate accumulation in previously depleted synaptosome and non-depleted, calculated by linear regression x analysis of variance ($F_{(3,8)}$=15.46; p<0.01; β=0.76) and $F_{(3,12)}$=10.20, P<0.01, β=0.81), respectively, depicted a dose-dependent effect.

The *in vitro* effect of GUO on basal and K^+-stimulated glutamate release was evaluated (Fig. 2). GUO increased basal glutamate release ($F_{(3,16)}$=24.53; p<0.01) and K^+-stimulated glutamate release ($F_{(3,16)}$=78.07; p<0.01). GUO *in vitro* was able to dose dependently increase vesicular glutamate uptake (Fig. 3) up to 71% ($F_{(1,26)}$=14,01; p=0.001; β=-0.59).

The effects of GUO i.c.v. administration (30 min after i.c.v. injection, 4 uL/660 uM) were also evaluated. Fig. 4-A illustrates a stimulatory effect over synaptosomal glutamate release up to 21% (basal) and 28% (K^+-stimulated release). We observed that the *ex vivo* experiments depicted an increase in the synaptosomal glutamate uptake up to 55% (p<0.05) (Fig. 4-B) when compared to control (saline group). Finally, GUO also increased the vesicular glutamate uptake by 44% (Fig. 4-C).

In the measured of CSF contents (GUO and GMP) collected 30 min after the i.c.v. treatment, no significant difference between control group and those that received GUO or

GMP was found. GMP was administered following the same i.c.v. protocol as the GUO treated group (Table 1).

Discussion

The results obtained in this study demonstrate the influence of GUO on the physiological tonus of glutamatergic system, affecting various glutamatergic parameters, such as the uptake and release of glutamate by synaptosomes, synaptic vesicles uptake *in vitro*, and for the first time in *ex vivo* experiments.

Extracellular effects of GBPs, along with evidence of specific binding sites for GUO (21), have strengthened the proposal of a specific guanine-based purinergic system in addition to the adenine-based purinergic system. In particular, GUO has been shown to exert *in vitro* trophic (17), neuroprotective activity (16) as well as to stimulate glutamate uptake (14,15). In additon, *in vivo* GUO has been shown to present anticonvulsant effect (7-10) and amnesic effects in rodents (10,33).

Considering that synaptosomal preparations have a glial component (26,27), the *in vitro* and *ex vivo* stimulatory effect of GUO on glutamate uptake by synaptosomal preparation corroborate with previous works showing that 1 μM GUO could increase glutamate uptake in astrocyte cultures and slice preparations (14,15). Very importantly, our experiments using synaptosomes previously and non-previously depleted from endogenous glutamate demonstrated that this GUO effect: i) is dose-dependently; ii) occurs at lower GUO concentrations; and iii) is capable to produce a higher increase in the glutamate uptake in previously depleted synaptosomal fraction. This could indicate that depletion procedure affects the synaptosomal preparations probably causing a decrease in the glutamate quanta. Interestingly, guanosine depicted a significant effect in both conditions tested, non-previous and previous depleted from endogenous glutamate. In cell cultures exposed to conditions as hipoxia/ischemia, extracellular purine levels are found to be

elevated and there is a higher increase of GBPs than adenine based purines (3.5 fold) (19). In addition, GUO levels are increased in ischemic focal insults and remain increased up to one week (20). These conditions are accompanied by a great discharge of glutamate in the synaptic cleft that could be excitotoxic to neurons. Our finding indicates that GUO really act on glutamate uptake in different conditions on CNS. These results corroborate with previous work (16) which GUO increase glutamate uptake in rat cortical brain slices in normal and exitotoxic conditions.

Tasca and collegues (34) do not found any effect for of GUO in the glutamate uptake by synaptic vesicles when tested at 10min of incubation time. However, when tested 100 μM and 1 mM, but now the experiment was carried out using a different time of incubation (1.5 min), GUO displayed an inhibitory effect (34). This inhibition seems to vanish with increasing time of experiment. In contrast, our work, using 10 min as the time for glutamate uptake, depicted that glutamate uptake by synaptic vesicles increased both *in vitro* and *ex vivo,* similarly to the synaptosomal preparations. Taken together, the described by Tasca (34) and our results indicated that, as observed with Na^+-dependent glial transporters, the synaptic vesicular transporters could be modulated by GUO. This modulation could be performed directly (*in vitro*) or by an intracellular pathway (*ex vivo*), the mechanisms that underline the GUO effect carry on unclear.

Previously, we demonstrated that i.p. GUO or GMP administration caused a slight increase in CSF of GUO after 30 min of administration. In this work, no difference was detected in GUO concentration after 30 min of GUO or GMP i.c.v. administration. This rapid return to basal levels indicates, as described earlier (17), a powerful homeostatic mechanism dealing with brain purines. Concurrently, *in vivo* GUO administration that affected glutamatergic parameters without alterations on GUO levels could indicate that GUO triggers a mechanism that remains functioning even though the return of GUO to extracellular basal levels.

Our group has given many evidences that GUO is a neuroprotective compound (5-10,16). In the present study, our results indicate that GUO, besides being neuroprotective, may be an overall activator of the glutamatergic system, specially of the glutamate/glutamine cycle, since the increase in glutamate taken up by cells may be used for metabolic purposes or be recycled as neurotransmitter (1,4,35,36). Both pathways require some energy, however the mechanism adopted could not include an additional glucose expend by the astrocytes, once the fuel for active glutamate uptake and glutamine formation could be derived from own glutamate oxidation (37). Thus, independently of the glutamate destination, GUO could act as an activator of the glutamate metabolism in the central nervous system, triggering the glutamate-glutamine cycle and increasing the metabolic use of glutamate.

These interactions of GUO with the glutamatergic parameters tested (*in vitro* and *ex vivo*) reinforce some specific connections (15) of the neurotransmitter pool of glutamate, in physiological concentrations, with GUO. In conclusion, we could hypothesize that GUO, besides being neuroprotective against toxic overstimulation of the glutamatergic system, could indicate other possible physiological role of GUO in strengthening the glutamatergic tonus. These modulatory effects of GUO on glutamatergic tonus could contribute to the study of processes produced physiologically by glutamate as neural plasticity or other pathological processes as neurodegenerative disorders.

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Acknowledgments

TABLE I – CSF contents of Guanosine and GMP of rats treated by i.c.v. procedures with saline, GUO or GMP.

Treatment Group	Purine measured	
	Guanosine (µM)	**GMP (µM)**
Saline	1.34 ± 0.13	0.66 ± 0.07
Guanosine	1.63 ± 0.31	0.62 ± 0.21
GMP	1.20 ± 0.16	0.44 ± 0.05

Experimental procedures are described in methods section. Data are mean ± S.E.M. from at least six independent experiments.
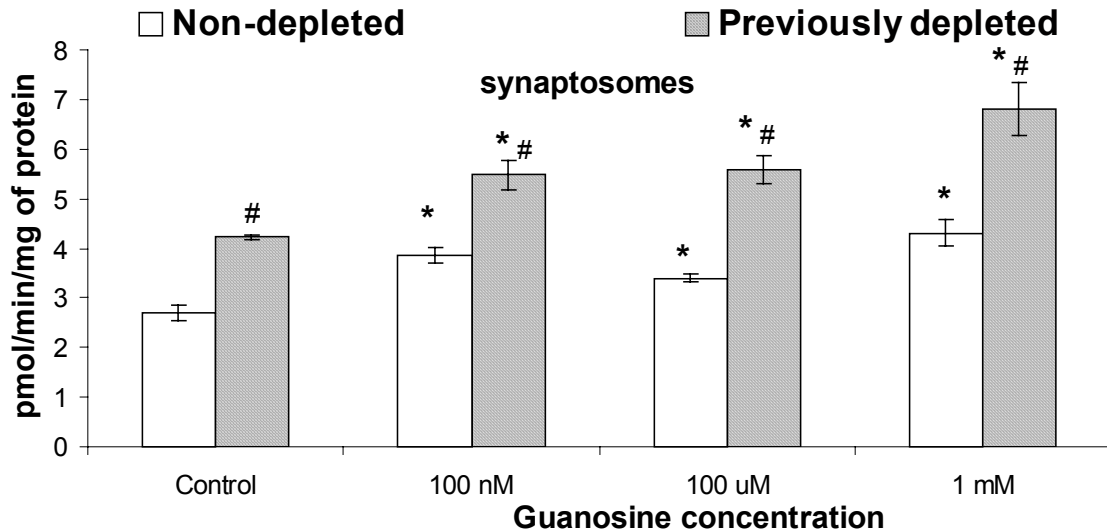
Figure 1

Figure 1 – Effect *in vitro* guanosine on glutamate accumulation into brain synaptosomes, previous or non-previous depleted from endogenous glutamate. Glutamate accumulation by synaptosomes is expressed pmol/min/mg of protein. Data are mean ± S.E.M. from at four independent experiments performed in triplicates. The symbol (*) represents statistically significant difference between the guanosine concentrations used when compared to the respective control group, (#) represents statistical significant difference to respectively non-previous depleted group. Differences were considered significant at $p < 0.05$ variance by ANOVA followed by the Duncan´s multiple range test.
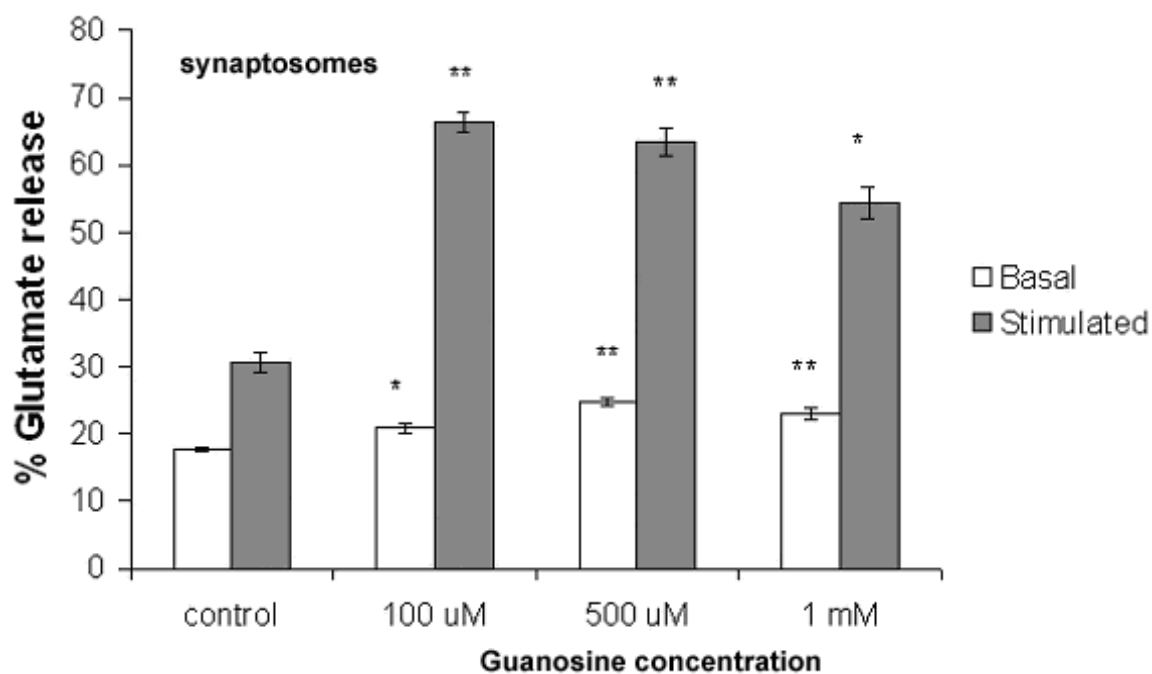
Figure 2

