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EFEITOS DA ADMINISTRAÇÃO ORAL AGUDA E CRÔNICA DE GUANOSINA SOBRE A MEMÓRIA E COMPORTAMENTO DE RATOS E CAMUNDONGOS

Tese de doutorado

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Juliana e Isabella e ao Roska

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Resumo

(Elsa Regina do Canto Vinadé, EFEITOS DA ADMINISTRAÇÃO ORAL DE GUANOSINA SOBRE A MEMÓRIA E COMPORTAMENTO DE RATOS E CAMUNDONGOS). Uma das formas utilizadas para se classificar memória é através do seu tempo de duração; ou seja, o tempo que transcorre entre a aquisição e a evocação desta. Sendo assim, a memória pode ser de curta ou longa duração. A memória de curta duração (STM) se mantém por segundos ou minutos até 3 - 6 horas após o aprendizado e a de longa duração (LTM) pode durar horas, dias, meses, ou anos. A guanosina, um nucleosídeo da guanina, tem sido descrita por exercer efeitos sobre a memória e neuroprotetores, os quais parecem estar relacionados com o antagonismo ao sistema glutamatérgico. Nessa tese, estudamos o efeito da administração oral (aguda ou crônica) de guanosina sobre a LTM utilizando a tarefa de esquiva inibitória. Além disso avaliamos também o seu efeito neuroprotetor sobre convulsões e morte induzidas por α-dendrotoxina, e sobre a atividade locomotora e ansiedade em ratos e/ou camundongos. Os resultados demonstram que duas semanas de tratamento com guanosina ingerida à vontade foram suficientes para atenuar a convulsão e a morte de camundongos induzidas por α-DTX, e também apresentou efeitos ansiolítico e amnésico, sem afetar a coordenação motora, temperatura retal, peso corporal ou consumo de água e alimento. O mesmo tratamento em ratos atenuou convulsões induzidas por ácido quinolínico e apresentou somente efeito amnésico. As evidências experimentais demonstram que os efeitos centrais da guanosina estão relacionados ao antagonismo do sistema glutamatérgico, devido ao aumento da captação do glutamato, ao invés de uma ação direta nos receptores glutamatérgicos. Além disto, uma série de estudos neuroquímicos e comportamentais sugerem que as ações da guanosina são independentes da adenosina, um nucleosídeo da adenina que possui efeitos semelhantes à guanosina. Conjuntamente, esses resultados obtidos com camundongos e ratos se tornam um bom indicativo para o uso oral da guanosina, que sendo um composto endógeno, poderia ser bem tolerada para uso humano com propósitos terapêuticos.

Abstract

(Elsa Regina do Canto Vinadé, EFFECTS OF ORAL ADMINISTRATION OF GUANOSINE ON MEMORY AND BEHAVIOR IN RATS AND MICE). One manner to classify memory is trough its time duration, as well, the time elapsed between aquisition and consolidation. Thus short term memory (STM) can be sustained by seconds minutes until 3 to 6 hours after learning, whereas the long term memory (LTM) is able to be maintened by hours, days, months or years. The nucleoside guanosine has been described to exert effects on memory and as a neuroprotective agent, and such actions appear to be related to an antagonism in the glutamatergic system. Here we studied the effect of oral administration of guanosine (acute or chronic) in the LTM, using an inhibitory task. Moreover, it was evaluated the neuroprotective effect of guanosine in seizures and death induced by α-dendrotoxin (α-DTX), as well as, in the locomotor activity and anxiety using rats and/or mice. The results demonstrated that after two weeks the free ingestion of guanosine was able to prevent seizure and death of mice induced by α-DTX. Also guanosine was anxiolitic and amnestic without influence in the motor coordination, retal temperature body weight or water consuption and breed. The same protocol prevented, in rats, the seizure induced by quinolinic acid and evoked an amnestic effect. Experimental evidences showed that the effects of guanosine are related the antagonism of the glutamatergic system , probably due to increase in glutamate uptake instead of a direct action on glutamate receptors. Additionally, several neurochemical and neurobehavioural studies suggested that the guanosine act independently of adenosine, a nucleoside that has similar as effects of guanosine. Altogether, these results indicate that guanosine, an endogen compound for humans, can be potentially used terapeutically when administered orally.

Lista de Abreviaturas

ADP	Adenosina difosfato
α-DTX	α-dendrotoxina
AMP	Adenosina monofosfato
AMPA	Ácido α-amino-3-hidroxi-5-metil-4isoxazol propiônico
ATP	Adenosina trifosfato
CR	Resposta condicionada
CS	Estímulo condicionado
EAAC1	Aminoácido excitatório
GABA	Ácido gama-aminobutírico
GBP	Bases purínicas da guanina
GDP	Guanosina difosfato
GLAST	Transportador de glutamato e aspartato em ratos
GLT-1	Transportador de glutamato em ratos
GMP	Guanosina monofosfato
GTP	Guanosina trifosfato
iGluR	Receptores glutamatérgicos ionotrópicos
KA	Ácido caínico
LTD	Depressão de longa duração
LTM	Memória de longa duração
LTP	Potenciação de longa duração
mGluR	Receptores glutamatérgicos metabotrópicos
NMDA	N-metil-D-aspartato
SNC	Sistema Nervoso Central
STM	Memória de curta duração
US	Estímulo incondicionado
WM	Memória de trabalho

Apresentação

O item I - **Introdução** contém dados da bibliografia corrente, de forma a fundamentar este estudo.

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos publicados e um submetido à publicação, os quais encontram-se organizados em anexos, no item II – **Parte Experimental** – Artigos Científicos. Material e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos e representam a integra deste estudo.

O item III - **Considerações Finais**, encontrado no final desta tese, apresenta interpretações e comentários gerais sobre todos os artigos científicos e manuscritos aqui apresentados.

No item IV - **Referências Bibliográficas** refere-se somente ás citações que aparecem na introdução e considerações finais desta tese.

I. Introdução

I.1. Memória

Uma das mais importantes capacidades do sistema nervoso central (SNC) é a de adquirir informações e armazená-las como memórias; a expressão das memórias previamente adquiridas é crucial para a sobrevivência e evolução das espécies. A memória permite ao indivíduo situar-se no presente, levando em consideração o passado e futuro. Assim, o acervo de nossas memórias faz com que cada um de nós seja o que é, com que sejamos, cada um, um indivíduo, um ser para o qual não existe outro idêntico (Izquierdo, 2002).

Por definição, memória é a aquisição, o armazenamento (consolidação) e a evocação (retrieval) de uma informação e de suas conseqüências, sendo a aquisição o momento no qual o indivíduo é apresentado ao evento a ser aprendido. Pode-se dizer que a consolidação é um processo de fixação da memória e envolve uma série de processos metabólicos no cérebro, uma vez que esta não é adquirida na sua forma final (Izquierdo e Mc Gaugh, 2000; Mc Gaugh, 2000; Izquierdo, 2002). Por último, a evocação é a "lembrança", ou seja, no momento da evocação o cérebro deve "recriar" as memórias formadas, e é importante ressaltar que os mecanismos de evocação são diferentes dos de formação da memória (Barros, et al., 2000; Szapiro, et al., 2000; Izquierdo, 2002).

As memórias podem ser classificadas de acordo com o seu conteúdo ou tempo de duração. Segundo seu conteúdo, elas podem ser classificadas como declarativas ou explícitas, e procedurais ou implícitas, sendo que as declarativas, relacionadas a fatos ou conhecimentos, são conscientemente adquiridas e evocadas, enquanto que as procedurais são memórias gradativamente adquiridas e relacionam-se com nossas capacidades ou habilidades, e uma vez adquiridas são extremamente duradouras. Além disso, a memória declarativa corresponde à "aprendizagem racional", uma forma de aprendizagem que permite ao indivíduo adquirir informações sobre pessoas, lugares, fatos e circunstâncias complexas, utilizando-se de mais de uma modalidade sensorial (Thompson & Kim, 1996). Por sua vez, a memória implícita é revelada quando a experiência prévia facilita o desempenho numa tarefa que não requer a evocação consciente ou intencional daquela experiência (Schacter, 1987). Mais recentemente, Danion et al.,(2001) descreveram que as memórias adquiridas inconscientemente são ditas implícitas e aquelas adquiridas conscientemente são explícitas.

De acordo com o tempo de duração, uma memória primária é aquela que estaria atuando enquanto a memória secundária ou principal seria formada (Willian James, 1890). Mais tarde essas denominações foram substituídas por memória de curta duração (STM – Short-Term Memory) e memória de longa duração (LTM – Long-Term Memory). Antecedendo a STM, ocorre a memória de trabalho (WM – Working Memory), que pode ser definida como uma memória imediata. Essa memória é dependente de uma atividade elétrica das células e persiste somente enquanto essa atividade existir, e sua função é completamente diferente da STM e LTM (Izquierdo, et al., 1998, Izquierdo, et al., 1999, McGaugh, 2000).

A memória de curta duração estende-se desde os primeiros segundos ou minutos após o aprendizado até 3-6 horas, e, durante esse tempo, ocorrem vários eventos bioquímicos que culminam com a transcrição gênica e síntese protéica necessárias para a consolidação da memória de longa duração (Medina, et al., 1999). A STM não é suscetível à extinção, mas é bastante resistente a muitos dos mecanismos que afetam a consolidação da LTM. Porém, é vulnerável a interrupções; por exemplo, pode ser "apagada" por traumas cranianos ou eletrochoque convulsivo. A LTM se processa lentamente, e nas primeiras horas após sua aquisição é lábil e suscetível à interferência de fatores como traumatismos, tratamentos farmacológicos e novidades. Sabe-se também que essas memórias não ficam estabelecidas na sua forma permanente imediatamente após sua aquisição (Izquierdo, et al., 1999; Vianna, et al., 2000; Nader, et al.,2000).

A diferença entre a STM e a LTM está nos mecanismos subjacentes a cada uma delas, já que seu conteúdo cognitivo é o mesmo. Em vários trabalhos publicados pelo grupo do Prof. Izquierdo foi demonstrado que a STM e a LTM são processos paralelos (Izquierdo, et al., 1998a-c). Atualmente já são conhecidos mais de 20 tratamentos que afetam apenas um dos tipos de memória, sem comprometimento do outro (Izquierdo, et al., 1999; Vianna, et al., 1999; Izquierdo e McGaugh, 2000).

I.1.1. Mecanismos de formação de memórias e consolidação

Muito do que se conhece atualmente sobre mecanismos envolvidos no fenômeno de plasticidade neuronal deve-se à descrição do fenômeno de potenciação de longa duração ou simplesmente LTP (Long Term Potentiation) por Bliss e Lomo, (1973). Este consiste em um aumento na eficiência da transmissão sináptica em neurônios de várias estruturas cerebrais, em resposta a um estímulo de alta freqüência. Vários trabalhos demonstraram que a LTP

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compartilha importantes características típicas com o aprendizado de novas memórias (Bliss e Colingridge, 1993; Eichenbaum, 1994, 1996; Izquierdo, 1994). Tanto a aquisição de novas memórias, como a indução da LTP são iniciadas na sessão de aprendizado, pela ativação de receptores glutamatérgicos dos tipos AMPA (responsáveis pela transmissão basal), NMDA e metabotrópicos (Huang, et al., 1996; Izquierdo e Medina, 1997; Malenka e Nicoll, 1999).

Apesar do processo de aquisição de uma nova memória ser intenso, com o correr do tempo, a intensidade e clareza poderão sofrer um decréscimo, já que não recordamos tudo o que nos sucede e daquilo que recordamos não temos todos os detalhes. Guardamos aquilo que, por determinadas circunstâncias, individuais e do contexto, parecem ser determinantes para que possamos recordar. Á esse "filtro" chamamos consolidação. O termo consolidação é usualmente empregado para designar o complexo de atividades bioquímicas que ocorrem após a aquisição. Durante a consolidação a informação armazenada pode ser modulada pelo componente emocional (com toda sua fisiologia hormonal), para posteriormente ser evocada quando o indivíduo necessitar dessa informação (McGaugh, 2000; Barros, et al., 2003).

I.1.2. Evocação (Retrieval) e extinção

Apesar de muitas das doenças psiquiátricas serem acompanhadas por déficits de memória, que na maioria das vezes só serão descobertas quando o indivíduo começa a apresentar dificuldades para evocar memórias, apenas recentemente foram estudados os mecanismos moleculares e as áreas cerebrais envolvidas no processo de evocação (Barros, et al., 2000; Izquierdo, et al., 2000; Sara, 2000; Szapiro, et al., 2000).

Algumas vezes as evocações de memória podem ocorrer espontaneamente como resultado de flutuações nos modelos de atividade neural, porém, a evocação usualmente ocorre como resultado da integração da chegada da informação do meio ambiente com a rede de memórias já existente (Tulving e Thomson, 1973). Segundo Tulving e Thomson, a evocação da memória leva à formação de novas memórias combinadas com memórias anteriores. Isso pode sugerir que cada evocação de memória poderia produzir um processo de reconsolidação (Nader, et al., 2000; Sara, 2000).

A memória só pode ser avaliada através da medida de sua evocação; por exemplo, pelo estudo do comportamento de um rato nadando numa piscina, ou através de perguntas a humanos, ou podem ser inferidas por uma medida eletrofisiológica (Sara, 2000).

No condicionamento proposto por Pavlov (1956) o aprendizado ocorre quando um estímulo condicionado (CS) é associado com um outro estímulo incondicionado (US), produzindo uma resposta curta e clara. O estímulo condicionado normalmente é simples ou neutro (luz, tom, odor) ou um conjunto (um ambiente, um aparato de treino) provocando uma resposta de orientação ou investigativa, enquanto que o estímulo incondicionado ao qual Pavlov chama "estímulo biologicamente significativo", pode ser um alimento, um choque, uma circunstância ou uma situação prazerosa ou não. A resposta ao CS muda quando este é associado ao US, deixando de ser uma resposta meramente de orientação e passa a ser chamada de resposta condicionada (CR) (Vianna, et al., 2003; Izquierdo, et al., 2002).

Também segundo Pavlov, a apresentação repetida do estímulo condicionado, sem a presença do incondicionado, resulta na extinção da resposta; assim, a evocação pode dar início ao processo de extinção da memória. Muitas evidências indicam que a extinção de um aprendizado também necessita de uma nova informação. Então, no caso de um aprendizado aversivo (como na esquiva inibitória), a ausência do choque na sessão de teste é a nova informação, não mais associada ao ambiente do treino (US) (Vianna, et al., 2003; Szapiro, et al., 2003; Izquierdo, et al., 2002; Vianna, et al., 2001).

I.2. Sistema Glutamatérgico

O glutamato é o principal neurotransmissor excitatório do Sistema Nervoso Central (SNC) e está presente na maior parte das sinapses (Cotman et al., 1995; Ozawa et al., 1998). Sendo responsável por respostas excitatórias pós-sinápticas, o glutamato desempenha um papel fundamental em diversos processos fisiológicos, como aprendizado e memória, cognição e na formação de redes neurais durante o desenvolvimento e envelhecimento de mamíferos (Collingridge e Lester, 1989; Izquierdo e Medina, 1997; Castellano et al., 2001; Segovia et al., 2001). O glutamato também produz alterações de longa duração na excitabilidade neuronal, como a indução da Potenciação de Longa Duração (LTP – Long Term Potentiation), na transmissão sináptica em neurônios do hipocampo e do córtex visual (Artola e Singer, 1987; Ito, 1989). Entretanto, em algumas situações patológicas, quando ocorre uma excessiva ativação de receptores glutamatérgicos, pode haver dano ou morte neuronal. Olney e Ho (1970) denominaram essa situação de excitotoxicidade. Vários trabalhos têm associado a excitotoxicidade do glutamato com patologias e desordens

neurodegenerativas como a isquemia cerebral, epilepsia, encefalopatias isquêmicas, doenças de Alzheimer e de Huntington (Lipton e Rosenberg, 1994).

I.2.1. Receptores glutamatérgicos

Existem dois tipos de receptores glutamatérgicos: os ionotrópicos (iGluR) e os metabotrópicos (mGluR). Os ionotrópicos são canais iônicos, ou seja, permitem a passagem de um íon específico quando ativados. São subdivididos, de acordo com sua sensibilidade a agonistas, em receptores N-metil-D-aspartato (NMDA), α–amino-3-hidroxi-5-metil-4-isoxazol-ácido propiônico (AMPA) e ácido caínico (KA). Todos os subtipos de receptores são ativados pelo glutamato, porém cada um deles é ativado seletivamente por um agonista diferente. Os receptores metabotrópicos (mGluR) são receptores que interagem com proteínas ligantes de nucleotídeos da guanina (proteína G), ativando ou inibindo eventos celulares pela modulação de efetores intracelulares (Ozawa et al., 1998; Bear et al., 2001).

Os receptores AMPA e NMDA medeiam a maioria das transmissões excitatórias rápidas no cérebro, enquanto que o receptor cainato não tem sua função bem clara ainda. Receptores AMPA são permeáveis tanto ao Na^+ como ao K^+ e, principalmente, não são permeáveis ao Ca^{2+}. Normalmente o efeito de ativação é um potencial de membrana negativo, que ao permitir a entrada de Na^+ na célula causam uma ampla e rápida despolarização. Receptores AMPA coexistem com os receptores NMDA em muitas sinapses, mas diferem entre si em dois pontos muito importantes: enquanto os NMDA são permeáveis ao Ca^{2+} e a entrada de íons é voltagem-dependente, os AMPA não são.

Os receptores NMDA estão distribuídos em todo o cérebro e apresentam importantes propriedades: i) a abertura dos canais iônicos requer a ocupação do sítio de união de NMDA/Glutamato por um agonista e do sítio de união para glicina que atua como co-agonista, da liberação via despolarização (voltagem dependente) e do bloqueio do canal iônico por Mg^{+2}; ii) sua ativação resulta em alta permeabilidade a Ca^{+2}; iii) são ativados por baixas concentrações de glutamato; iv) o tempo de abertura do canal é prolongado; v) possuem mecanismos modulatórios múltiplos (glicina, zinco, poliaminas, etc...). A entrada de Ca^{+2} na célula, através dos receptores NMDA, pode causar mudanças que levam à LTM, por exemplo (Bear, et al., 2001).

A ativação do receptor NMDA, assim como o influxo de íons, só ocorre se a membrana neuronal for previamente despolarizada pela ativação do receptor AMPA, permitindo a saída de Mg^{2+} do canal, uma vez que em repouso o canal NMDA está bloqueado por Mg^{2+}.

I.2.2. Captação de glutamato

Uma vez sintetizado no citosol, o glutamato é estocado em vesículas sinápticas no terminal pré-sináptico, até ser liberado destas na fenda sináptica por um processo chamado de exocitose, que é dependente da concentração de Ca^{2+}. Após interação com seus receptores pré- e pós-sinápticos, o glutamato é removido da fenda sináptica por sistemas de transporte dependentes de Na^{+}, localizados na glia, principalmente (Anderson e Swanson, 2000; Amara e Fontana, 2002).

São conhecidos cinco subtipos de transportadores de glutamato (EAAT 1-5): GLT-1, localizado exclusivamente em astrócitos; GLAST, expresso em glia e em neurônios, com distribuição predominante em astrócitos; EAAC1, localizados em neurônios; EAAT4, transportador limitado às células de Purkinje do cerebelo e EAAT5, presente na retina e células bipolares (Maragakis e Rothstein, 2004). A quantidade de GLT-1 e GLAST em astrócitos é particularmente alta nos astrócitos de terminações nervosas e cordão dendrítico, o que é consistente com o papel destes transportadores na "captação" do glutamato liberado sinapticamente (Chaudhry, et al., 1995; Rothstein, et al.,1994). O sistema de captação de glutamato é responsável pela inibição momentânea da ação glutamatérgica, uma vez que os transportadores retiram o glutamato da fenda sináptica. Após ser captado, o glutamato, pela ação da glutamina sintetase dos astrócitos, é transformado em glutamina. Esta, por sua vez, através da glutaminase presente nos neurônios, é transformada em glutamato, que será vesiculado e liberado novamente, reiniciando o processo (Anderson e Swanson, 2000; Amara e Fontana, 2002).

I.2.3. Sistema glutamatérgico e memória

É bem estabelecido que a aquisição de novas memórias, assim como a indução da LTP, é iniciada, durante a sessão de aprendizado, pela ativação de receptores glutamatérgicos NMDA e metabotrópicos, além dos receptores AMPA, responsáveis pela transmissão sináptica basal (Izquierdo e Medina, 1997; Malenka e Nicoll, 1999). Os eventos bioquímicos envolvidos na formação da memória de esquiva inibitória, no hipocampo, envolvem primeiramente uma

ativação dos receptores glutamatérgicos NMDA, AMPA e metabotrópicos, além de um pequeno aumento dos níveis de NMDA1 e um aumento tardio, maior, dos níveis de GluR1 (Izquierdo e Medina, 1997).

Existe uma infinidade de trabalhos, utilizando diferentes métodos, em diferentes estruturas cerebrais, que demonstram a importância dos receptores glutamatérgicos sobre a memória (Izquierdo, et al., 1992; Jerusalinsky, et al., 1992; Izquierdo, et al., 1997; Izquierdo e Medina, 1997; Izquierdo e Mc Gaugh, 2000; Mc Gaugh, 2000; McKay, et al., 2002; Nishiga, et al., 2002). Por outro lado, a extinção da memória parece ser independente de receptores NMDA (Berman e Dudai, 2001).

Izquierdo et al. (1997) demonstraram que os receptores NMDA são necessários, inicialmente no hipocampo e amídala, depois no córtex entorrinal e parietal, para o processamento pós-treino da memória, enquanto que para o mecanismo de "retrieval" são necessários os receptores AMPA. Existem várias similaridades entre o efeito de receptores glutamatérgicos sobre a LTP e outros tipos de memória (esquiva inibitória e "water maze"), enquanto a intervenção de receptores glutamatérgicos NMDA ou não-NMDA (dependendo da região) na LTP é restrita a um breve período de indução (segundos). A intervenção destes mesmos receptores nos estágios iniciais das outras memórias tem duração maior (minutos), (Izquierdo and McGaugh, 2000).

Segundo Castellano et al. (2001), pode haver uma discrepância nos resultados devido a diferentes modalidades de administração (crônica/aguda), e/ou à influência de fatores não específicos (administração periférica ou central) ou ainda devido ao grau de stress do animal experimentado.

I.3. Sistema purinérgico

As purinas desempenham uma ação ampla e específica na sinalização extracelular, regulando assim diversas funções em tecidos de vertebrados e invertebrados. Além disso, desempenham um papel chave no desenvolvimento, proliferação e diferenciação celular (Abbracchio and Burnstock, 1999). Os efeitos extracelulares das bases púricas da adenina são exercidos através de receptores específicos: P1 para a adenosina e P2 que são seletivos para ATP (Gendron et al., 2002). Entretanto, apesar de efeitos extracelulares já descritos, ainda não foram identificados receptores para os derivados das bases púricas da guanina.

O sistema purinérgico é composto pelos derivados da adenina (ATP, ADP, AMP e adenosina) e os derivados da guanina (GTP, GDP, GMP e guanosina).

I.3.1. Guanosina

A guanosina, nucleosídeo foco desta tese, embora menos estudada que os demais derivados da guanina, tem atualmente recebido muita atenção com o intuito de elucidar seu papel e mecanismo de ação no SNC. Em cultura de astrócitos, a exposição a condições de hipóxia associada à hipoglicemia 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). Uemura e col. (1991), demonstraram que uma isquemia cerebral produziu um aumento de aproximadamente 140 % dos níveis de guanosina por mais de uma semana. Além disso, um estudo de microdiálise em tálamo de ratos demonstrou que a despolarização in vivo por K^+, cainato e oubaína elevam a concentração de guanosina e adenosina (Dobolyi, et al., 2000). Fisiologicamente 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 serem mediados em parte pela adenosina, já que são atenuados por antagonistas de receptores de adenosina do tipo P1 e pela enzima adenosina deaminase. Além disso, foi visto que a guanosina promove a liberação de adenosina em astrócitos (Cicarelli, et al., 2001). 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; Cicarelli, et al., 2001). Em várias publicações do grupo do Prof Diogo Souza têm sido proposto que a guanosina atua modulando os efeitos do neurotransmissor glutamato no SNC.

I.3.2. Adenosina e o sistema glutamatérgico

A adenosina tem um papel importante na modulação da transmissão sináptica e excitabilidade neuronal, e medeia esses efeitos através de 4 tipos de receptores: A_1, A_{2A}, A_{2B} e A_3, nos quais a proteína G é acoplada. Os receptores A_1 são os mais abundantes e tem maior afinidade entre os receptores de adenosina no SNC (Pereira et al., 2002), enquanto os A_2 são mais concentrados em áreas ricas em dopamina. Agonistas destes dois tipos de receptores de adenosina produzem alterações comportamentais e neurofisiológicas induzidas por

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antagonistas de receptores NMDA; por outro lado, a ativação de receptores NMDA induz a liberação de adenosina no hipocampo e estriado, *in vivo* e *in vitro* (Dall'Igna, et al., 2003). Com relação à ansiedade, a adenosina têm mostrado ter uma atividade ansiolítica, que foi confirmada pelo uso de camundongos nocautes para receptores A_1 de adenosina, que demonstraram um aumento no comportamento de ansiedade (Ribeiro et al., 2003).

Os receptores A_1 inibem a liberação de neurotransmissores e afetam a plasticidade sináptica no hipocampo de maneira atividade-dependente, atenuando a depressão de longa duração (LTD) e inibindo a potenciação de longa duração (LTP) (Pereira et al., 2002).

A adenosina exerce função de neuroprotetor endógeno, graças à inibição da liberação pré-sináptica e da ação pós-sináptica do glutamato, por hiperpolarização neuronal (Brundege e Dunwiddie, 1997). Outro fato importante é que seus níveis aumentam em situações de excitotoxicidade, onde há um hiperatividade glutamatérgica (Lipton e Rosenberg, 1994; Ralevick e Burnstock, 1998). A adenosina inibe a liberação de glutamato, que por sua vez promove a liberação de adenosina (Hoehn et al., 1990; Craig e White, 1993).

Tem sido demonstrado que vários agonistas e antagonistas de receptor A_1 de adenosina, quando administrados sistemicamente, alteram o aprendizado de esquiva inibitória (Pereira et al., 2002). Nesta tese usamos a cafeína, um antagonista não seletivo A1/A2 dos receptores de adenosina, conhecida por melhorar a performance cognitiva em humanos, para reverter o efeito da adenosina sobre a memória de esquiva inibitória em camundongos (Kopf, et al., 1999).

I.4. Epilepsia e convulsões

O termo *epilepsia* se refere a uma ampla categoria de sintomas complexos em torno de qualquer número de funções cerebrais desordenadas, decorrentes de uma anormalidade e hipersincronia de atividade neuronal, podendo ser secundárias a uma variedade de processos patológicos, não sendo esta uma doença específica ou uma síndrome simples (Engel Jr e Pedley, 1997). Os termos *desordem convulsiva e convulsão cerebral* são sinônimos de epilepsia quando caracterizadas clinicamente por alterações subjetivas de comportamento súbitas (crises epiléticas), podendo se repetir ao longo da vida do paciente (Duncan et al., 1995).