Figure 2 - Effect of *in vitro* guanosine on basal and stimulated glutamate release from brain synaptosomes. Glutamate release is expressed as a percentage of total radioactivity content incorporated by synaptosomes. Data are expressed as mean ± S.E.M. from five independent experiments performed in triplicates. The symbols (*) and (**) represent values significantly different from the control group, $p < 0.05$ and $p < 0.01$, respectively, by ANOVA followed by Duncan´s multiple range test.
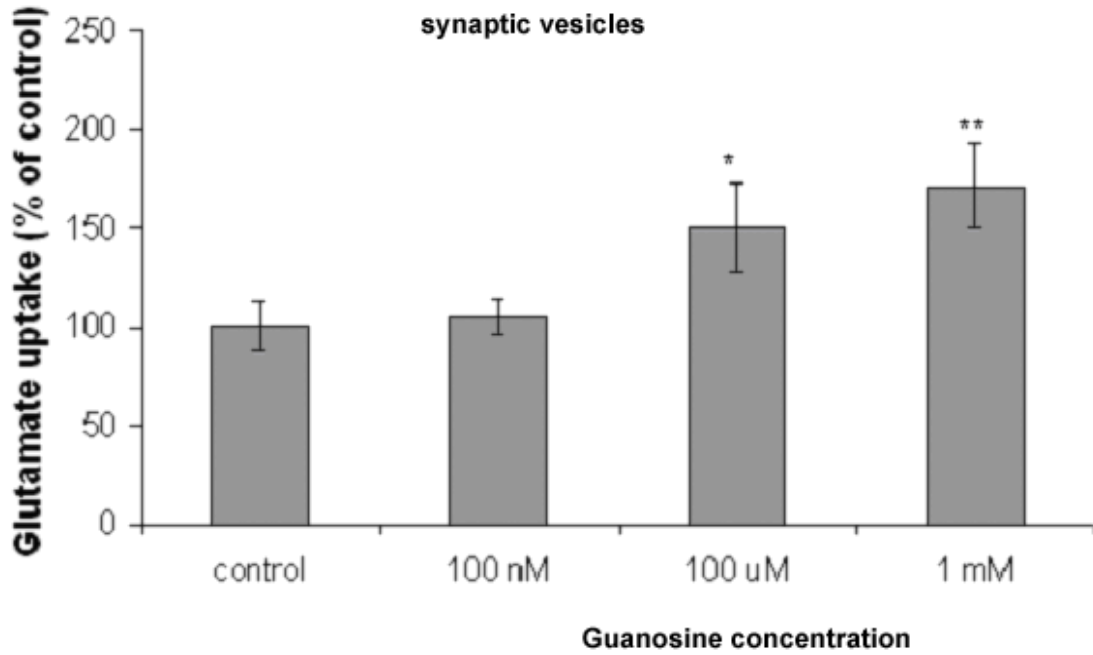
Figure 3

Figure 3 - Effect of *in vitro* guanosine on glutamate uptake into brain synaptic vesicles.
Results represent the mean ± S.E.M. from seven independent experiments performed in triplicates and are expressed as a percentage of control (0.147 nmol/min per mg protein). The symbols (*) and (**) represent values significantly different from the control group, $p<0.05$ and $p<0.01$, respectively, by ANOVA followed by Duncan´s multiple range test
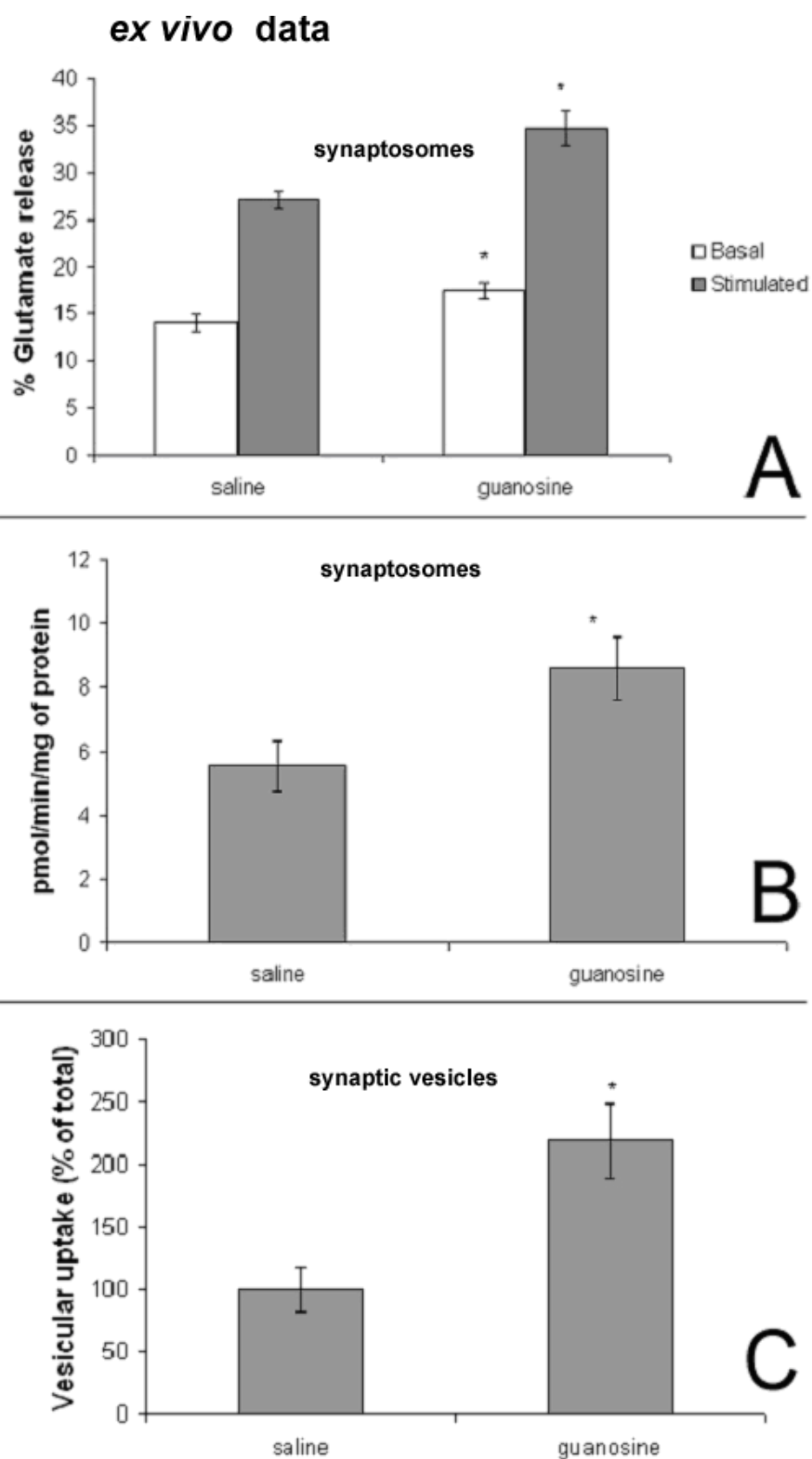
Figure 4

ex vivo data

Figure 4 - *Ex-vivo* **experiments using guanosine i.c.v. administration in rats**. **A: Glutamate release** is expressed as a percentage of total radioactivity content incorporated by synaptosomes. **B: Glutamate uptake** is expressed as pmol/min/mg of protein. **C: Vesicular glutamate uptake** is expressed as a percentage of control (0.083 nmol/min per mg protein). Data are mean ± S.E.M. from five independent experiments performed in triplicates. The symbol (*) represents values significantly different between saline (control group) and guanosine group. Differences were considered significant at $p < 0.05$ by *Student´s t*-test.

3.4 – ARTIGO IV

Artigo a ser submetido à Glia

Astrocytes are the possible location for [^{3}H]-Guanosine binding site

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Running title: Guanosine specific binding

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Key words: Guanosine, glial plasmatic membrane, Guanosine binding, purine receptors, astrocytes.

Abstract

The main objective of our work was to determine the presence of a specific binding site for guanosine in rat brain plasmatic membrane. We performed a glial plasmalemmal vesicle preparations to obtain plasmatic membrane enriched with glial fraction and synaptosomal and mitochondrial fractions as well. The guanosine binding in plasmatic membranes is inhibited by adenosine, but not by ATP,caffeine and by classical purine receptor antagonist. GMP and GTP-N also inhibit the guanosine binding, but AOPCP reverts GMP inhibitory effect. GMP should be converted to guanosine to inhibit the guanosine binding. In contrast, glutamate do not affect the guanosine binding. Surprisingly the mitochondrial membranes are able to binding guanosine. Collectively, the observations demonstrate that guanosine binding site 1) are preferentially astrocytic; 2) seem to be G-protein linked and 3) responsive to guanosine and adenosine. In addition, we depicted the necessity of a purification processes to work with guanosine binding, since mitochondria has binding sites to guanosine. To our knowledge, it is the first evidence for the presence of a guanosine receptor in astrocytes.