A prevalência na população humana de epilepsia é aproximadamente de 1% (Shorvon, 1990; Palmini e Costa, 1998; Sander e Sillanpää, 1998), acreditando-se que, de toda a população mundial, 3% desenvolverão epilepsia em algum momento de sua vida (Chang e Lowenstein, 2003). Destes números, aproximadamente 70% dos pacientes epilépticos têm suas crises controladas através de medicamentos com drogas antiepilépticas. O restante dos pacientes constitui um grupo de alta morbidade, uma vez que suas crises epilépticas não são controladas através do uso de medicamentos (Palmini e Costa, 1998).

O termo convulsões refere-se aos sintomas que ocorrem em doenças agudas (convulsões provocadas) ou em epilepsia (convulsões não provocadas). Este constitui episódio estereotipado envolvendo alterações sensoriais, das funções autonômicas motoras e de consciência, devido a uma descarga elétrica anormal no SNC (Fischer e Maslah, 2003). Convulsões representam uma disfunção neurológica em que os sintomas incluem manifestações positivas que podem ocorrer durante a convulsão: percepção de luzes piscando ("flashes") e movimento involuntário dos membros ("jerking"); e manifestações negativas: lentidão da função cerebral resultando em perda da consciência, cegueira transiente e paralisia. A manifestação destes sintomas é dependente da região afetada. Em humanos, recorrência espontânea de convulsões não provocadas é o principal sintoma de epilepsia, no qual síndromes específicas podem ser definidas por diversos fatores, incluindo achados em exames clínicos, eletroencefalograma e estudo neurológico por imagem (Shneker e Fountain, 2003; Chang e Lowenstein, 2003; Doman e Pelligra, 2003).

Os mecanismos envolvidos nesta desordem estão diretamente relacionados com a neurotransmissão do SNC, podendo ser esta devido a diversos fatores (genéticos ou patológicos). Geralmente, as epilepsias de origem genética aparecem na infância, e as decorrentes de algum processo patológico aparecem na vida adulta (Chang e Lowenstein, 2003). Anormalidades na neurotransmissão inibitória ou de ambos eventos ocorrem concomitantemente (Meldrum, 1994).

Diversos modelos de convulsões e epilepsias são descritos na literatura (Mody e Schwartzkroin, 1997; Naffah-Mazzacorati, 1998). Os modelos de convulsões, também chamados modelos agudos, são aqueles em que o animal apresenta episódios convulsivos somente durante a ação do agente indutor. Estes agentes indutores podem ser químicos, no qual compostos são aplicados por via tópica ou injetável, interferindo no balanço neuroquímico da atividade neuronal, ou elétricos em que é aplicado um choque

eletroconvulsivo no animal, alterando a atividade elétrica cerebral (Mody e Schwartzkroin, 1997). Os modelos de epilepsias, modelos crônicos, são aqueles em que as crises epilépticas surgem em intervalos de tempos variados, sem um estimulo exógeno antes de cada crise convulsiva. A característica principal destes modelos é a apresentação de um fator incidente conhecido, capaz de induzir a epileptogênese; após certa latência, ocorrem crises epilépticas espontâneas (Naffah-Mazzacorati, 1998). Considerando estes modelos de epilepsia, os mais utilizados são o modelo da pilocarpina (Turski, et al., 1983), do ácido caínico (Bem-ari, 1985) e o abrasamento (kindling) (Goddard, 1967), sendo que todos mimetizam a epilepsia de lobo temporal, o tipo mais freqüente na população humana.

I.5. Avaliação da memória pela tarefa de esquiva inibitória

Nessa tarefa o animal aprende a não descer de uma plataforma para evitar um leve choque nas patas. Usualmente o aprendizado se dá em uma única sessão (treino), ideal para estudar processos inicializados pelo treino sem a contaminação por sessões prévias ou subseqüentes, como ocorre em outras tarefas. Esta tarefa foi escolhida por várias razões. Primeiramente, porque proporciona uma rápida aquisição (segundos), facilitando a análise do tempo de ocorrência de eventos pós-treino; suas bases farmacológica e molecular já foram bastante estudadas; com um único treino é possível distinguir, farmacologicamente, a memória de trabalho e a memória de curta duração; com um pequeno choque é possível se obter tempos de retenção muito curtos ou suficientemente longos (teto) que permitem a comparação de tratamentos estimulantes ou depressivos pós-treino. Além disso, com pequenas modificações no protocolo da tarefa, também é possível determinar a extinção de memórias (Izquierdo e Medina, 1997; Izquierdo, et al., 1999; Vianna, et al., 2001; Vianna, et al., 2003).

I.6. Outras tarefas comportamentais

I.6.1. Exploração ao campo aberto (Open field)

Este teste consiste em colocar o animal em um novo ambiente onde se pode observar tanto a atividade locomotora, (através da contagem do número de cruzamentos) quanto sua atividade exploratória, avaliada pelas respostas de orientação (rearings). O teste tem duração

de cinco minutos e a diminuição das respostas ao longo da sessão evidencia a habituação ao ambiente.

I.6.2. Labirinto em cruz elevada (Plus-maze)

Baseado no princípio de aversão natural de roedores contra altura e espaços aberto; este método pode ser considerado como o paradigma padrão para testar respostas relacionadas com ansiedade (Gaalen e Streckler, 2000). O comportamento padrão medido pelo labirinto em cruz elevada mostra uma distribuição espacial típica, onde a preferência por braços abertos pode representar o efeito ansiolítico de um tratamento, tornando os animais menos apreensivos em relação ao ambiente novo e à altura, enquanto que a maior exploração e permanência nos braços fechados podem indicar um efeito ansiogênico do tratamento, que leva o animal a preferir um local seguro, fechado e escuro (Graeff, et al., 1998).

No labirinto em cruz elevada, o decréscimo da ocupação dos braços abertos e/ou redução do número de entradas nesses braços em relação ao número total de entradas fornece a medida da inibição do medo-induzido da atividade exploratória, a qual é atenuada por agentes ansiolíticos e aumentada por ansiogênicos (Sonavane, et al., 2002).

O procedimento é simples e não requer equipamentos caros; é baseado em um comportamento espontâneo do animal; por isso, não necessita treinos prolongados, nem utiliza estímulos (por exemplo choque elétrico) ou privação de alimento: É capaz de identificar o efeito ansiolítico de drogas clássica; assim como identificar tanto efeitos ansiolíticos ou ansiogênicos de drogas utilizadas nas mesmas condições experimentais (Pellow, et al., 1985).

I.6.3. Placa perfurada (Hole-board)

O modelo da placa perfurada ou hole-board é um método simples para medir a resposta do animal a um ambiente diferente. A principal vantagem deste teste é que diferentes comportamentos podem ser observados e quantificados, porém essa vantagem pode se tornar um problema se não forem identificados os comportamentos que são afetados pela ansiedade e/ou um estado ansiolítico (Takeda, et al., 1998). Tem sido sugerido que este teste permite investigar o comportamento exploratório, independentemente da atividade locomotora tanto de ratos como de camundongos. A atividade ansiolítica de drogas benzodiazepínicas está relacionada com o aumento do número de “visitas aos poços” (Gaalen e Streckler, 2000).

I.7. Objetivos

Nessa tese utilizamos a tarefa de esquiva inibitória para estudar os aspectos relacionados à memória. E para estudar os demais parâmetros utilizamos outros modelos experimentais: para atividade locomotora utilizamos o teste de campo aberto; para avaliar a ansiedade, usamos o modelo de placa perfurada (camundongos) e labirinto da cruz elevada (ratos). As convulsões foram induzidas por α-dendrotoxina (bloqueador de canais de potássio e estimulador da liberação de glutamato endógeno) ou ácido quinolínico (agonista dos receptores NMDA).

No primeiro artigo, o objetivo foi determinar se a administração crônica de guanosina, oferecida "*ad libitum*" aos animais, teria efeito sobre convulsões e morte induzidas por α-dendrotoxina e investigar o efeito da guanosina sobre a memória e ansiedade.

No segundo artigo, para investigar se o efeito da administração aguda de guanosina na memória é via adenosinérgica, utilizamos adenosina e seu antagonista cafeína, individualmente e co-administradas com a guanosina no modelo de esquiva inibitória. Também avaliamos o perfil de locomoção e ansiedade em ratos e camundongos.

No terceiro artigo estudamos o efeito comportamental da administração oral crônica de guanosina e sua possível correlação com a captação cerebral de glutamato. Também avaliamos se o efeito da administração sistêmica da guanosina se reflete nos níveis centrais deste nucleosídeo.

Anexo I

Chronically administered guanosine is anticonvulsant, amnesic and anxiolytic in mice

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Research report

Chronically administered guanosine is anticonvulsant, amnesic and anxiolytic in mice

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Abstract

Acute administration of intraperitoneal and oral guanosine has been shown to prevent quinolinic acid and α-dendrotoxin-induced seizures in rats and mice. In this study, we investigated the effects of 2 weeks ad libitum consumption of guanosine (0.5 mg/ml) added to mice water supply on seizures and lethality induced by the α-dendrotoxin, hole-board behavior, inhibitory avoidance task, locomotor activity, motor coordination, rectal temperature, body weight, and water and food consumption. Guanosine prevented seizures in 40% and death in 50% on mice treated with i.c.v. α-dendrotoxin; it also impaired inhibitory avoidance memory and increased head-dipping behavior and locomotor activity on the hole-board test. Guanosine consumption did not alter any of the other parameters evaluated. The anticonvulsant, amnesic, and anxyolytic-like effects may be associated with the ability of guanosine in modulating the glutamatergic excitatory system. Adding to previously reported data, these findings suggest a potential role for chronic guanosine in the management of diseases associated with glutamatergic excitotoxicity, including epilepsy and anxiety.
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Theme: Neurotramsmitters, modulators, transporters, and receptors

Topic: Excitatory amino acids: excitotoxicity

Keywords: α-Dendrotoxin; Guanosine; Glutamate; Seizure; Excitotoxicity; Adenosine; Anxiety; Memory

1. Introduction

Adenine-based purines (ABP), namely the nucleoside adenosine and the nucleotide ATP, are considered the main effectors of the purinergic system [25]. Via P1 receptors, adenosine acts as an inhibitory neuromodulator and via P2 receptors, ATP acts as an excitatory neurotransmitter [29]

Glutamate is the main excitatory neurotransmitter in mammalian CNS, being involved in plastic processes, such as development and ageing, learning and memory and adaptation to environmental stimuli [16,27,36]. However, overstimulation of the glutamatergic system (inducing excitotoxicity) is involved in many acute and chronic brain diseases [23,27]; accordingly, adequate modulation of the glutamatergic tonus is essential to normal brain function.

Glutamate and adenosine interact for modulating CNS functions; glutamate stimulates adenosine release, which acts on presynaptic A_1 receptors inhibiting glutamate release [2]. Recently, extracellular guanine-based purines (GBP), namely the nucleoside guanosine and the nucleotides GTP, GDP and GMP, have also been shown to antagonize the glutamatergic activity [3,13,24,38]. Moreover, adenosine and guanosine are released under ischemic conditions [6,7,11], a process that involves glutamatergic excitotoxicity [10]. In addition, guanosine protected brain slices submitted to hypoxia/hypoglycemia [14], and culture medium from astrocytes treated with guanosine prevented NMDA-induced toxicity in neurons [4]. In vitro, GBP inhibit the binding of glutamate and analogs and

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prevent cell responses to excitatory amino acids [1,11,30,40]; GBP also stimulate glutamate uptake by astrocyte cell cultures [13,12], an effect involved in neuroprotection [14]. Regarding seizures, adenosine agonists and glutamate antagonists are anticonvulsants while adenosine antagonists and glutamate agonists promote convulsions [2,25]. Behaviorally, i.p. acute administration of guanosine impaired inhibitory avoidance performance in rats [33], and i.p. and p.o. acute administration were effective anticonvulsants in mice against overstimulation of the glutamatergic system induced by quinolinic acid and the glutamate releaser α-dendrotoxin [21,35], being ineffective against seizures induced by the GABA antagonist picrotoxin [21,35], pentilenotetrazol and transcorneal eletroshock (data not published). These behavior profile, similar to that of NMDA antagonists, indicate some degree of specificity towards the glutamatergic system.

In view of the reported anticonvulsant action of acute guanosine administration, the aim of this study was to investigate the effects of chronic oral consumption of guanosine on lethality and seizures induced by the endogenous glutamate releaser α-dendrotoxin (α-DTX). The potassium channel blocker α-DTX promotes the release of endogenous neurotransmitters, including glutamate, leading to seizures and death [8]. These effects can be attenuated by the anticonvulsants phenytoin, carbamazepine, valproate and phencyclidine. We have previously shown that the NMDA receptor antagonist MK801 prevented death caused by this dose of alpha-DTX [21]. Additionally, we investigated guanosine effects on inhibitory avoidance memory and the hole-board model of anxiety. Furthermore, rectal temperature, motor coordination, locomotor activity, body weight, and water and food consumption were evaluated.

2. Materials and methods

2.1. Materials

Guanosine was obtained from Sigma Chemicals (St. Louis, MO, USA). α-DTX, isolated from *D. angusticeps* venom (J.L., Kenya), was a generous gift from Dr C. Cerveñansky. The anesthetic sodium thiopental was obtained from Cristália (Itapira, SP, Brazil).

2.2. Animals

Male adult Swiss albino mice (25–35 g) were kept on a 12-h light/dark cycle (light on at 07:00 h) at a constant temperature of 22±1 °C. Animals were housed for 2 weeks in plastic cages (five per cage), with water (controls) or 0.5 mg/ml guanosine (GUO) and commercial food available ad libitum. Institutional protocols for experiments with animals, designed to minimize suffering and limit the number of animals killed, were followed throughout the experiments.