Introduction

Studies on the purinergic system normally deal with adenine–based purines, namely adenine nucleotides (e.g. ATP) and adenosine (Zimmermann and Braun 1996). Others and we have suggested that a guanine-based purinergic system has also important neuromodulatory roles, which, similarly to the adenine-based purinergic system, are exerted by guanine nucleotides and the nucleoside guanosine (GUO) (Rathbone et al., 1999, Frizzo et al., 2003, Soares et al., 2004, Schmidt et al., 2005).

Guanine-based purines (GBPs) have been demonstrated to be able to modulate the glutamatergic system in varied conditions. It was shown that GBPs inhibit cell responses to glutamatergic ligands (Tasca et al., 1995; Regner et al., 1998; Burgos et al., 2000; Tasca and Souza 2000) and have the capacity to act as anticonvulsant by preventing seizures induced by quinolinic acid (QA), kainate and α-dendrotoxin (Lara et al., 2001; Schmidt et al., 2000, 2005; Vinade et al., 2003; Soares et al., 2004). Furthermore, the inhibitory effect of GBPs on the binding of glutamate and analogs in different preparations from chick, rat and goldfish brain was verified (Baron et al., 1989; Gorodinsky et al., 1993; Paas et al., 1996; Paz et al., 1994, Porciuncula et al., 2002). GBPs have been demonstrated to enhance glutamate uptake in astrocyte cultures (Frizzo et al., 2001, 2003) and brain cortical slices (Frizzo et al., 2002). Moreover, GUO has been shown to play trophic effects on neural cells (Rathbone et al., 1999), to preserve viability in mouse spinal cord cultures during chemical

hypoxia (Litsky et al., 1999), to promote myelination and functional recovery in chronic spinal injury (Jiang et al, 2003), and to stimulate neurite outgrowth in PC2 cells (Bau et al., 2005).

The presence of nucleotides, nucleosides and their metabolites in cerebrospinal fluid has been earlier described (Rodriguez-Nunez et al., 1993, 2000). *In vivo*, the extracellular concentration of purines depends on a balance between the cell release and uptake as well as on extracellular metabolism (Rathbone et al., 1999; Ciccarelli et al., 2001; Latini et al., 2001). It has been shown that purine concentrations are elevated during ischemic events (Uemura et al., 1991; Ciccarelli et al., 1999), besides other works reveal that in elevated concentrations of nucleotides, the ratio of GMP and GUO production is higher when compared to the AMP and adenosine ratio by its respective hydrolysis cascade (Portela et al., 2002).

Recently, our group demonstrated that glutamate uptake by astrocyte cultures was increased by GUO and by guanine nucleotides (Frizzo et al., 2000, 2003). In addition, we established that GBPs possess anticonvulsant effect (Soares et al., 2004; Schmidt et al., 2005). However, the inhibition of the breakdown of GMP into GUO, through addition of the ecto-5'-nucleotidase inhibitor α-β-methyleneadenosine 5'-diphosphate (AOPCP), prevented the effects of guanine nucleotides (Frizzo et al., 2003; Soares et al., 2004). Additionally, the poorly hydrolysable guanine nucleotide derived from GTP (GTP-N) also failed to stimulate glutamate uptake and to be anticonvulsant, reinforcing the hypothesis

that GUO is the final mediator of such effects of guanine nucleotides (Frizzo et al., 2003, Schmidt et al., 2005)

Another issue that has been addressed is the relative contribution of adenosine to the effects of GUO. Whereas adenosine may participate in the trophic effects of GUO (Ciccarelli et al., 2000, 2001; Rathbone et al., 1999), non-selective adenosine receptor antagonists, such as caffeine and theophylline, did not affect the effects of GUO on glutamate uptake (Frizzo et al., 2001), seizures (Lara et al., 2001), and amnesic effect (Vinadé et al., 2004). Furthermore, adenine-based purines are not able to increase the glutamate uptake in astrocyte cultures (Frizzo et al., 2003). As well, GUO is depicted as an antiapoptotic agent, conversely to what is presented by adenosine (Di Orio et al., 2002, 2004).

In view of the presented it is reasonable to suppose that GUO has a different site of action to that classical proposed to Adenine-based purines. Recently, in an interesting work by Traversa and colleagues (2002) demonstrated the presence of a specific guanosine binding in rat brain membranes and another work (Traversa et al., 2003) made an improvement in suggesting the model of a pseudo-receptor to guanosine and the probable participation of G-proteins as effectors of the guanosine binding. In this study we try to bring an upgrading into the GUO binding studies. We depicted that guanosine binding site is preferentially glial, and performed a new methodology to obtain an improved glial plasmatic membrane to guarantie a more accurate binding assay.

The relevance of our findings involve the real improvement in the binding assay, by the use of a plasmatic membrane with less mitochondrial membrane contamination, and that guanosine binding site appears to be preferentially glial. In addition, a valuable methodology that could be a helpful tool in the neurochemical research concerning specific glial plasmatic membranes aspects (membrane bound enzymes, specific neurotransmitter binding sites, transporters) without the necessity to use just cell cultures. This new protocol makes possible studies with glial components that undergo all the transformations that occurred during the development of central nervous system (CNS).

Material and Methods

Animals and reagents

Two month old Wistar rats (180-250g) were maintained at 25°C in a 12:12h light/dark cycle, with tap water and standard lab chow *ad libitum*. They were housed in plastic cages (five per cage). Our institutional protocols for experiments with animals were followed to minimize suffering and limit the number of animals sacrificed.

[^{3}H]-Guanosine (15 Ci/mmol) was purchased from American Radiolabeled Chemicals, (St Louis, MO, USA). Guanosine (GUO), guanosine 5'-monophosphate (GMP), 5´-guanylylimidodiphosphate (GTP-N), αβ-methyleneadenosine 5'-diphosphate (AOPCP), 2,6-dichloroindophenol (DCIP), ouabain, and adenosine 5´-thriphosphate (ATP) were

obtained from Sigma Chemicals (St Louis, MO, USA). Other chemicals were of analytical reagent grade and purchased from local suppliers.

Astrocyte cultures

Primary astrocyte cultures were prepared as described previously (Frizzo et al., 2001) from the cortices of 1-3 day-old Wistar rats. Briefly, the plating medium was MEM (Eagle´s minimum essential medium) with 10% FBS (fetal bovine serum) (Cultilab, Campinas, SP, BR) and the cells were seeded at a density of 12 x 10^5 cells/cm^2 in three cell cultures flasks. Astrocyte cultures were maintained in an incubator at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. The cultures were used at 12-14 days *in vitro* (DIV) with astrocytes showing a confluent monolayer aspect, being >95% of glial fibrillary acidic protein (GFAP)-positive cells. In order to use the cultures in the protocol of membrane preparation, the medium was washed with phosphate buffer saline (PBS) with 0.4 mM of ethylenediaminetetraacetic acid (EDTA). After this, the medium was replaced to PBS with 0.4 mM of EDTA and 0.5% of trypsin. The medium was gently shacked to detach the cells. After this procedure, horse serum (HS) 0.5% was used to inactivate trypsin and the cell suspension was centrifuged at 30,000 x g for 15 min. The pellet was resuspended in Tris-HCl 5mM and used in the membrane preparation protocol.

Neuronal Cultures

Primary cultures of neuronal cells were prepared from 8-day-old Wistar rats (Sanz et al., 1996). Briefly, freshly dissected cerebella were incubated with 0.025% trypsin solution for 15 min at 37^OC and disrupted mechanically in the presence of 0.1 mg/ml DNase and 0.05% trypsin inhibitor. For the experiments, the cells were seeded at a density of 2.5-3 x 10^5 cell/cm^2 in a 6-well dish, coated with 10 mg/ml poly-D-lysine in MEM supplemented with 10% FBS, 50 mg/ml garamicine and 25 mM KCl. The growth of non-neuronal cells was inhibited by addition of 20 mM cytosine arabinofuranoside 18-20 h after seeding and the medium was maintained without change during the culture period. At 8-9 DIV, cultures were rinsing in order to use the cultures in the protocol of membrane preparation. The medium was washed with PBS with 0.4 mM of EDTA. After this the medium was replaced by PBS with 0.4 mM of EDTA and 0.5% of trypsin. The medium was gently shacked to detach the cells. After this procedure, HS 0.5% was used to inactivate trypsin and the cell suspension was centrifuged at 30,000 x g for 15 min. The pellet was resuspended in Tris-Hcl 5mM and used in the membrane preparation protocol.

Glial plasmalemmal vesicle (GPV) and synaptosomal preparations

Animals were decapitated and the whole brain was used to prepare GPV, synaptosomal and mitochondrial fractions that will be used throughout the experiments. We used a discontinuous Percoll gradient according to Nakamura et al (1993). Briefly, the CNS tissue (about 0.5 g) were homogenized with 5 ml of 0.32 M sucrose containing 1mM of EDTA (SE solution) using a Teflon-glass homogenizer, and centrifuged at 1,000 x g for 10 min. The supernatant (S1) was held in reserve. The pellets (P1) were rehomogenized with 20 ml of SE solution and centrifuged again under the same condition. The resulting supernatant (S1') were combined with S1 and centrifuged at 15,000 g for 30 min. The pellet then obtained (P2) was homogenized with 8 ml of SEDH solution (0.32 M sucrose, 1 mM of EDTA, 0.25 mM dithiothreitol, and 20 mM Hepes, pH 7.4). The obtained supernatant (S3) was layered onto a 4-step discontinuous gradient composed by 20, 10, 6, and 2 % of Percoll in SEDH solution. The tubes were centrifuged at 35,000 x g for 5 min. The turbid layer between 2 and 6% Percoll was collected as GPV fraction. Synaptosomal fraction was collected from the interface of 10 and 20% Percoll. Mitochondrial fraction was collected at the end of 20% layer. All fractions were diluted in SEDH and centrifuged at 30,000 x g for 15 min. The obtained pellet was resuspended in SEDH and centrifuged again at same condition. The final pellets of all fractions were used in the membrane protocol preparation.

Immunocytochemistry for GFAP

Immunocytochemistry for GFAP was carried out as described previously (Gottfried et al., 2003). Briefly cells were fixed with 4% paraformaldehyde in PBS and permeabilized for 10 min in PBS containing 0.2% Triton X-100. Fixed cells were then blocked with 0.5% bovine serum albumin for 60 min and incubated overnight with polyclonal anti-GFAP (DAKO), followed by peroxidase-conjugated IgG (Amersham) for 2 h and 0.05% diaminobenzidine (Sigma) containing 0.01% hydrogen peroxide for 10 min. Cells were viewed with a Nikon inverted microscope and images transferred to computer with a digital camera (Sound Vision Inc, Wayland, MA, USA).

Enzyme Linked Immunosorbent Assay for GFAP

ELISA for GFAP was carried out by coating the microtiter plate with 100 uL samples containing 40 ug of protein for 48 h at 4°C. Incubation with a polyclonal anti-GFAP (DAKO) for 2 h was followed by incubation with a peroxidase-conjugated IgG for 1 h, at room temperature. The color reaction with o-phenylenediamine was measured at 492 nm.

Membrane Preparation

The fractions were submitted to the protocol of Jones and Matus (1974) in order to obtain plasmatic membranes. The membranes were stored at $-70^{\circ}C$ for up to 2 months. On the day of binding assay the membranes were rapidly thawed in a water bath (37°C) for 30 min and centrifuged at 27,000 x g for 15 min. The final pellet was resuspended in the same buffer used to binding assay to yield a protein concentration of 0.8 – 1.2 mg/mL and was added to binding assay. Mitochondrial fraction was submitted to the same protocol as GPV and synaptosomal fractions. The membranes obtained by this protocol were used in the enzyme studies of all fractions.

Determination of Complex II (succinate: DCIP oxireductase, SDH) activity

We used the method described by Brusque et al. (2002). Briefly, the samples were collected and diluted in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 UI/ml heparin). The homogenates were centrifuged at 800 x g for 10 min and the supernatants were kept at $-70^{O}C$ until used for enzyme activity determination. SDH activity was measured by following the decrease in absorbance due to the reduction of DCIP at 600 nm with 700 nm as reference wavelength ($\varepsilon=19.1$ mM^{-1}cm^{-1}). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 5 mM succinate and 8 uM DCIP was preincubated with 40–80 ug homogenate protein at $30^{O}C$ for 20 min.

Subsequently, 4 mM sodium azide and 7 uM rotenone were added and the reaction was initiated by addition of 40 uM DCIP and was monitored for 5 min.

Determination of Na^+,K^+-ATPase activity

The reaction mixture for the *Na^+,K^+-ATPase* assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20 mM KCl, and 40 mM Tris–HCl buffer, pH 7.4, in a final volume of 200 uL. The reaction was started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. Controls were assayed under the same conditions with the addition of 1 mM ouabain. *Na^+,K^+-ATPase* activity was calculated by the difference between the two assays (Tsakiris and Deliconstantinos, 1984). Released inorganic phosphate (Pi) was measured by the method of Chan et al., 1986.

Binding assay

On the day of assay, the membranes were thawed in water bath (36^OC). All the tubes used were pre-treated with a silicone solution by at least 30 min prior to use. The binding assay was based on the method of Traversa et al (2002). The standard measurement of [^{3}H]-guanosine binding performed throughout the experiments was performed using 200 nM of [^{3}H]-guanosine with 45 - 60 ug of protein by 20 min at 36^OC in a total volume of

500 uL of PBS buffer. All the solutions used in the binding assay were diluted in the PBS buffer before use. The reaction was started by the addition of [^{3}H]-guanosine in the medium. After 20 min of incubation the membranes were then kept at 4°C and centrifuged at 14,000 x rpm in an Eppendorf centrifuge (5402) during 10 min. The pellet and the wall of the tube were gently and quickly washed with ice-cold Milli-Q water. SDS (0.1%) and NaOH 1mM was added to the dry pellet. After dissolution of the pellet we added scintillation liquid and the radioactivity incorporated was measured in a Wallac scintillation counter (1409). The non-specific binding was determined using unlabelled guanosine at 2 mM in the binding assay in a parallel assay. Non-specific binding amounted around of 15% to 30% of total binding. Specific binding was considered the difference between total and non-specific binding.

Measurement of protein content

Protein content was determined by the method of Lowry et al (1951), using serum bovine albumin as standard.

Statistics

All experiments were performed at least in triplicate and the mean was used for the calculations. Data were analyzed by one-way ANOVA, followed by the Duncan post hoc test when necessary. Significance occurred when $p<0.05$.

Results

Figure 1 depicted the immunoreactivity content of GFAP in the astrocyte and neuronal cultures used in our binding experiments. The astrocyte cultures (Panel A) presents 95% of purity and the neuronal (Panel B) 2%-3% of contamination with glial components. The binding experiments performed with astrocyte (0.212 ± 0.014 pmol of GUO/ ug of protein) and neuronal (0.029 ± 0.008 pMol of GUO/ ug of protein) cultures demonstrate a preference of [^{3}H]-guanosine binding at least 7 times more in glial plasmatic membrane than in the neuronal plasmatic membrane (Fig 2).

The fractions used in our experiment were tested in order to determine the capacity to obtain purified plasmatic membrane. The ratio of Na^+,K^+-ATPase/SDH indicates the purified grade of the plasmatic membrane obtained, since that Na^+,K^+-ATPase is a marker enzyme for plasma membranes and SDH activity is higher in mitochondrial membrane (Nakamura et al., 1993). Figure 3 demonstrates that synaptosomal and GPV fractions were more enriched in the plasma membranes than S1+S1´ and mitochondrial fractions tested. Figure 4 illustrates the specific [^{3}H]-guanosine binding in the membranes obtained in our

experiments. It is very important to note that GPV plasmatic membrane and mitochondrial membrane demonstrate a similar [^{3}H]-guanosine specific binding and synaptosomal plasmatic membrane possess as a minimum 10 times less specific [^{3}H]-guanosine binding when compared to the other two fractions tested.

Figure 5 shows the GFAP content of synaptosomal, GPV obtained in our procedures. The GPV fraction obtained demonstrated a very clear increased in GFAP content indicating a higher glial content in this fraction when compared to the synaptosomal fraction.

Figure 6 indicates that both total and non-specific binding of 200 nM [^{3}H]-guanosine increase in a linear range of protein content. Figure 7 shows that the concentration selected to our experiments was performed in a non-saturated condition once the total and non-specific binding increased linearly with [^{3}H]-guanosine concentration used in the experiment.

Figure 8 indicates the displacement curve for guanosine when the highest displacement was obtained with 2mM of guanosine. An approach of the specificity of the binding site to guanosine was shown in the figure 9 when was used other purines and glutamate to test the capacity of these molecules to affect the specific [^{3}H]-guanosine binding. Adenosine, GMP and the poor hydrolysable GTP derived (GTP-N) depicted a similar effect in the inhibition of [^{3}H]-guanosine binding. However, caffeine, a well-described non-selective adenosine receptor antagonist, does not affect the specific [^{3}H]-

guanosine binding in the same way as 2 mM of adenosine when both are compared to specific [^{3}H]-guanosine binding. ATP reveals an inability to affect the specific [^{3}H]-guanosine binding. The addition of the ecto-5'-nucleotidase inhibitor (AOPCP) did not affect the specific [^{3}H]-guanosine binding in GPV plasmatic membrane in the concentration tested. In contrast with the observed with GMP alone, when the same concentration of AOPCP was added in an assay with GMP, the specific [^{3}H]-guanosine binding was not affected. Glutamate seems not to affect the specific [^{3}H]-guanosine binding in the tested concentration.

Discussion

The present results clearly indicate the preference of guanosine binding site to the glial membrane. The higher specific [^{3}H]-guanosine binding found in astrocytic and in the GPV plasmatic membranes when compared to neuronal and synaptosomal plasmatic membranes strongly confirms this hypothesis. We have to consider that GPV is more enriched in glial components than synaptosomal fraction, once the GFAP content is higher in GPV. The GPV could be considered a better substitute of glial cells than synaptosomes. These findings corroborate previous works in which the effect of guanosine is related to glutamate transport in the astrocyte cultures (Frizzo et al., 2001, 2003). Since astrocytes are the main responsible by taking out the glutamate from the synaptic cleft (Danbolt et al.,

2001), it is reasonable to suppose that other protective effects observed by guanosine which involves the glutamatergic system (Schmidt et al., 2000, 2005; Frizzo et al., 2002; Vinadé et al., 2003, 2004; Soares et al., 2004) are carried out by the enhancement in the glutamate uptake caused by guanosine. With so many works indicating that guanosine interacts with astrocytic glutamate transport it is very rational to presume that the preferred site to the guanosine binding should be found in the glial plasmatic membrane.

In addition, the methodology used to obtain the GPV plasmatic membrane depicted a real improvement in the plasma membrane content and that GPV plasmatic membrane could be considered a satisfactory instrument in the studies of glial membrane aspects preventing the use of astrocytic membrane. While cultured glial cells provide the benefits of homogenous population, it is possible that some cellular propreities could be altered under culture conditions. In view of this, our protocol provide a useful tool to study membranes obtained from tissues of experimental animals in any age of development, whereby the effects of aging or experimental manipulation on cellular or molecular events can be evaluated in the CNS plasmatic membranes, in contrary these effects could not occur in cultured cells (Daniels and Vickroy 1998). On the other hand, we can separate in the same preparation the neuronal components by using the synaptosomal fraction, which could be used in a comparative study (Nakamura et al., 1993).

Other important point to be ruled out is the purity of the membrane used in our experiments. The GPV membrane demonstrates to be enriched in plasma membranes,

despite other works that do not describe any enrichment procedure (Traversa et al., 2002,2003). Considering that mitochondrial membrane is able to binding GUO in the same levels of the GPV plasmatic membranes, we strongly recommend that experiments concerning the study of GUO receptors in the SNC should be done with a similar procedure that could separate the mitochondrial membrane to the plasmatic membranes that will be used in the experiments. The procedure without any purification step could produce a super estimation in the results obtained. Conversely, the use of regular plasmatic membranes of SNC in the study of GUO receptors could lead to a lower binding levels, once that synaptosomal plasmatic membrane presented 10 times less specific [^{3}H]-guanosine binding than GPV plasmatic membrane. In view of this, two procedures, should be assumed in the studies of GUO receptors in glial components: a) the separation of fractions enriched with plasma membranes, and b) separation of glial and neuronal contents.