2.3. Hole-board

The hole-board apparatus (Ugo Basile, Varese, Italy) consisted of gray Perspex panels (40×40 cm, 2.2 cm thick) with 16 equidistant holes 3 cm in diameter in the floor. Photocells below the surface of the holes measured the number of head-dips. The board was positioned 15 cm above the table and divided into nine squares of 10×10 cm with a water-resistant marker. Each animal was placed singly in the center of the board facing away from the observer and its behavior recorded for 5 min. The number of head-dips, crossings (number of squares crossed with all four paws), rearings, groomings, and defecations was recorded, as well as the latency to start locomotion.

2.4. Inhibitory avoidance

The apparatus was a 50×25×25 cm acrylic box whose floor consisted of parallel caliber stainless steel bars (1 mm diameter) spaced 1 cm apart. A 10-cm-wide, 2-cm-high, acrylic platform was placed in the center of the floor. Animals were placed on the platform and the latency to step down to the grid with all four paws was measured automatically. In the training session, immediately after stepping down to the grid the animals received a 2.0-s, 0.3-mA, scrambled foot shock; retention test sessions were procedurally identical except that no foot shock was given. The latency to step-down in retention test sessions performed 24 h after training was taken as a measure of long-term memory. A ceiling of 180 s was imposed for test sessions. Data are shown as median (interquartile ranges) of training and test step-down latencies.

2.5. Surgical procedure and treatments

After 2 weeks of guanosine treatment, animals were anesthetized with sodium thiopental (60 mg/kg i.p.) and a cannula for intracerebroventricular (i.c.v.) infusion was implanted as previously described [31]. After 2 days for recovery, 0.1 nmol of α-DTX (2.4 μl, dissolved in saline solution 0.9% and adjusted to pH 7.4 with NaOH) was i.c.v. infused [8,20]. Control i.c.v. saline (2.4 μl) did not produce seizures. After experiments with α-DTX, methylene blue (2.4 μl) was injected i.c.v. and that animals lacking dye in the lateral brain ventricle were discarded.

2.6. Evaluation of seizures and lethality

Animals were placed in plexiglass chambers. After administration of α-DTX, mice were observed for 90 min for the occurrence of wild running, clonic, tonic or tonic–clonic seizures lasting more than 5 s; latencies for the first

seizure and death as well as the time of seizures were also noted [21].

2.7. Rotarod performance

Rotarod performance was determined before and after 2 weeks of oral guanosine treatment, as detailed elsewhere [22].

2.8. Rectal temperature

Rectal temperature was measured with a rectal probe before and after 2 weeks of oral guanosine treatment.

2.9. Statistical analysis

Statistical analysis between groups was performed by the Fisher exact test for the occurrence of seizures or death, the Mann–Whitney U-test for inhibitory avoidance performance, rotarod performance and rectal temperature and the Student's unpaired t-test for latencies for first seizure or death and the time of seizures, hole board test, weight measurement, water and food consumption. All results with $P<0.05$ were considered significant.

3. Results

As previously observed with acute guanosine p.o. [21], 2 weeks ad libitum guanosine 0.5 mg/ml was effective against α-DTX, preventing seizures in 40% ($P=0.05$, Fisher Exact Test) and lethality in 50% ($P=0.05$, Fisher Exact Test) of the subjects (Fig. 1). Guanosine had no effect on latencies for seizures—mean±S.D., in seconds: (444±373 and 295±213 for control and guanosine, respectively) or death (1812±1113 and 1469±1435 for control and guanosine, respectively) nor on duration of seizures (828±377 and 747±329 for control and guanosine, respectively).

Ad libitum guanosine 0.5 mg/ml impaired memory of the inhibitory avoidance task in mice ($P<0.05$, Mann–Whitney U-test) (Fig. 2), significantly reducing step-down latency of test session.

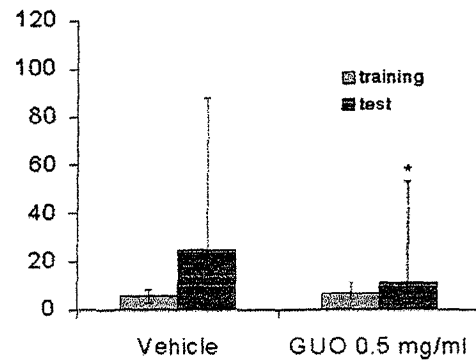

Fig. 2. Effect of GUO on inhibitory avoidance task. Mice were treated p.o. during 2 weeks with ad libitum GUO 0.5 mg/ml before the training session. Data are medians (interquartile ranges) of the step-down latencies on training (light gray columns) and test (dark gray columns. ($n=20$ mice/group). *$P<0.05$ (Mann–Whitney U-test), as compared with vehicle group.

In the hole-board model (Fig. 3), ad libitum guanosine 0.5 mg/ml increased the number of head-dips ($t=1.763$, $P=0.0431$) and crossings ($t=2.812$, $P=0.0078$), indicating an anxiolytic effect compared to Diazepan [37]. Guanosine did not affect the number of rearings ($t=0.8258$, $P=0.4142$), groomings ($t=0.7734$, $P=0.6498$), defecations ($t=0.3241$, $P=0.7477$), nor the latency to start locomotion ($t=1.478$, $P=0.1478$).

Ad libitum guanosine 0.5 mg/ml had no effect on water and food consumption, body weight, rotarod performance, or rectal temperature (data not shown).

4. Discussion

Two weeks ad libitum guanosine 0.5 mg/ml attenuated seizures and lethality induced by α-DTX in mice. Additionally, guanosine presented anxyolytic-like and amnesic effects without affecting motor coordination, rectal temperature, body weight, water and food consumption; no obvious behavioral disturbances were noted in the

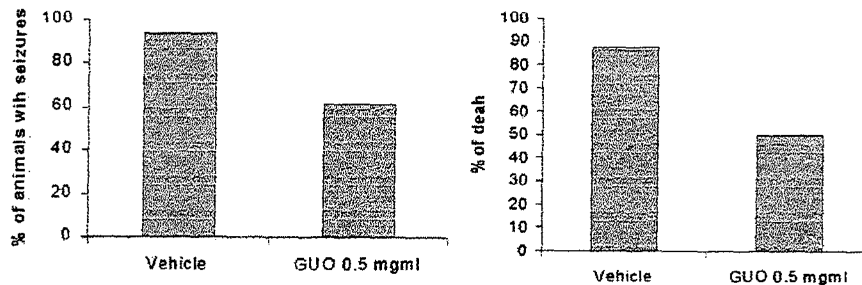

Fig. 1. Protection against convulsion and lethality caused by α-DTX. Mice were treated p.o. during 2 weeks with ad libitum GUO 0.5 mg/ml before i.c.v. injection of α-DTX (0.1 nmol), and observed for 90 min for occurrence of seizures and death ($n-18$ mice/group). *$P<0.05$ (Fisher's exact test), as compared with vehicle group.

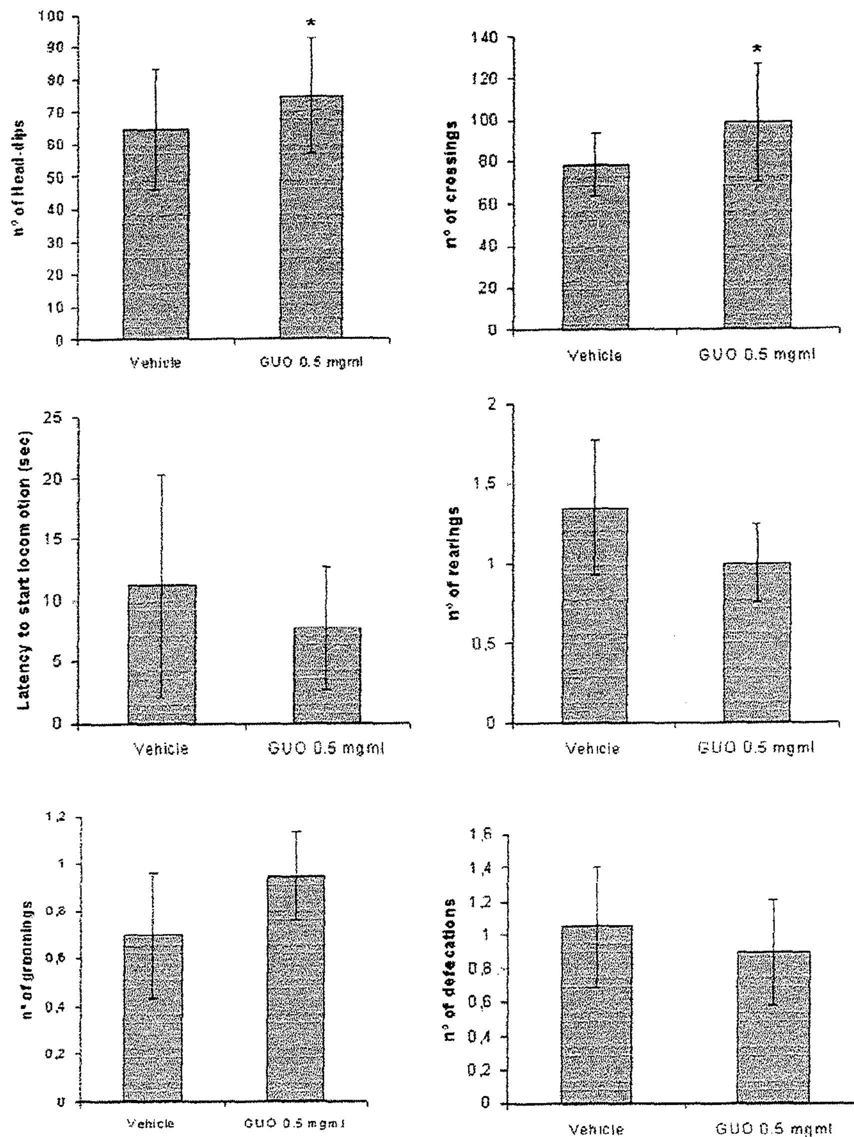

Fig. 3. Effects of GUO on the hole-board test. Mice were treated p.o. during 2 weeks with ad libitum GUO 0.5 mg/ml before testing anxiety-related behavior (number of head-dips) and exploratory behavior (crossing, rearing, grooming, defecation and latency to star locomotion). Data are mean±S.D. (n=20 mice/group). *P<0.05 (Student's unpaired t-test), as compared with vehicle group.

guanosine treated group. Regarding memory impairment, several anxyolytic drugs used in humans, such as benzodiazepines, exhibit a similar profile in animal models [5].

Since nucleoside transport has been evidenced in intestinal cells [28], in cerebral micro vessels and in the blood–brain barrier [19], and guanosine is taken up by nucleoside transporters [17,18], it is not surprising that ad libitum administered guanosine was orally active.

Although evidence indicates that guanosine is anticonvulsant due to its antagonism against overstimulation of the glutamatergic system [24,33–35,38], the mechanisms underlying guanosine effects on other behavioral parameters remains to be clarified. Previous results [33] indicate that, using the same protocol, guanosine and glutamatergic antagonists are amnesic. Since guanosine is a poor displacer of glutamate ligands, a direct antagonism on glutamate receptors is unlikely [39]. Considering that guanosine stimulates the release of adenosine in cultured astrocytes [31], and that both are released under ischemic conditions [7], a relevant contribution of adenosine to the anticonvulsant guanosine effect cannot be ruled out. However, several lines of neurochemical [6,13,41] and behavioral [13,15] evidence suggest that guanosine acts independently from adenosine.

In vitro, guanosine prevented ischemic injury [14] and NMDA-induced excitotoxicity [7]. Recently, we showed

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that acute oral administration of guanosine prevents seizures and lethality induced by quinolinic acid and α-DTX, as well as the potentiation of kainate toxicity by MK-801, parameters related to glutamate release [9,20,26]. Both neuronal and astrocytic cell cultures are able to release guanosine under basal and toxic conditions [6,7], and we recently found that even at 1 μM guanosine significantly stimulates glutamate uptake in cultured astrocytes [13], a physiological process preventing glutamate toxicity. Therefore, we suggest that in vivo effects reported in this study are related to the stimulatory action of guanosine on glutamate uptake [14,38], rather than a direct antagonism of glutamatergic receptors.

It is noteworthy that other GBP, such as GMP and GTP, reported to possess anticonvulsant [1,21,35], trophic [31,41] and neuroprotective [21,31,34,35] effects are hydrolyzed to guanosine by ecto-nucleotidases [41]. In light of our data, one cannot exclude that these GBP properties are not exerted through guanosine.

The results reported here provide in vitro and in vivo evidence for an antagonism of guanosine on the glutamatergic system activity, both in excitotoxic (e.g. seizures) and physiologic (e.g. memory) conditions. We further suggest that such antagonism involves a guanosine-induced stimulation of glutamate uptake by astrocytes.

Guanosine is a naturally endogenous compound that, like adenosine, shows a wide spectrum of biological activities. Interestingly enough, it has been recently shown that in children affected with meningitis, purine levels, including guanosine are increased in CSF [32]. This study adds new data on the tolerability and efficacy of this compound, suggesting that oral supplementation of guanosine may be useful for treating diseases associated with glutamatergic excitotoxicity (e.g. stroke and neurodegenerative disorders). Additional data concerning guanosine efficacy in other animal models of glutamatergic excitotoxicity, its mechanism of action, toxicity and biodisponibility, as well as the role of other GBP and nucleotidases, all currently under extensive investigation in our laboratory, will be useful to substantiate the potential of guanosine in therapeutics.