Considering the well-documented action of GUO in the glutamatergic system, it is important to note that glutamate was unable to affect the specific guanosine binding in the GPV plasmatic membrane, indicating that there were no interactions between the glutamate and the guanosine binding sites. These findings are in accordance with an early study (Souza and Ramirez, 1991) in which guanosine was the unique GBPs that did not interfere in the binding of glutamate ligands. Otherwise, another report (Porciuncula et al., 2002) depicted a little decrease in the [^{3}H]-glutamate binding at post-synaptic density (PSD) preparations, but guanosine showed no effect when was tested at the MK-801 binding in

the PSD preparation. These works demonstrated that guanosine does not displace glutamate in an important way from its binding sites. The same effect occurs when the binding site is related to guanosine and the displacer was glutamate. This finding indicates that guanosine effects, in the glutamate uptake at the astrocyte (Frizzo et al., 2001, 2003) and cortical slices (Frizzo et al., 2002) transporters of glutamate, occur in a different binding site from glutamate, probably in the GUO receptor.

Our findings concern the GTP-N inhibition are in agreement with other works that suggest that the guanosine binding site posses a G-protein coupled (Traversa et al., 2003). It was described that GTP-N could bind in G-protein in a very stable GTP-N/G-protein complex, since the stimulated adenylate cyclase activity was maintained by a long period of time (Rotta et al., 2004). The effect depicted by GMP is not surprisingly since GMP is able to mimic some effects of GUO (Frizzo et al., 2003; Soares et al., 2004). GMP seems to be able to displace GUO from the binding site. On the other hand, the use of AOPCP, a classical inhibitor of 5′-nucleotidase, reverts the effect of GMP on the specific GUO binding. This is in accordance with our previous results that indicate a necessity of GMP to be converted in GUO to exert the effects in the glutamate uptake (Frizzo et al., 2003) and the anticonvulsant activity (Soares et al., 2004).

The effect of adenosine in the GUO binding is a great surprise and it does not match with previous results for guanosine binding in the rat brain membranes (Traversa et al., 2002, 2003). Adenosine presents a similar displacement power that GTP-N and GMP. The

effect of adenosine in the specific GUO binding point to a possible new purinergic family receptor, since caffeine, a classical unspecific antagonist of adenosine receptor, at 2mM concentration is unable to reach a similar pattern of inhibition that we found for adenosine. Besides, caffeine was found to inhibit in the range of uM concentrations the adenosine receptors; the highest K_D found to the A_3 receptor was 80 uM in humans and 190 uM in rats (Fredholm et al., 1999). It is a very low concentration when compared to the 2mM used in our experiments. ATP (2mM), the other possible displacer of a purine receptor that was used in this study, was ineffective in displacing the 200 nM [^{3}H]-guanosine binding in the GPV plasmatic membrane. This effect could be attributed to the use in our experiments GPV plasmatic membrane; since the other works (Traversa et al., 2002, 2003) carried out the experiments with crude membrane preparations. This difference could be crucial, once we used a membrane enriched in glial plasma membrane and the GUO binding is preferentially glial. Our results putting simultaneously with the previous effects depicted by GUO and adenosine could indicate that the intracellular action of the receptor depending on the agonist that is bound on the binding site.

In summary, our results produced a significant contribution to the idea that GUO has a specific binding in the SNC and that GBPs must be considered an important part of the purinergic system. The data obtained indicate some important approaches in the field of purine receptors, as follows: a) this new receptor is preferentially glial, b) this new receptor seems to be responsive to GUO and adenosine, c) ATP does not affect this binding, d) the

possibility of a new purine receptor to be affected by caffeine at high doses, e) the intracellular mechanism of the new receptor seems to be G-protein coupled, f) this receptor should trigger different intracellular mechanisms depending on the agonist used, and g) membranes used to study GUO binding should be plasmatic membranes enriched with glial membranes. These findings reinforce the possibility of guanine-based purinergic system independent of the adenosine classical receptors. The studies of GUO receptor bring a new frontier in the purine system, demanding the development of a new class of specific antagonists/agonists. These new drugs could be useful to modulate the glutamatergic system in order to prevent the development of neurological disorders related to the high levels of glutamate or modulate physiologically the glutamatergic tonus.

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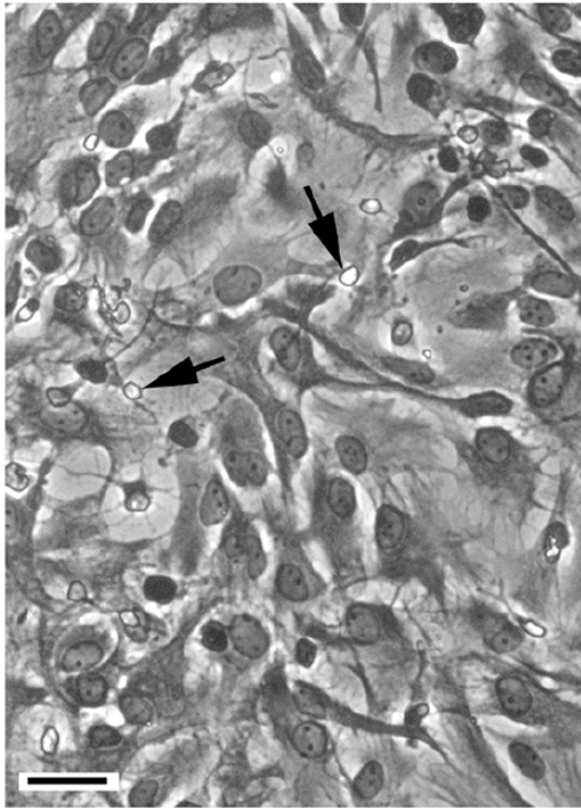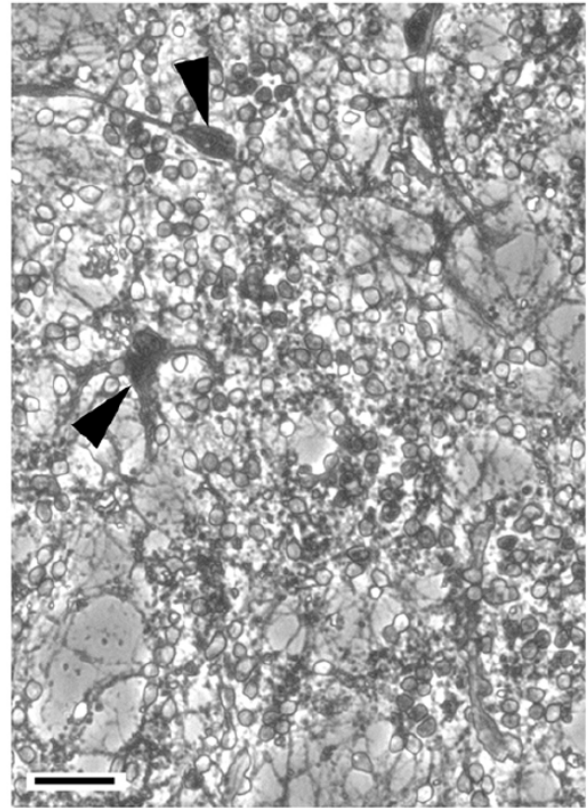
Figure 1 – Representative images of astrocyte and neural cultures after GFAP immunocytochemistry assay. Arrows show neurons and arrowheads show astrocytes. Astrocyte and neuronal cultures are 12 DIV and 9 DIV, respectively. Scale bar = 50 μm
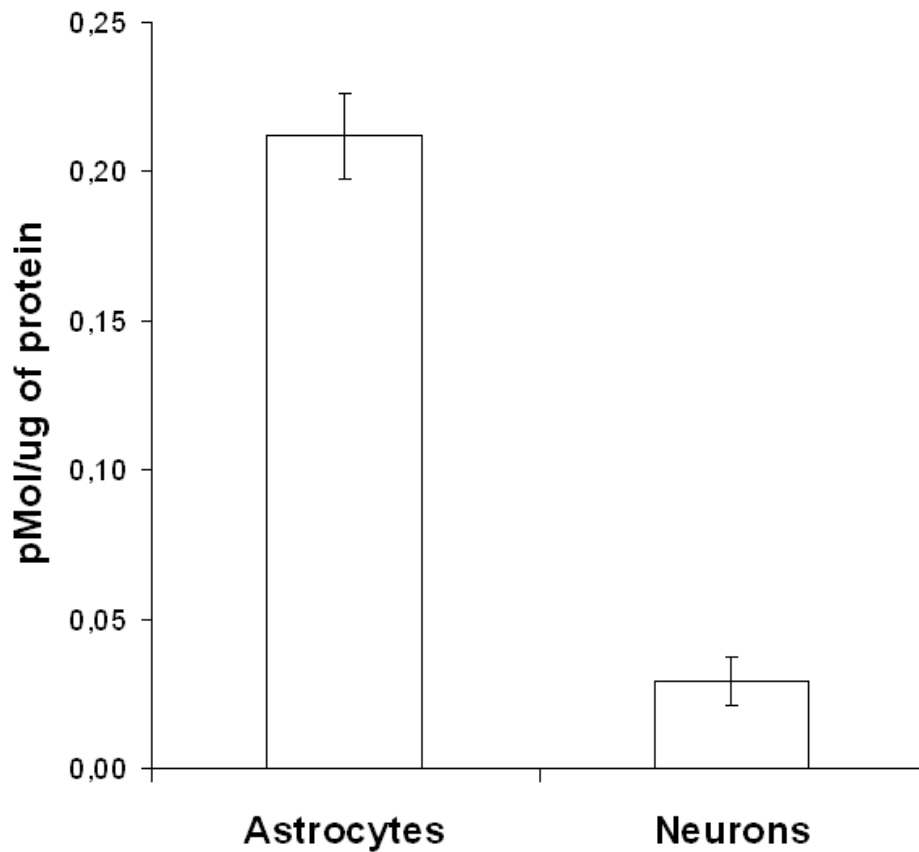
Figure 2 – Specific [³H]-guanosine binding in astrocyte and neuronal plasmatic membranes. Results represent the mean ± S.E.M. from four independent experiments performed in triplicate. The binding experiments are assayed with 10 – 15 ug of protein and 200 nM of [³H]-guanosine by 20 min. Specific binding was obtained by the subtraction of the total binding by the non-specific binding. The non-specific binding was obtained using 2 mM of non-labeled guanosine in the binding assay.
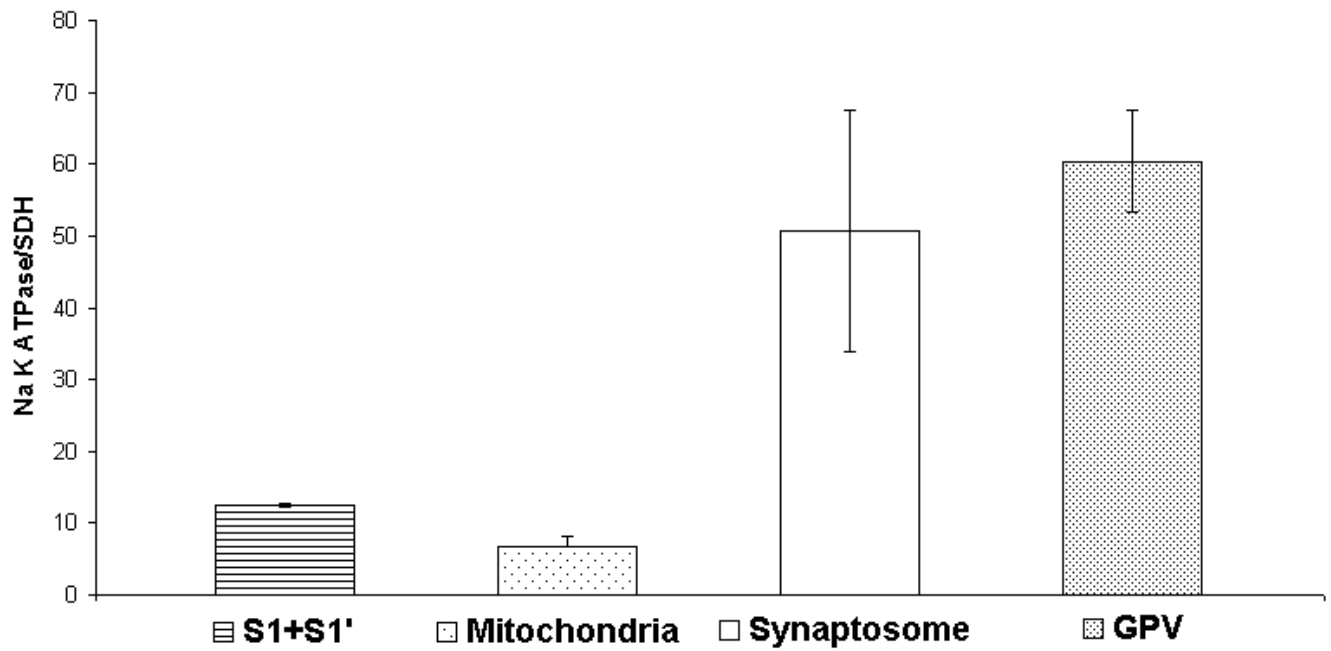
Figure 3 – Ratio of Na$^+$,K$^+$-ATPase/SDH activity in the membranes of the different preparations tested. Results represent the mean ± S.E.M. from four independent experiments performed in triplicate. S1+S1´ represents the supernatant fraction used to obtain the other fractions tested.
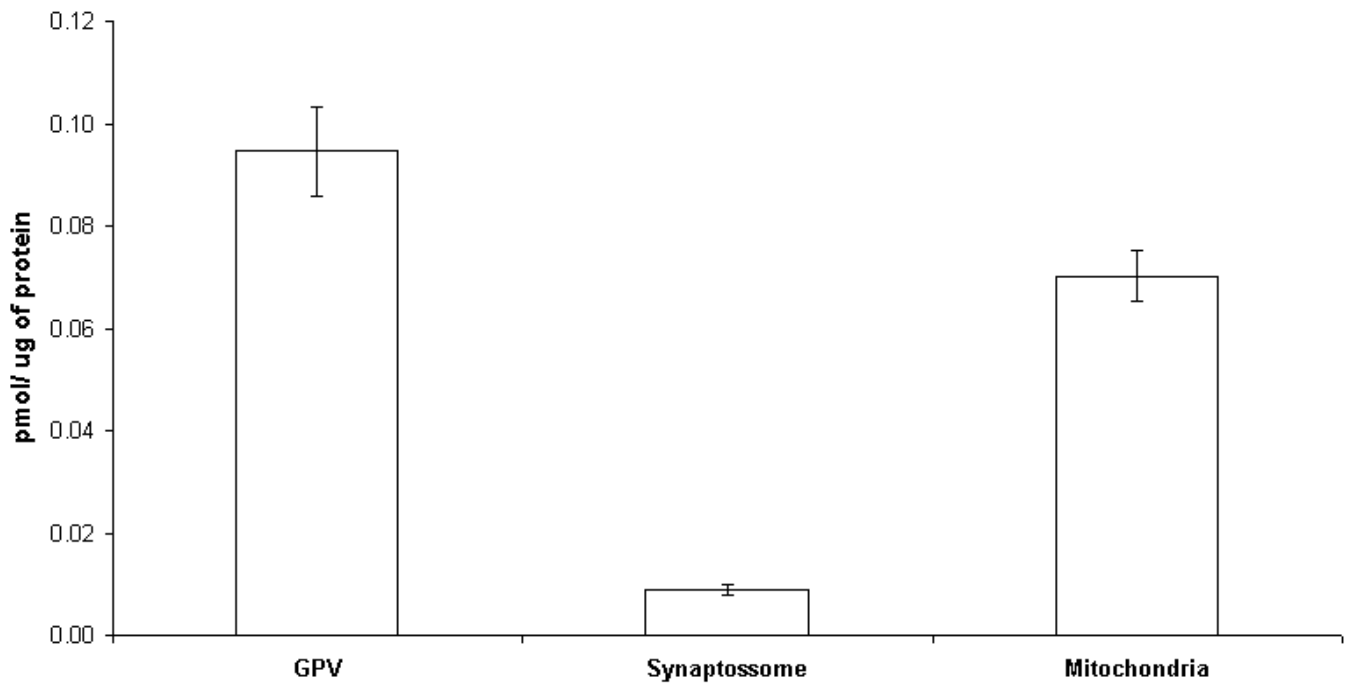
Figure 4 – Specific [³H]-guanosine binding at the GPV, synaptosomal, and mitochondrial membranes. Results represent the mean ± S.E.M. from four independent experiments performed in triplicate. In experiments to assay the [³H]-guanosine binding were used 45 – 60 ug of protein by 20 min of the correspondent membrane preparation. Specific binding was obtained by the subtraction of the total binding by the non-specific binding. The non-specific binding was performed using 2 mM of guanosine as displacer.
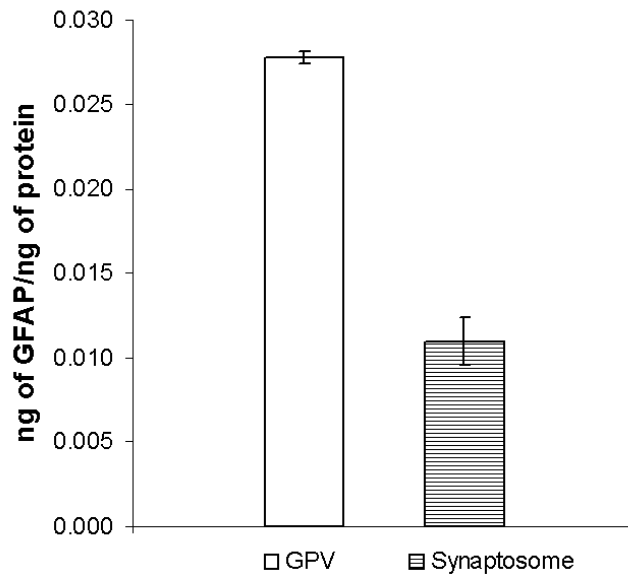
Figure 5 – Content of GFAP in fractions used in the plasmatic membrane preparation. Results represent the mean ± S.E.M. from two independent experiments performed in duplicate. The experimental procedure was described in material and methods section. In each experiment we used 40 ng of protein of the respective fraction.
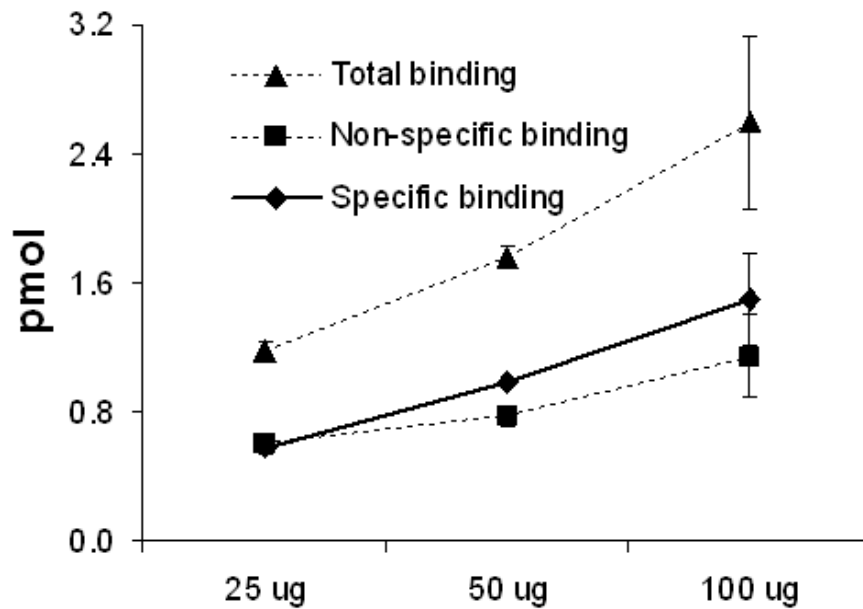
Figure 6 – [³H]-guanosine binding in the GPV plasmatic membrane as a function of the amount of protein. Results represent the mean ± S.E.M. from three independent experiments performed in triplicate. The experimental procedure was performed using 200 nM of [³H]-guanosine by 20 min as described in material and methods section. Specific binding was obtained by the subtraction of the total binding by the non-specific binding. The non-specific binding was achieved performed binding assay using 2 mM of guanosine.
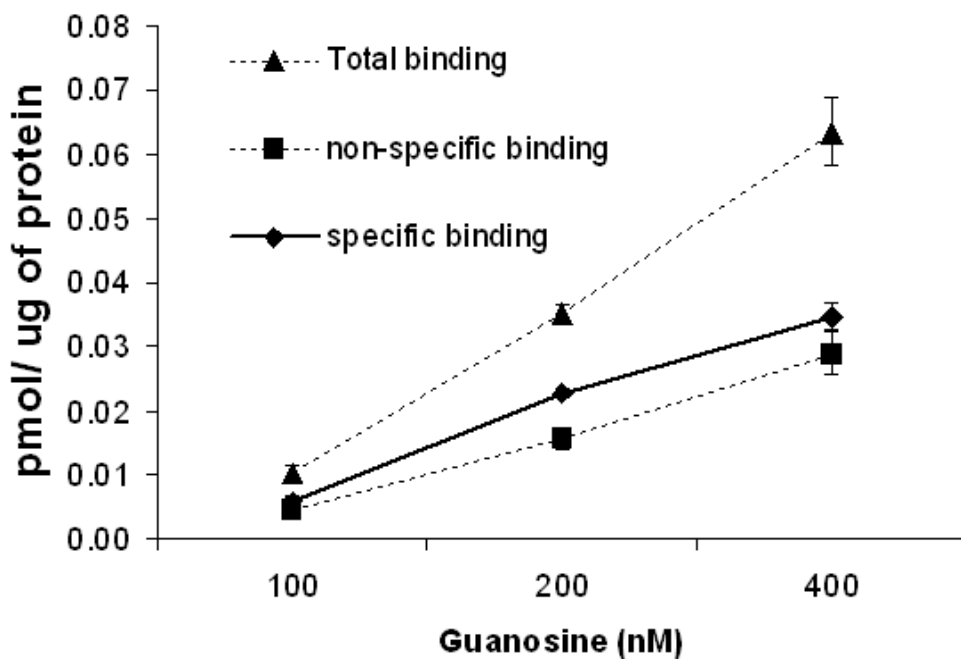
Figure 7 – Effect of [H³]-guanosine concentration on the guanosine binding using plasmatic membrane from GPV. Results represent the mean ± S.E.M. from four independent experiments performed in triplicate. [³H]-guanosine binding assay was performed using 50 ug of protein by 20 min under standard assay as described in material and methods section. Specific binding was obtained by the subtraction of the total binding by the non-specific binding using 2 mM of guanosine as displacer.
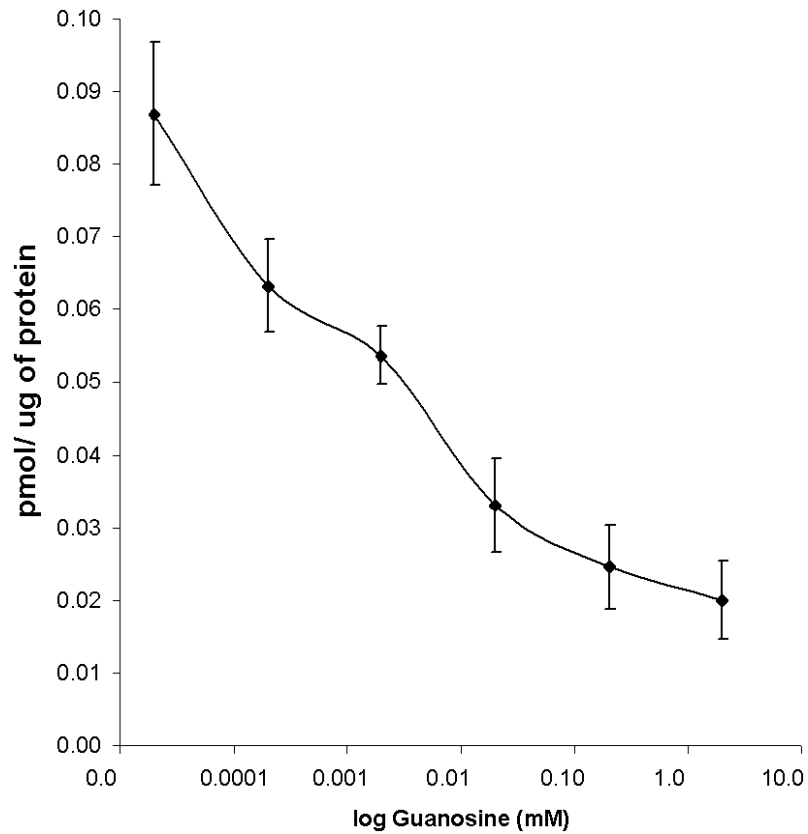
Figure 8 – Displacement of 200 nM of [H³]-guanosine binding from GPV plasmatic membrane. Results represent the mean ± S.E.M. from four independent experiments performed in triplicate. The binding assay was performed using 50 ug of protein by 20 min as described in material and methods section.