Acknowledgements

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Anexo II

Orall administration of guanosine impairs inhibitory avoidance performance in rats and mice

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Oral administration of guanosine impairs inhibitory avoidance performance in rats and mice

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Abstract

Extracellular guanine-based purines, mainly the nucleoside guanosine, have recently been shown to exert neuroprotective effects, which seem to be related to antagonism of the glutamatergic system. In this study, we investigated the effects of acute oral administration of guanosine on inhibitory avoidance task in rats and mice. We also studied its effects on locomotor activity, anxiety-related behaviors and mechanisms of action involving the purinergic system. Guanosine (2.0 and 7.5 mg/kg, per os), administered 75 min pretraining, dose-dependently impaired retention of the inhibitory avoidance task in rats and mice, an effect not prevented by the adenosine receptor antagonist caffeine. Guanosine presented no effects on locomotor activity and anxiety-related behaviors. This amnesic effect of guanosine may be compatible with inhibition of glutamatergic system and seems to be not mediated by adenosine.
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Keywords: Guanosine; Glutamate; Adenosine; Caffeine; Memory; Inhibitory avoidance

1. Introduction

Glutamate, the major excitatory neurotransmitter in the mammalian central nervous system (CNS) (Ozawa, Kamiya, & Tsuzuki, 1998), modulates several brain functions, and is also implicated in acute and chronic brain disturbances, both in animals and humans (Lipton & Rosemberg, 1994; Rathbone et al., 1999). Physiologically, glutamate plays a crucial role in learning and memory processes. Specifically, NMDA receptors are essential to the initiation of cellular and molecular mechanisms responsible for memory formation (Izquierdo & Medina, 1997; Morris & Davis, 1994), as evidenced by the amnesic effect of NMDA antagonists on inhibitory avoidance (Izquierdo & Medina, 1997; Miserendino, Sananes, Melia, & Davis, 1990; Morris, Anderson, Lynch, & Baudry, 1986).

Intracellular guanine-based purines (GBPs) have been shown to modulate cell transmembrane signals via G-proteins activity (Gudermann, Schorneberg, & Schultz, 1997; Morris & Malbon, 1999). More recently, extracellular GBPs (mainly the nucleoside guanosine) were shown to exert biological effects without a direct interaction with G-proteins, including trophic role in neural cells (Rathbone et al., 1999) and antagonism of the glutamatergic activity (Baron et al., 1989; Frizzo et al., 2001; Frizzo et al., 2002; Frizzo et al., 2003; Lara et al., 2001; Malcon et al., 1997; Paas, Devillers-Thiery, Changeux, Medevielle, & Teichberg, 1996; Paz, Ramos, Ramirez, & Souza, 1994; Roesler et al., 2000; Rubin et al., 1996; Schmidt, Lara, Maraschin, Perla, & Souza, 2000; Tasca, Wofchuk, Souza, Ramirez, & Rodnight, 1995). Concerning the neuroprotective role of GBPs, in vivo studies have shown that guanosine administered intraperitoneally (i.p.) or orally (p.o.) in mice, prevented seizures induced by quinolinic acid and α-dendrotoxin (compounds that overstimulate the glutamatergic system) (Lara et al., 2001; Schmidt et al., 2000; Vinadé et al., 2003), whereas intrastriatal GMP protected against quinolinic acid-induced cell death in rats (Malcon et al., 1997). These results point to a neuroprotective

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role of GBPs, mainly guanosine, possibly by modulating the glutamatergic neurotransmission.

We have previously shown that i.p. guanosine exerted an amnesic effect on inhibitory avoidance task in rats (Roesler et al., 2000), similar to NMDA antagonists (Izquierdo & Medina, 1997), that was present when guanosine was administered before training, but not after training or before testing. In this study, we investigated the effect of guanosine on inhibitory avoidance task, performing an acute oral administration of the drug, a more promising therapeutic route. We further investigated the role of adenosine and its receptor antagonist caffeine in the mechanism of action of guanosine, since there is evidence that guanosine might act by releasing adenosine (Rathbone et al., 1999). We also studied the effects of p.o. guanosine on locomotor activity (hole board to mice and open-field to rats) and anxiety-related behaviors (hole board to mice and elevated plus-maze to rats).

2. Materials and methods

2.1. Animals

Male adult Wistar rats (250–350 g) were used in the inhibitory avoidance task, exploration of open-field and elevated plus maze and male adult Swiss albino mice (35–45 g) were used in inhibitory avoidance and hole board test. They were kept on a 12 h light/dark cycle (light on at 7:00 am) at a constant temperature of 22 ± 1 °C, in plastic cages (five per cage) with tap water and commercial food ad libitum. All behavioral procedures were conducted between 1:00 and 5:00 pm.

2.2. Drugs

Guanosine, adenosine, and caffeine were obtained from Sigma Chemicals, St. Louis, MO, USA.

2.3. Drugs administration

In the first experiment (Fig. 1), animals (rats and mice) received an oral solution of water (vehicle) or guanosine 0.75, 2.0 or 7.5 mg/kg for 75 min before the first behavioral session. In the further experiment, with mice (Fig. 2), we orally administered vehicle, guanosine (7.5 mg/kg), adenosine (7.5 mg/kg or 100 mg/kg) or intraperitoneally administered caffeine (5 mg/kg). All groups received the drugs 75 min before training sessions. In attempt to clarify the mechanisms involved in guanosine effects, we simultaneously administered caffeine i.p. at a concentration without amnesic effect (5 mg/kg), with the highest concentration of guanosine (p.o. 7.5 mg/kg) or with adenosine (p.o. 100 mg/kg).

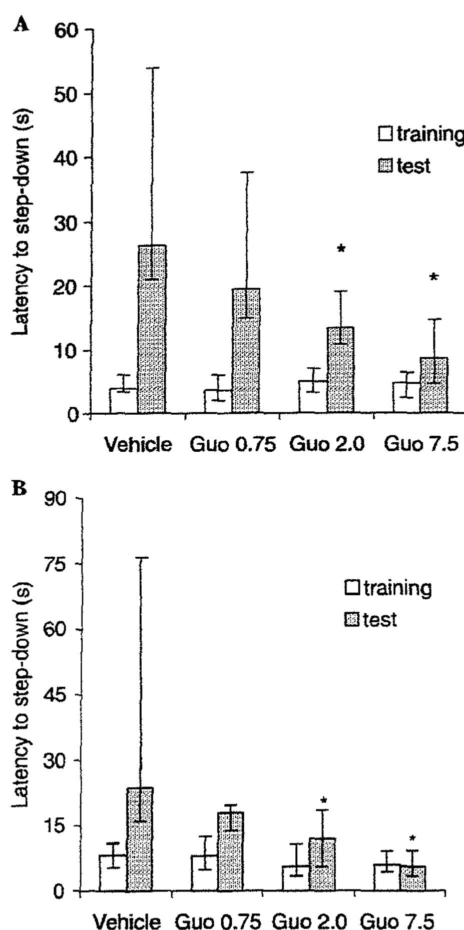

Fig. 1. Effects of pretraining guanosine administration on inhibitory avoidance of rats (A) and mice (B): animals received orally water (vehicle) or guanosine at doses 0.75, 2.0 or 7.5 mg/kg, 75 min before training session. Data are medians (interquartile ranges) of the step-down latencies on training and test sessions. $n = 13–16$ animals per group. Statistical comparison between groups was performed with Kruskal–Wallis test followed by Mann–Whitney test. *Significant difference from vehicle group (Mann–Whitney test $p < 0.001$).

2.4. Inhibitory avoidance

The inhibitory avoidance procedure is described in previous reports (Roesler et al., 2000). The apparatus was a 50 × 25 × 25 cm acrylic box whose floor consisted of a parallel caliber stainless-steel bars (1 mm diameter) spaced 1 cm apart. A 7 cm wide, 2.5 cm high platform was placed against the left wall of the box. Animals were placed on the platform and their latencies to step-down on the floor with the four paws were measured with an automatic device. In training sessions, immediately after stepping-down animals received a 0.5 mA, 2 s footshock. In test sessions, carried out 24 h after training, no footshock was given and the step-down latency (180 s ceiling) was taken as a measure of retention. The same apparatus was adapted to mice, using a 10 cm^2 and

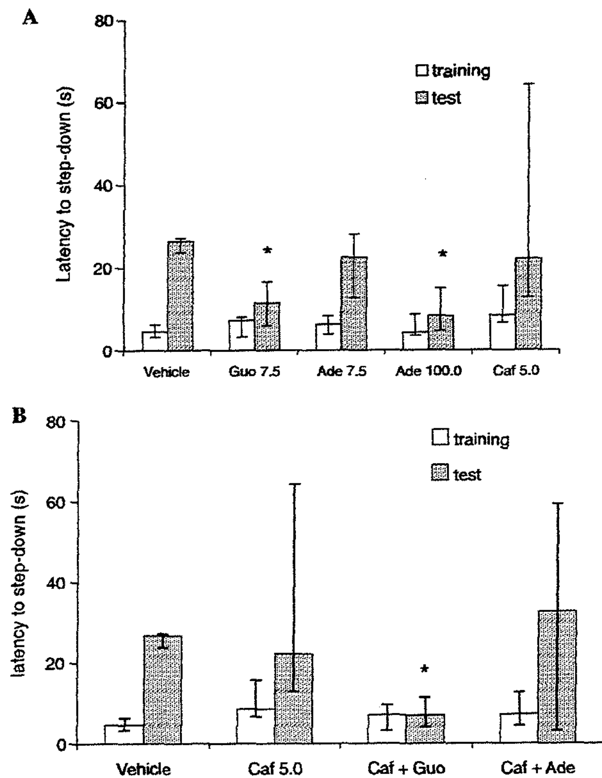

Fig. 2. Effects of pretraining administration of guanosine, adenosine or caffeine on inhibitory avoidance in mice. (A) Animals received orally water (vehicle), guanosine (7.5 mg/kg), adenosine (7.5 or 100 mg/kg), or i.p. caffeine (5 mg/kg), 75 min before training session. (B) Animals received orally vehicle, guanosine (7.5 mg/kg) or adenosine (100 mg/kg), co-administered with i.p. caffeine (5 mg/kg), 75 min before training session. Data are medians (interquartile ranges) of the step-down latencies on training and test sessions; $n = 13–16$ animals per group. Statistical comparison between groups was performed with Kruskal–Wallis test followed by Mann–Whitney test. *Significant difference from vehicle group (Mann–Whitney test $p < 0.001$).

2.0 cm high platform placed in the center of the box and in training sessions, immediately after stepping-down on grid, the animals received a 0.3 mA, 2 s footshock. Data for inhibitory avoidance are shown as median (interquartile ranges) of training and test latencies to step-down on the grid. Comparisons among groups were performed using a Kruskal–Wallis analysis of variance followed by a Mann–Whitney U test if necessary. Differences between training and test latencies to step-down within each group were performed using a Wilcoxon test.

2.5. Exploration of open-field

To evaluate the effects of guanosine on general locomotor activity, rats were submitted to 5 min exploration of an open-field 75 min after receiving an oral solution of vehicle or guanosine 7.5 mg/kg. The open-field was a 50 × 25 × 50 chamber made of brown polywood with frontal glass wall. The floor of the open-field was divided into 12 equal squares by black lines. Animals were placed on the left rear quadrant and left to explore the arena for 5 min. The number of crossings of the black lines, rearings and fecal boli were counted, as well as the latency to start locomotion. Data for the open-field sessions are shown as mean ± SD. Comparison among groups was performed by unpaired t test.

2.6. Hole board

The hole-board apparatus (Ugo Basile, Varese, Italy), used exclusively for mice, consisted of gray Perspex panels (40 × 40 cm, 2.2 cm thick) with 16 equidistant holes 3 cm in diameter in the floor. Photocells below the surface of the holes measured the number of head-dips. The board was positioned 15 cm above the table and divided into 9 squares of 10 × 10 cm with a water-resistant marker. Then, 75 min after an oral infusion of vehicle or guanosine 7.5 mg/kg, each mouse was placed singly in the center of the board facing away from the observer and its behavior recorded for 5 min. The latency to the first head-dip was measured using a stopwatch. Rearing, the number of fecal boli and spontaneous movements (number of squares crossed with all four paws) were also recorded to evaluate locomotor activity of mice. Data for the hole board sessions are shown as means ± SD. Comparison among groups was performed by unpaired t test.

2.7. Elevated plus-maze

The elevated plus-maze test was used exclusively for rats, as previously described (Pellow, Chopin, File, & Briley, 1985; Price, 1999). The apparatus consists of two opposite open arms ($50 \times 10\,cm^2$) and two opposite enclosed arms ($50 \times 10 \times 40\,cm^3$). The maze is 70 cm high and the sessions were conducted under dim red light. The rats received an oral infusion of vehicle or guanosine 7.5 mg/kg, 75 min before sessions, and observed during a 5 min trial for the number of entries, the time spent within open and enclosed arms, and the exploratory behavior (total number of arms entries), which were recorded by two observers. Data for the elevated plus-maze are shown as means ± SD. Comparison among groups was performed by unpaired t test.

3. Results

Figs. 1A and B show that pretraining p.o. administration of guanosine dose-dependently impaired retention of the inhibitory avoidance task in rats and mice, respectively. Guanosine 2.0 mg/kg ($U = 34.5$, $p < 0.001$) and 7.5 mg/kg ($U = 10$, $p < 0.001$) in rats, 2.0 mg/kg

($U = 24.5$, $p < 0.001$) and 7.5 mg/kg ($U = 11$, $p < 0.001$) in mice significantly reduced test latency to step-down, on Mann–Whitney test when compared to vehicle group.