Figure 9 – Effects of purines and other agents on displacing 200 nM [³H]-guanosine binding from GPV plasmatic membranes. Equivalent signals indicate statistically homogenous groups. The differences between the groups are significant with $p < 0.05$. Results are expressed in % of specific binding (0.072 ± 0.009 pmol/ ug of protein). The results represent means $\pm$ SEM at least three experiments performed in triplicate.

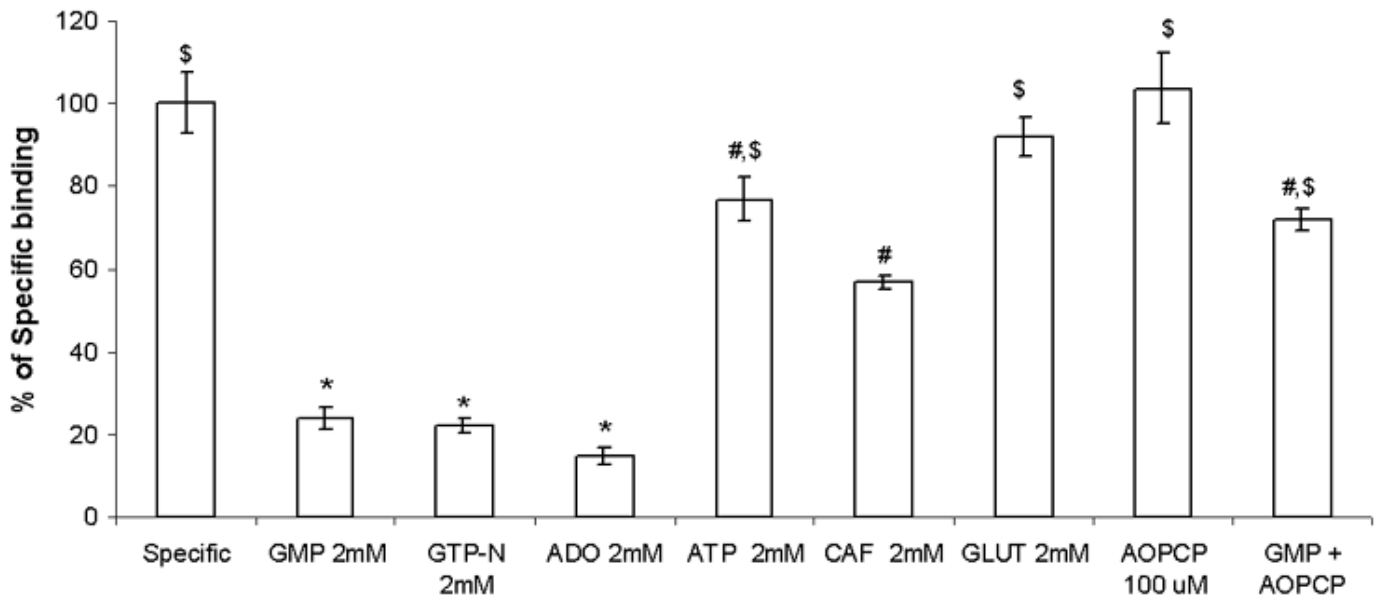## 4 – DISCUSSÃO & CONCLUSÕES

Os resultados apresentados nesta tese vêm corroborar com achados anteriores que demonstraram a interação dos derivados da guanina extracelulares com o sistema glutamatérgico. De acordo com trabalhos já publicados por nosso grupo os derivados da guanina têm uma ação específica sobre o sistema de captação do glutamato em astrócitos (Frizzo et al., 2001, 2003). Sendo que esse efeito é específico do nucleosídeo guanosina, uma vez que GMP e GTP não são capazes de apresentar um resultado semelhante quando as suas degradações são impedidas (Frizzo et al., 2003). Igualmente, o efeito anticonvulsivante parece ser também uma ação direta do nucleosídeo (artigo I), já que a ação do GMP é substancialmente diminuída quando sua conversão a guanosina é impedida pelo AOPCP. Um trabalho posterior do nosso grupo (Schmidt et al., 2005) ajuda a demonstrar que essa degradação dos nucleotídeos é necessária para seu efeito anticonvulsivante. Esse trabalho usa análogos pouco hidrolisáveis do GTP e do GDP que não são capazes de mimetizar os efeitos anticonvulsivantes dos nucleotídeos e da própria guanosina. Esse efeito anticonvulsivante da guanosina não é afetado, assim como a captação de glutamato, por antagonistas clássicos de receptores purinérgicos como a cafeína e a teofilina (Lara et al., 2000, Frizzo et al., 2000, 2003). Ainda a adenosina e outros derivados da adenina não conseguem alterar a captação do glutamato (Frizzo et al., 2003). Dessa forma esse grupo de pesquisa pode, a partir de seus resultados obtidos, sugerir

um papel modulador exercido pelos derivados da guanina no sistema glutamatérgico sobre a captação do glutamato. Efeito esse exercido, a princípio, pelo nucleosídeo guanosina, embora os nucleotídeos tenham efeitos próprios em outras funções que não se assemelham com as da guanosina (Rathbone et al., 1999).

A captação de glutamato tem um papel essencial na finalização da ação do neurotransmissor, uma vez que o mesmo não possui um sistema enzimático capaz de degradar o aminoácido na fenda sináptica (Danbolt, 2001). Uma série de doenças neurodegenerativas parece ter na sua origem problemas relacionados com a captação de glutamato em diferentes áreas do cérebro, áreas essas que são o foco das doenças (Sims & Robinson, 1999; Maragakis & Rothstein, 2001, 2004). Dessa maneira, o estudo do efeito de substâncias capazes de alterar a captação de glutamato pode ser uma alternativa para o estudo da prevenção dessas doenças. Assim a guanosina surge como uma molécula endógena com um potencial muito grande para ser utilizada com essa finalidade.