Fig. 2A shows that the guanosine 7.5 mg/kg ($U = 1.0$, $p < 0.001$) and adenosine 100 mg/kg ($U = 6.0, p < 0.001$) were amnesic, while adenosine 7.5 mg/kg ($U = 19.0$, $p > 0.05$) and caffeine 5 mg/kg ($U = 29.0, p > 0.05$), had no effect, when compared with vehicle. The co-administration of i.p. caffeine 5 mg/kg prevented the amnesic effect of adenosine but not of guanosine, as shown in Fig. 2B ($U = 9.0, p < 0.001$).

In the open-field model (Fig. 3), guanosine did not affect the number of crossings ($t = 3.985$, $p = 0.063$), rearings ($t = 5.370$, $p = 0.34$), fecal boli ($t = 0.457$, $p = 0.581$) or the latency to start locomotion in rats ($t = 0.127$, $p = 0.726$).

In the hole board model (Fig. 4), guanosine 7.5 mg/kg did not affect the number of head-dips ($t = 1.223$, $p = 0.2287$), crossings ($t = 1.803$, $p = 0.0794$), rearings ($t = 0.7156$, $p = 0.4786$), fecal boli ($t = 0.3664$, $p = 0.7161$), groomings ($t = 0.7789$, $p = 0.4409$) or the latency to start locomotion ($t = 0.698$, $p = 0.4894$).

Furthermore, in the elevated plus-maze test (Table 1), guanosine did not affect the number of the entries in open arms ($F = 2.723$, $p > 0.05$), the time spent within open arms ($F = 2.219$, $p > 0.05$), the number of the entries in enclosed arms ($F = 0.95$, $p > 0.05$), and the time spent within the enclosed arms ($F = 1.757$, $p > 0.05$).

4. Discussion

Glutamate plays a key role in plastic events on CNS, including learning and memory processes. Accordingly, blockade of NMDA and AMPA glutamatergic receptors is amnesic for the inhibitory avoidance task (Izquierdo & Medina, 1997). Besides these physiological roles of glutamate, overstimulation of the glutamatergic system is involved in various chronic and acute brain disorders such as epilepsy, stroke, and neurodegenerative disorders (Lipton & Rosemberg, 1994; Ngo, Patil, & Unadkat, 2001). Consequently, inhibition of glutamatergic activity is a strategy used to overcome these disorders (Lipton & Rosemberg, 1994).

It has been shown that guanosine protects neural cells from in vitro hypoxia/hypoglycemia (Frizzo et al., 2002). In vivo studies on GBPs protection against glutamatergic neurotoxicity have shown that GMP antagonizes NMDA-induced neural cell loss in rats (Malcon et al., 1997), and guanosine and GMP act as anticonvulsants against quinolinic acid or α-dendrotoxin in mice (Lara et al., 2001; Schmidt et al., 2000). In this study, extending previous reports (Roesler et al., 2000; Rubin et al., 1996; Vinadé et al., 2003), we show that acute orally adminis-

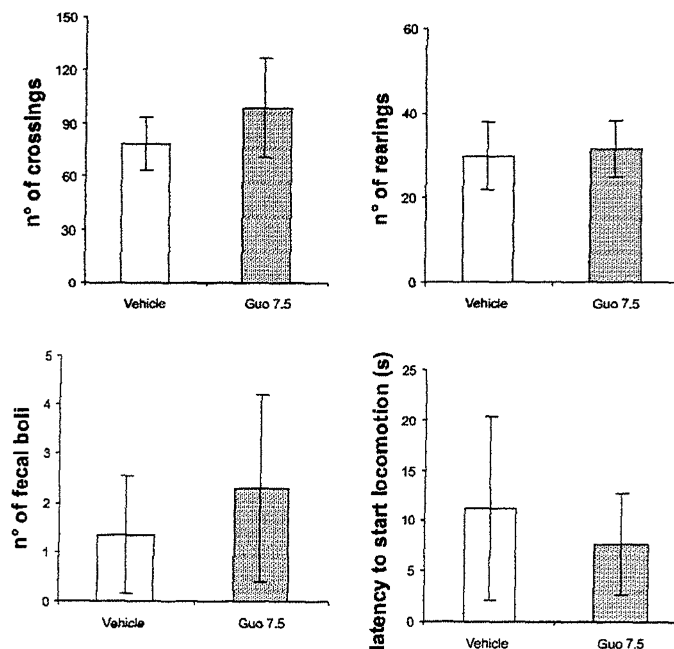

Fig. 3. Effects of pretraining administration of guanosine on locomotor activity in rats. Animals received p.o. vehicle or guanosine 7.5 mg/kg, 75 min before exploratory behavior session (number of crossings, rearings, fecal boli, and latency to start locomotion). Data are means ± SD; $n = 13$–16 animals per group. Statistical comparison between groups was performed with Student's t test.

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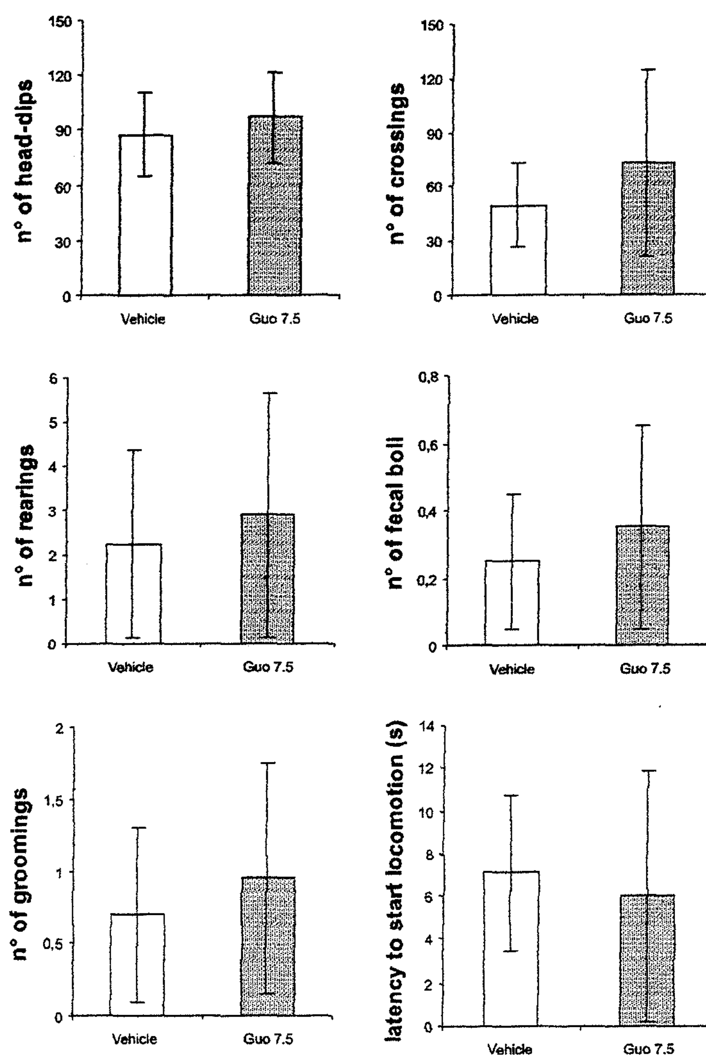

Fig. 4. Effects of pretraining administration of guanosine on anxiety-related behaviors and locomotor activity in mice. Animals received p.o. vehicle or guanosine 7.5 mg/kg, 75 min before anxiety-related behaviors session (number of head-dips, groomings, and latency to first head-dip) and locomotor activity (crossings, rearings, and fecal boli). Data are means ± SD; $n = 13–16$ animals per group. Statistical comparison between groups was performed with Student's t test.

Table 1
Effects of pretraining guanosine administration (7.5 mg/kg) on the elevated plus-maze test

Group	Number of entries		Time spent (s)		Exploratory behavior
	Open arms	Closed arms	Open arms	Closed arms	
Vehicle	1.5 ± 0.6	4.0 ± 0.7	28.3 ± 14.3	264.8 ± 13.8	13.7 ± 1.7
GUO	2.5 ± 0.8	3.5 ± 0.5	48.6 ± 16.4	255.6 ± 16.8	11.2 ± 1.0

Values are expressed as means ± SD.

tered guanosine impaired inhibitory avoidance task, in rats and mice without affecting either locomotor performance or anxiety-related behaviors in both animals. Furthermore, Roesler et al. (2000) have previously demonstrated that footshock sensivity was not altered by guanosine. Taken together, these results reinforce the idea that guanosine exerts a primary amnestic effect. Since transport of nucleosides has been evidenced in intestinal cells (Ngo et al., 2001) and they cross the blood–brain barrier via a carrier-mediated process in cerebral microvessels (Price, 1999), is reasonable to expect that guanosine can be active orally.

Adenosine modulates glutamatergic activity and impairs memory formation for inhibitory avoidance task in mice and rats (Normile, Gaston, Johnson, & Barraco, 1994; Pereira et al., 2002; Tchekalarova, Kambourova, & Georgiev, 2002). Since guanosine stimulates the release of adenosine in cultured astrocytes (Rathbone et al., 1999) and both are released under excitotoxic ischemic conditions (Ciccarelli et al., 2001), a contribution of adenosine to the amnesic effect observed here could not be ruled out. In this study, adenosine at 100 mg/kg, but not at 7.5 mg/kg, presented an amnesic effect in inhibitory avoidance task, an effect prevented by the adenosine receptor antagonist caffeine. However, caffeine failed to prevent the amnesic effect of guanosine, strenghting previous evidence that both nucleosides act through different mechanisms. The previously observed protective effect of guanosine against seizures induced by glutamatergic agents probably is also not mediated by adenosine (Lara et al., 2001). Taken together, these results and the recent identification of a high affinity binding site for guanosine in rat brain membranes (Ciccarelli et al., 2001; Traversa et al., 2002) reinforce the hypothesis that guanosine acts independently from adenine-based purines.

Regarding putative mechanisms involved in this effect, guanosine presented similar effects to glutamatergic antagonists using in the same protocol (Roesler et al., 2000). A direct antagonist action on glutamatergic receptors is unlikely, since guanosine is a poor displacer of glutamate agonists (Souza & Ramirez, 1991). However, we recently found that guanosine even at 1 μM significantly stimulated glutamate uptake in cultured astrocytes (Frizzo et al., 2001, 2003) and in rat brain slices (Frizzo et al., 2002), as well as prevented neurotoxicity induced by in vitro hypoxia–hypoglycemia. Thus, it is tempting to explain the present results by a stimulatory action of guanosine on glutamate uptake, perhaps by acting on its specific membrane transporter (Frizzo et al., 2001).

In conclusion, these results suggest that p.o. guanosine modulates, independently from adenosine, the activity of the glutamatergic system, impairing memory in the inhibitory avoidance task. This deserves to be considered in studies addressing the putative neuroprotective role of GBPs against glutamate toxicity.

Acknowledgments

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Anexo III

Effects of chronic administered guanosine on behavioral parameters and brain glutamate uptake in rats

Submetido à Experimental Neurology

2004

Effects of Chronic Administered Guanosine on Behavioral Parameters and Brain Glutamate Uptake in Rats

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Abstract:

Oral and intraperitoneal administration of the nucleoside guanosine have been shown to prevent quinolinic acid (QA) and α-dendrotoxin-induced seizures, impair memory and anxiety in rats and mice. In this study, we investigated the effect of 2 weeks *ad libitum* orally administered guanosine (0.5 mg/ml) on seizures induced by quinolinic acid, inhibitory avoidance memory and locomotor performance in rats. We also aimed to study the mechanism of action of guanosine through the measurement of its concentration in the cerebrospinal fluid (CSF) and its effect on glutamate uptake in cortical slices of rats. QA produced seizures in 85 % of rats, an effect partially prevented by guanosine (53% of seizures – $p = 0.0208$). Guanosine also impaired retention on the inhibitory avoidance task ($p = 0.0278$) and decreased locomotor activity on the open field test ($p = 0.0101$). The CSF concentration of guanosine increased 2-fold in the treated group comparing to vehicle group (p = 0.0178). Additionally, QA promotes a 30% decrease in glutamate uptake as compared to i.c.v. saline administration, an effect prevented by guanosine in animals protected against QA-induced seizures. Altogether, these findings suggest a potential role of guanosine for treating diseases involving glutamatergic excitotoxicity such as epilepsy. These effects seem to be related to modulation on glutamate uptake.

Keywords: Guanosine; Glutamate uptake; Seizures; Epilepsy; Excitotoxicity; Adenosine; Memory.

1. Introduction

Glutamate is the main excitatory neutransmitter in mammalian Central Nervous System (CNS), being essential for normal brain functions [Ozawa *et al.*, 1998]. However, overstimulation of the glutamatergic system is involved in many acute and chronic brain diseases (excitotoxicity) [Castellano *et al.*, 2001; Lipton *et al.*, 1994; Price, 1999]. Furthermore, it has been suggested that glutamate plays an essential role in epileptic activity [Meldrum, 1994]. Quinolinic acid (QA), an over stimulator of the glutamatergic system [e.g. as endogenous agonist of N-methyl-D-aspartate (NMDA) receptors], induces seizures and has been proposed to be involved in the etiology of epilepsy [Heyes *et al.*, 1990; Nakano *et al.*, 1993]. Accordingly, the main process responsible by maintaining the extracellular glutamate levels below toxic levels if the Na^+-dependent glutamate uptake, carried out mainly by transporters located on the astrocytic cell membranes (Chen and Swanson, 2003; Danbolt, 2001).

Adenine-based purines (ABPs), namely the nucleotide ATP and the nucleoside adenosine, are considered the main effectors of the purinergic system [*Ralevic et al.*,1998]. Glutamate and adenosine closely interact in modulating CNS functions. Glutamate stimulates adenosine release, which acts on presynaptic A_1 receptors inhibiting glutamate release [Brundege *et al.*, 1997].

Intracellular guanine-based purines (GBPs) (the nucleotides GTP, GDP, GMP and the nucleoside guanosine) exert regulatory roles on cellular functions, as evidenced by the modulation of G-proteins activities [Gudermann *et al.*, 1997]. Similarly to ABPs, GBPs have also been shown to exert extracellular effects, not directly related to the modulation of G-proteins activity, such as trophic effects on neural cells [Ciccarelli *et al.*, 2001] and

antagonism of the glutamatergic system [Baron *et al.*, 1989; Burgos *et al.*, 1998; Malcon *et al.*, 1997; Regner *et al.*, 1998; Roesler *et al.*, 2000; Rubin *et al.*, 1996]. *In vitro*, GBPs inhibit the binding of glutamate and analogs, prevent cell responses to excitatory amino acids and increase the glutamate uptake by astrocytes, a process involved in neuroprotection [Frizzo *et al.*, 2001, 2002, 2003]. Moreover, adenosine and guanosine are released under ischemic conditions [Ciccarelli *et al.*, 1999, 2001; Dobolyi *et al.*, 2000], a process that involves glutamatergic excitotoxicity [Dirnagl *et al.*, 1999]. Guanosine protected brain slices exposed to hypoxia/hypoglycemia [Frizzo *et al.*, 2002], and medium from astrocytes treated with guanosine prevented NMDA-induced toxicity in neurons [Ciccarelli et al, 2001]. *In vivo*, GBPs, administered intracerebroventricularly (i.c.v.), intraperitonially (i.p.), or orally (p.o.) protected against seizures induced by glutamatergic agents in mice [Baron *et al.*, 1989; Lara *et al.*, 2001; Schmidt *et al.*, 2000]. Additionaly, chronic orally administered guanosine (*ad libitum*) seems to be anticonvulsant, amnesic and anxiolytic in mice [Vinadé *et al.*, 2003]. These behavioral effects seem to be related to an antagonism of the glutamatergic system.

Extending previous reported effects of chronic guanosine administration in mice, the aim of this study was to evaluate the effects of chronic (two weeks *ad libitum*) orally administered guanosine on locomotor activity, inhibitory avoidance memory and seizures induced by QA (an over stimulator of the glutamatergic system) in rats, additionally investigating the involvement of glutamate uptake by brain cortical slices. Furthermore, body weight, water and food consumption during the treatment were also evaluated.

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2. Materials and methods

2.1. Chemicals:

Guanosine, quinolinic acid (QA) and all other reagents were purchased from Sigma Chemicals (St Louis, MO, USA). The anesthetic sodium thiopental was obtained from Cristália (Itapira, SP, Brazil). QA used for i.c.v. infusion was dissolved in saline 0.9% and adjusted to pH 7.4 with NaOH or HCl when necessary. Guanosine (0.5 mg/ml) was prepared in water and the concentration was limited by its poor water solubility.

2.2. Animals:

Male adult Wistar rats (250-350 g) were kept on a 12 hour 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 water or GUO (0.5 mg/ml) and commercial food *ad libitum* for 2 weeks. Our institutional protocols for experiments with animals, designed to minimize suffering and to limit the number of animals sacrificed, were followed throughout. All behavioral procedures were conducted between 1:00 p.m. and 5:00 p.m.

2.3. Exploration of open-field:

In order to evaluate the effects of guanosine on general locomotor activity, animals were submitted to 5 min exploration of an open-field. The open-field apparatus was a 50 x 25 x 50 chamber made of brown polywood with frontal glass wall. The floor of the open-field was divided into 12 equal squares by black lines. Animals were placed on the its left rear quadrant for exploring the arena for 5 min. The number of crossings of the black lines, rearings and fecal boli were counted, as well as the latency to start locomotion. Data are shown as mean ± Standard Deviation.

2.4. Inhibitory avoidance:

The apparatus was a 50 x 25 x 25 cm acrylic box whose floor consisted of a parallel caliber stainless-steel bars (1 mm diameter) spaced 1 cm apart. A 7 cm wide, 2.5 cm high platform was placed against the left wall of the box. Animals were placed on the platform and their latencies to step-down on the floor with the four paws were measured with an automatic device. In training sessions, immediately after stepping – down, animals received a 0.5 mA, 2 s footshock. In test sessions, carried out 24 h after training, no footshock was given and the step-down latency (180 s ceiling) was taken as a measure of retention. Data of inhibitory avoidance are shown as median (interquartile ranges) of training and test latencies to step-down on the grid.

2.5. *CSF sampling:*

Animals were anesthetized with sodium thiopental (40 mg/kg, 1 ml/kg, i.p.). In a stereotaxic apparatus, the CSF was drawn (40 - 60 μl per rat) by direct puncture of the cisterna magna with an insulin syringe (27 gauge×1/2" length), as described elsewhere [Portela *et al.*, 2002]. Individual samples that presented visible blood contamination were discarded. Samples were centrifuged at 4500 g at 4 °C for 5 min in order to obtain cell-free supernatants and stored in single tubes at -70 °C for quantification of purines.

2.6. *High Performance Liquid Cromatography (HPLC) analysis:*

HPLC analysis of guanosine was performed with aliquots obtained of the cell-free supernatants from CSF. The measurement of guanosine has been described elsewhere [Frizzo *et al.*, 2003]. Briefly, separation was carried out with a reverse phase column (Supelcosil LC-18, 25 cm x 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 on the basis of the absorption at 254 nm. The retention time of standards was used as an identification and quantification parameter.

2.7. Surgical procedure:

Immediately after CSF sampling, still in the stereotaxic apparatus, the skin of the skull was removed and a 27 gauge 9 mm guide cannula was placed at 0.9 mm posterior to bregma, 1.5 mm right from de midline and 1.0 mm above the 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 jeweler acrylic cement.

2.8. Evaluation of seizures and toxicity:

Experiments were performed 72 hours after surgery. A 30 gauge cannula was fitted into the guide cannula and connected by a polyethylene tube to a microsyringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula aiming the right lateral brain ventricle. Then, 239.2 nmol of QA (4 μl) were infused i.c.v. Control i.c.v. saline (4 μl) did not produce seizures.

Animals were placed in plexiglass chambers. After administration of QA, rats were observed for 10 minutes for the occurrence of tonic-clonic seizures lasting more than 5 seconds, latency to start seizure, time of seizure and death [Rotta *et al.*, 2003].

After experiments with QA, methylene blue (4 μl) was injected i.c.v. and animals without dye in the lateral brain ventricle were discarded.

2.9. Glutamate Uptake:

The animals were divided in five groups for glutamate uptake experiments: vehicle-vehicle, vehicle-QA, guanosine-vehicle, guanosine-QA-protected and guanosine-QA-not protected (animals treated with guanosine that exhibited seizures after QA administration). After 10 minutes of QA or vehicle infusion and subsequent behavioral observation, the animals were decapitated, the brain immediately removed and submerged in Hank's balanced salt solution (HBSS), pH 7.2. Cortices were dissected in petri dish filled with HBSS (room temperature) to obtain the parietal area. Coronal slices (0.4 mm) were achieved using a McIlwain tissue chopper and the sections were finally separated with the help of a magnifying glass. Then, cortical slices were transferred to 24-multiwell dishes, each one washed with 1 mL HBSS and immediately replaced by 280 µL HBSS (35°C). Uptake assay was assessed by addition of 20 µL HBSS containing 0.33 µCi mL^{-1} L-[2,3-^{3}H] glutamate with 100 µM unlabeled glutamate (final concentration), at 35°C. Incubation was stopped after 7 minutes by two ice-cold washes with 1 mL HBSS immediately followed by addition of 0.5 N 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 sodium dependent glutamate uptake, parallel assays were done under ice and using N-methyl-D-glucamine instead of sodium chloride in the incubation medium (0.023 ± 0.006 nmol mg^{-1} min^{-1}), being subtracted from the uptake at 35 °C with sodium chloride.

2.10. Protein determination.

Determination of protein was assessed using the method of Lowry *et al.* [1951] using bovine albumin as standard. The experiments were done in triplicate and performed in the linear range of the time curve.

2.11. Statistical analysis.

Statistical analysis between groups was performed by the Fisher exact test for the occurrence of seizures, the Mann-Whitney *U* Test for inhibitory avoidance performance, and the Student's unpaired *t*-test for open field test, weight measurement, water and food consumption, and CSF concentration of nucleosides. ANOVA plus Duncan test were used to evaluate glutamate uptake by cortical slices. All results with $p < 0.05$ were considered significant.

3. Results

QA caused seizures in 85% of rats, an effect partially prevented by oral administration of guanosine 0.5 mg/ml *ad libitum* (53% of seizures, $p = 0.0208$) (Figure 1). Guanosine had no effect on latency for starting or time of seizure, nor on death (data not shown).

As previously described with mice, *ad libitum* guanosine impaired the memory of inhibitory avoidance task in rats, measured 24 h after training session ($p = 0.0278$) (Figure 2), reducing significantly the test latency to step-down, compared to vehicle group.

In the open field session (Figure 3), guanosine decreased the number of crossings (t = 2.759, $p = 0.0101$) and rearings (t = 3.199, p = 0.0034), an opposed effect of previously

observed with mice. Guanosine did not affect the number of defecation ($t = 1.178$, $p = 0.2488$) nor the latency to start locomotion ($t = 0.5652$, $p = 0.5765$).

Furthermore, guanosine had no effect on body weight as well as on water and food consumption (data not shown).

The CSF concentration of guanosine increased 2-fold in treated group comparing to vehicle group [from 0.46 ± 0.40 to 1.05 ± 0.60 (mean $\pm$ SD), $p = 0.0178$] (Figure 4).

Figure 5 shows the effects of chronic oral administration of guanosine and/or i.c.v. QA on glutamate uptake by brain cortical slices from rats. QA promotes a 30 % decrease in gutamate uptake as compared to i.c.v. saline administration, an effected prevented by guanosine, only in animals protected against QA-induced seizures ($p < 0.05$). However, this effect did not occur when animals treated with guanosine presented seizures.

4. Discussion

Two weeks *ad libitum* guanosine 0.5 mg/ml attenuated QA-induced seizures in rats. Further, guanosine presented amnesic effects without affecting body weight, water and food consumption, as previously described in mice [Roesler *et al.*, 2000; Vinadé *et al.*, 2003]. Regarding memory impairment, several drugs used in humans, such as benzodiazepines, and in rats, as glutamatergic antagonists [Izquierdo *et al.*, 1997], exhibit a similar profile in animal models [Cahill *et al.*, 1986]. In this study, rats presented a decrease in locomotor activity, as evidenced by fewer number of crossings and rearings in the open field model. This effect was contradictory to the previously observed increased locomotion and anxiolytic profile caused by guanosine in mice [Lara *et al.*, 2001; Vinadé *et al.*, 2003], but resembles that of some glutamatergic antagonists [Danysz *et al.*, 1994]. Although

guanosine presents some contradictory locomotor effects, this action seems to be weak, without affecting motor coordination [Lara *et al.*, 2001; Vinadé *et al.*, 2003], the ability to consume food and water or the body weight gain, as evidenced with most antagonists of NMDA receptors. In fact, locomotor effects of chronic administered guanosine remains to be clarified and should be further investigated.

Animals treated with oral guanosine presented 2-fold increase in CSF concentration of guanosine as compared to vehicle group, an effect previosly described by our lab with acute i.p. administration in rats (data not published). Guanosine is taken up by nucleoside transporters [Jones *et al.*, 1995; Jurkowitz *et al.*, 1998], evidenced in intestinal cells [Patil *et al.*, 1997], in cerebral microvessels and in the blood-brain-barrier [Kalaria *et al.*, 1988]. Altogether, these results give good reason why guanosine is orally active. Therefore, being an endogenous compound and orally active, guanosine could be supposed safe and well tolerated for human use with therapeutic proposes.

In vitro, guanosine was shown to prevent ischemic injury [Frizzo et al, 2002] and NMDA-induced excitotoxicity [Ciccarelli *et al.*, 2001]. Acute and chronic administration of guanosine prevents seizures and toxicity induced by quinolinic acid or α-dendrotoxin, drugs that over stimulate the glutamatergic system, reproducing *in vivo* excitotoxicity [Baron *et al.*, 1989; Lara *et al.*, 2001; Schmidt *et al.*, 2000; Vinadé *et al.*, 2003]. Although *in vivo* and *in vitro* guanosine effects seem to be related to avoiding overstimulation of the glutamatergic system, the mechanism of action of guanosine is still unclear. Since guanosine is a poor displacer of glutamate ligands, a direct antagonism on glutamatergic receptors is unlikely [Souza and Ramirez, 1991]. Both neurons and astrocytes are able to

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release guanosine under basal and toxic conditions [Ciccarelli *et al.*, 1999, 2001] and guanosine significantly stimulated glutamate uptake in cultured rat astrocytes [Frizzo *et al.*, 200, 2003] and rat brain slices [Frizzo *et al.*, 2002], a physiological process which contributes to prevent glutamate toxicity. In this study, *in vivo* oral administration of guanosine prevented the decrease of glutamate uptake induced by QA in brain slices of rats only when it presented anticonvulsant action. Thus, this *in vivo* neuroprotective effect could be due to stimulatory action of guanosine on glutamate uptake [Frizzo *et al.*, 2001, 2002], and not as direct antagonist of glutamatergic receptors.