A guanosina não possui ainda um mecanismo descrito de como ela é capaz de agir sobre os transportadores gliais de glutamato, entretanto nosso trabalho (artigo II) traz uma direção possível para explicar o que pode ter acontecido. Uma vez que a alteração acontece na V_{max} da captação e não no Km dos transportadores, isso sugere um aumento na apresentação de transportadores de glutamato na membrana plasmática. O mecanismo de aumento da translocação ou da diminuição da retirada de transportadores da membrana plasmática que aumentaria a V_{max} já foi observado anteriormente (Duan et al., 1999; Munir

et al., 2000). De qualquer maneira, a ação da guanosina parece aumentar a capacidade de transporte de glutamato. Ainda, esse efeito da guanosina sobre a captação de glutamato em fatias de córtex cerebral acontece em altas concentrações de glutamato, embora em outro resultado (artigo III), temos um aumento na captação de glutamato em concentrações mais fisiológicas do glutamato. Além disso os efeitos da guanosina parecem persistir após sua remoção do meio (artigo II e III). No primeiro artigo temos uma exposição de 60 minutos das fatias de córtex a guanosina e a sua remoção antes do experimento de captação. No artigo III temos a exposição a um pico de concentração de guanosina administrada intracerebroventricularmente e após 30 minutos os animais são submetidos aos protocolos experimentais. Dessa forma a guanosina parece ativar alguma via de sinalização intracelular que leva ao aumento da captação de glutamato de maneira mais permanente após a exposição do tecido a uma determinada concentração do nucleosídeo.

Já foi descrito anteriormente, que os transportadores de glutamato podem sofrer o controle de suas atividades em diversos níveis: transcrição, fosforilação direta, ação de segundos mensageiros aumentando o número de transportadores na membrana plasmática (Sims & Robinson, 1999). Em um artigo, que mostra uma ação antiapoptótica da guanosina (Di Orio et al., 2004), ação esta oposta da apresentada pela adenosina (Di Orio et al., 2002), temos que uma via de sinalização intracelular ativada pela guanosina (PI3-K/AKT/PKB) é a responsável pelo efeito observado da guanosina. A guanosina ativa outras vias de sinalização intracelular como a cascata da MAPK e ainda aumenta a concentração de

AMPc (Rathbone et al., 1999). A guanosina ainda leva a um aumento persistente do cálcio intracelular livre em culturas de astrócitos (Chen et al., 2001). Além disso a guanosina é capaz de estimular a liberação de diversos fatores de crescimento (Rathbone et al., 1999). Os transportadores de glutamato são controlados pela ação de diversas quinases, entre elas PKA, PKC e PI3K (Guillet et al., 2005) representando uma regulação rápida do sistema de transporte de glutamato para prevenir possíveis danos excitotóxicos. Essas quinases sofrem a ação de diversos fatores intracelulares, entre eles os níveis de cálcio, AMPc e vias de sinalização como a cascata das MAPK (Guillet et al., 2005). Os transportadores de glutamato ainda podem ter sua atividade alterada pela ação dos fatores de crescimento (Danbolt, 2001). Considerando que a guanosina é capaz de afetar as cascatas de sinalização, a produção de segundo mensageiros e ainda a liberação de fatores de crescimento, é possível sugerir que uma das vias de sinalização ativadas pela guanosina seja capaz de ativar os transportadores, ou ainda ativar um aumento da sua translocação, uma vez que a rota da PI3K é conhecida por aumentar essa translocação de receptores para a membrana (Holman & Cushman, 1994). A ação de fatores de crescimento como reguladores da captação de glutamato pode nos levar a pensar em alterações mais permanentes, como uma defesa do organismo contra possíveis danos futuros (Figiel et al., 2003).

Diante de tantas ações no SNC é de grande importância a descrição da existência ou não de um receptor para a guanosina. Dessa maneira Traversa (2002) demonstrou a existência de um local de ligação específica pra guanosina em preparações de membranas

de cérebro de ratos. Esse sítio de união foi descrito como específico para guanosina e ainda ser acoplado a uma proteína G. Em nossos resultados (artigo IV) encontramos um sítio de união da guanosina que parece ser preferencialmente astrocitário em detrimento das preparações neuronais e ainda mostramos a importância do uso de preparações de membranas plasmáticas para esse tipo de experimentos, uma vez que as membranas de mitocôndrias demonstraram possuir um sitio de união para guanosina. Em nossos resultados o sitio de união para guanosina mostrou ser sensível ao GMP, entretanto o impedimento da conversão do GMP para GUO parece interferir no efeito encontrado para o GMP. O glutamato, AOPCP e ATP não foram capazes de afetar significativamente a união especifica da guanosina em seu sitio de união. A cafeína não demonstra o mesmo potencial de inibição que nos receptores clássicos para purinas (Fredholm et al., 1999). O sitio de união parece ser metabotrópico, ou seja possuir proteínas G acopladas, uma vez que o GTP-N é capaz de diminuir a união da guanosina no seu sitio. Esse achado está de acordo com os achados de Traversa e colaboradores (2002).

As diferenças entre o nosso trabalho e o apresentado por Traversa e colaboradores se dá nos seguintes parâmetros: demonstramos que o sitio de união da guanosina é preferencialmente astrocitário, o uso de uma preparação de membrana plasmática proveniente de uma preparação enriquecida em membrana de astrócitos, as diferenças na sensibilidade do sitio de união, principalmente no que diz respeito à adenosina. A utilização de membranas diferentes pode ser uma explicação para o resultado econtrado na

sensibilidade. Entretanto, nossa preparação de membrana parece ser mais ajustada ao que se poderia esperar de um sitio de união com tanta importância neuroquímica. Devemos lembrar que o processo pelo qual as membranas utilizadas passam, permite uma separação de membranas mitocôndrias e plasmáticas, permitindo um melhor estudo dos receptores encontrados na superfície das membranas celulares. Isso vem corroborar com alguns resultados do nosso grupo, que apontam para uma ação extracelular da guanosina, pelo menos no que diz respeito ao aumento da captação de glutamato (Frizzo et al., 2001). Ainda os achados referentes ao GMP e glutamato parecem estar de acordo com trabalhos anteriores. Os efeitos do GMP devem-se à sua conversão a guanosina (Frizzo et al., 2003, artigo I). Além disso a guanosina é o único derivado da guanina que não interfere na união de ácido caínico (Souza & Ramirez 1991), mostrando não haver uma relação de efeitos baseada na ação de deslocamento do sitio de união tanto da guanosina como do glutamato. Isso posto, a ação da guanosina sobre a captação de glutamato parece ser mediada via ação desse novo receptor e não uma ação direta no transportador ou em algum receptor de glutamato. Devido esse receptor ser sensível à adenosina e guanosina acreditamos que a ação desencadeada dependeria do ligando que estivesse presente no receptor, uma vez que adenosina e guanosina desempenham algumas ações bem diferentes no sistema nervoso central (Frizzo et al., 2003; Chen et al., 2001; Di Orio et al., 2002, 2004).

Diante de tudo que já foi exposto é necessário repensar o papel da guanosina como uma mera coadjuvante das ações da adenosina dentro do sistema purinérgico. É preciso

lembrar que em situações de desequilíbrios no sistema nervoso ou mesmo em condições normais que levam à liberação de purinas, a guanosina é liberada em maior quantidade e ainda permanece por mais tempo com sua concentração aumentada (Uemura et al., 1991; Ciccarelli et al., 1999; Dolbolyi et al., 2000). Algumas dessas situações assemelham-se com as que encontramos grandes liberações glutamato, como é caso de eventos hipóxico-isquêmicos ou mesmo em traumas cranianos (Sims & Robinson 1999). Dessa maneira parece haver uma associação entre os eventos possivelmente excitotóxicos, que levariam a uma descarga massiva de glutamato, com a liberação purinas nas sinapses centrais. Considerando todos os fatos já expostos sobre a guanosina temos que considerar o que possuímos de evidências para um papel importante da guanosina dentro do sistema purinérgico:

1)   a guanosina exerce um papel antiapoptótico, ao contrário do apresentado pela adenosina;

2)   a guanosina aumenta a captação de glutamato e a adenosina e outros derivados da adenina não afetam a captação astrocitária de glutamato;

3)   as condições que levam a grandes liberações de glutamato também levam a liberação de purinas; que as concentrações de guanosina liberadas são maiores que as da adenosina, e ainda permanecem por mais tempo elevadas;

4) a guanosina é capaz de alterar a concentração intracelular de cálcio em astrócitos em pequenas concentrações, e essas alterações são mais duradouras que as provocadas pela adenosina;

5) a guanosina demonstrou ser capaz de prevenir por diversas vias de administração (o.p., i.c.v., i.p.), situações de hiperatividade do sistema glutamatérgico, sendo estas ações independentes dos receptores clássicos das purinas;

6) os efeitos sobre captação de glutamato parecem acontecer em situações fisiológicas e em situações de altas concentrações de glutamato.

7) parece haver um sítio de união específico para a guanosina em membranas celulares no SNC

Uma vez que estes resultados são indicativos de uma real associação da guanosina com a modulação do sistema glutamatérgico, é possível pensar na guanosina como um fator difusível para modular o sistema glutamatérgico fisiologicamente e em situações não fisiológicas. Uma vez que a captação de glutamato deve ter um ajuste muito preciso, muitas opções devem ser oportunizadas para a execução dessa tarefa, essas opções podem atuar individualmente ou em associações. Dentro do sistema purinérgico a guanosina parece ser a mais indicada para participar deste controle do sistema glutamatérgico nas diferentes formas apresentadas. Desta maneira se faz necessário que tenhamos um novo olhar sobre o sistema purinérgico, não mais como um sistema de ações restrita aos derivados da adenina

(adenosina e ATP), mas com um novo e importante grupo de atores nesse sistema que são os derivados da guanina, com suas ações específicas e com um receptor apropriado para suas ações no sistema nervoso central. Tal importância parece que começa a ser reconhecida por trabalhos que mostram que a deficiência no metabolismo da guanosina, pode estar associada com anormalidades no neurodesenvolvimento, na neuromodulação e neurotransmissão associados com a síndrome de Lesch-Nyhan (Deutsch et al., 2005). Esses estudos desenvolvidos até aqui somados a essa nova perspectiva no sistema purinérgico abrem todo um novo campo de estudo. Essa nova fronteira abrange desde a modulação da captação de glutamato para fins terapêuticos utilizando a guanosina em várias doenças neurodegenerativas e outros eventos que envolvem a excitotoxicidade do glutamato, até a produção de novas moléculas capazes de atuar similarmente à guanosina ou mesmo a produção de antagonistas específicos para as ações desempenhadas pela guanosina.

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