The nucleosides guanosine and adenosine closely interact on modulation of the glutamatergic system. Glutamate stimulates the release of adenosine, which acts on presynaptic A_1 receptor, inhibiting glutamate release (negative feedback) [Brundege *et al.*, 1997]. Similarly, kainate stimulates the release of guanosine that presents a modulatory effect on glutamate uptake [Dobolyi *et al.*, 2000]. Therefore, a contribution of adenosine to the behavioral and neurochemical effects of guanosine cannot ruled out, since guanosine stimulates the release of adenosine in cultured astrocytes and both are released under ischemic (excitotoxic) conditions [Ciccarelli *et al.*, 1999, 2001]. However, the adenosine antagonist caffeine did not inhibit the anticonvulsant effect of guanosine, suggesting that adenosine A_1 and A_{2a} receptors are not involved in this effect [Lara *et al.*, 2001]. Also, we have previously shown that the amnesic effect of guanosine is independent from adenosine (submitted data). The recent identification of a high affinity binding site for guanosine in rat brain membranes [Traversa *et al.*, 2002] reinforce that guanosine is acting independently from adenosine.

To our knowledge, this study provides new evidence on the mechanism of action of guanosine, showing that behavioral effects of oral guanosine seem to be related to a stimulatory effect on brain glutamate uptake. Also, the increased levels of guanosine on CSF demonstrated that this compound crossed the blood brain barrier. Since guanosine is an endogenous compound and seems to be well tolerated with no obvious CNS toxic effects, this drug could be useful in the future studies on treatment or prevention of brain diseases associated with overstimulation of the glutamatergic system.

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Figure Legends:

Figure 1. Protection against QA-induced seizures. Rats were treated during two weeks with orally *ad libitum* guanosine (GUO) 0.5 mg/ml before i.c.v. injection of QA (239,2 nmol) and observed for 10 min for the presence of tonic-clonic seizures. (n = 30 rats/group). * = $p < 0.05$ (Fisher's exact test), as compared with vehicle group.

Figure 2. Effect of guanosine (GUO) on inhibitory avoidance task. Rats were treated during two weeks with orally *ad libitum* GUO 0.5 mg/ml before training session. Data are medians (interquartile ranges) of the step-down latencies on training (white columns) and test (gray columns); (n = 30 rats/group). * = $p < 0.05$ (Mann-Whitney U test), as compared with vehicle group.

Figure 3. Effects of guanosine (GUO) on the open-field session. Rats were treated during two weeks with orally *ad libitum* GUO 0.5 mg/ml before testing exploratory behavior

(crossing, rearing, defecation or latency to start locomotion). Data are mean ± SD; (n = 30 rats/group). * = $p < 0.05$ (Student's unpaired *t*-test), as compared with vehicle group.

Figure 4. Cerebrospinal fluid concentration of guanosine (GUO). Rats were treated during two weeks with orally *ad libitum* GUO 0.5 mg/ml before CSF sampling. Measurement of GUO was performed by HPLC. Data are mean ± SD; (n = 20 rats/group). * = $p < 0.05$ (Student's unpaired *t*-test), as compared with vehicle group.

Figure 5. Effects of guanosine (GUO) and/or i.c.v. QA on glutamate uptake by cortical slices from rats. Rats were treated during two weeks with orally *ad libitum* GUO 0.5 mg/ml before evaluation of seizures induced by QA. After the behavior evaluation, rats were sacrified and their cortical slices processed for glutamate uptake assay *in vitro*. GUO-QA-p: animals treated with guanosine protected against QA-induced seizures; GUO-QA-np: animals treated with guanosine displaying seizures after QA administration. Data are mean ± SD; (n = 30 rats/group). * = $p < 0.05$ (ANOVA plus Duncan test), as compared with vehicle group.

Figure 1:

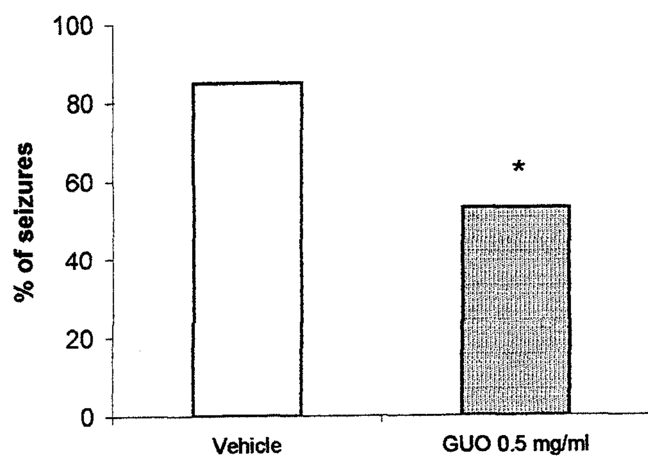
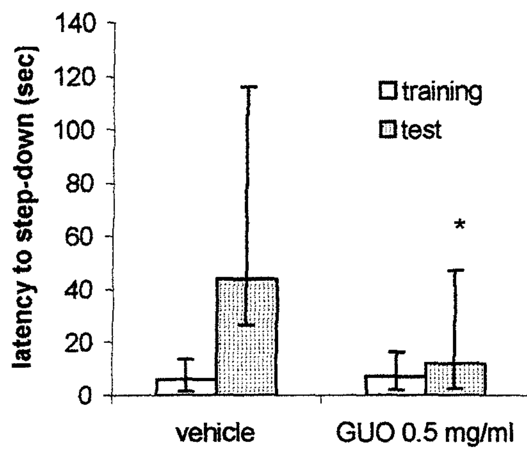


Figure 2:



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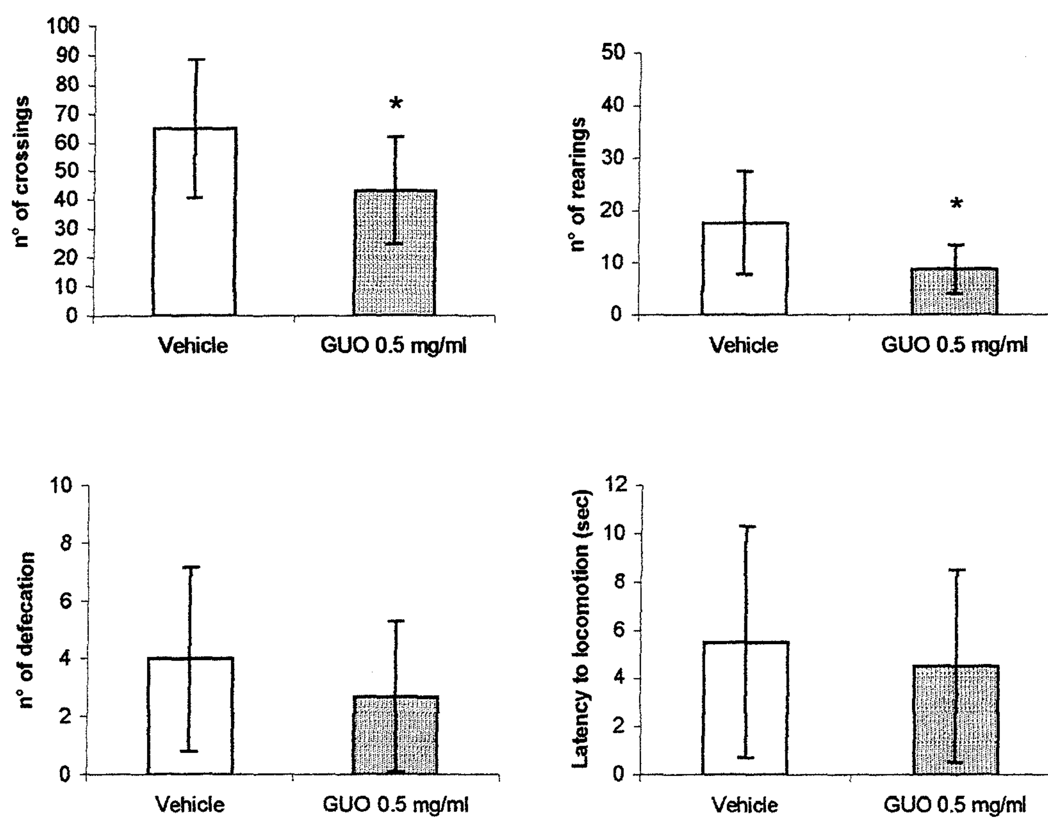

Figure 4:

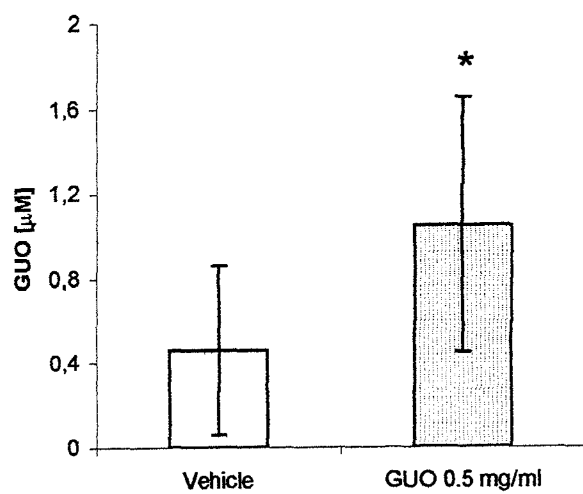

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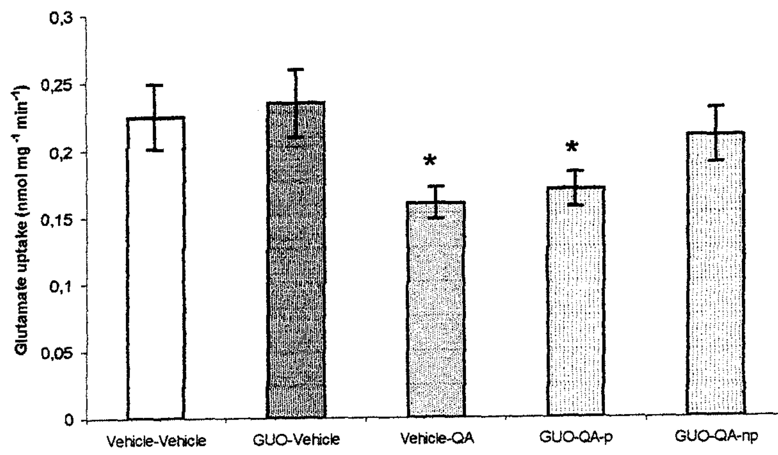
Figure 5:



III. Considerações Finais

Vários estudos têm demonstrado que os aminoácidos excitatórios (EAAs) estão envolvidos em processos associados à plasticidade neuronal. Os agonistas de receptores NMDA exercem ação facilitatória nos processos de aprendizado e memória, enquanto que os antagonistas de NMDA prejudicam os processos de aprendizado e memória em diferentes condições experimentais, tanto em tarefas aversivas como "apetitosas" (Castelano, et al., 2001).

Nesta tese estudamos o efeito da guanosina em parâmetros comportamentais como memória, ansiedade e como neuroprotetor de injúrias no SNC provocadas por convulsão. Também propomos experimentalmente que os efeitos da guanosina no cérebro são devido ao antagonismo que este nucleosídeo exerce sobre o neurotransmissor glutamato, que, por sua vez, atua em receptores NMDA, entre outros. Para isso, utilizamos protocolos de tratamento crônico e agudo em camundongos e/ou rato, em que a guanosina foi administrada por via oral.

3.1. Tratamento Crônico - Camundongos e ratos

Duas semanas de tratamento com guanosina 0,5 mg/ml (ingerida à vontade) foram suficientes para atenuar a convulsão e a morte de camundongos induzidas por α-DTX. Adicionalmente a guanosina apresentou efeitos ansiolítico e amnésico, sem afetar a coordenação motora, temperatura retal, peso corporal ou consumo de água e alimento.

Também em ratos, duas semanas de tratamento com guanosina 0,5 mg/ml (ingerida à vontade) atenuaram convulsões induzidas por ácido quinolínico. Embora tenha havido efeito amnésico, não houve alteração de peso corporal ou de consumo de água ou alimento nos animais tratados.

Com relação ao efeito sobre a memória, observado tanto em camundongos como em ratos, já foi relatado que algumas drogas utilizadas em humanos, tais como benzodiazepínicos e os antagonistas glutamatérgicos, exibem o mesmo perfil quando utilizadas em modelos animais (Cahill,et al., 1986). Entretanto, nesse estudo os ratos apresentaram um decréscimo na atividade locomotora, evidenciado pelo baixo número de cruzamentos e rearings no campo aberto. Esse efeito foi contraditório em relação ao aumento da locomoção e perfil ansiolítico causado pela guanosina em camundongos (Lara, et al. 2001; Vinadé, et al. 2003), mas

semelhante ao observado com antagonistas glutamatérgicos (Danysz, et al., 1994). Embora a guanosina apresente efeito contraditório em relação à locomoção, esse efeito é fraco e não altera a coordenação motora (Lara, et al., 2001; Vinadé, et al. 2003). Tanto os camundongos como os ratos não apresentaram alterações em relação ao consumo de alimento ou água, nem no peso corporal (Vinadé, et al. 2003), como ocorre com a maioria dos antagonistas de receptores NMDA.

O transporte de nucleosídeos tem sido evidenciado em células intestinais (Patil and Unadkat, 1997) e em micro-vasos cerebrais e barreira hemato-encefálica (Kalaria and Harik, 1988). Como a guanosina utiliza-se desses transportadores de nucleosídeos (Jones and Hammond, 1995; Jurkowitz, et al., 1998), não chega a ser surpreendente que ocorra a absorção intestinal e distribuição tecidual, inclusive no cérebro, quando se administra guanosina via oral. De acordo com isso, os ratos tratados com guanosina aumentaram a concentração deste nucleosídeo no líquor quando comparados ao grupo controle.

Embora as evidências indiquem que a guanosina é um anticonvulsivante, devido ao seu antagonismo frente à hiper estimulação do sistema glutamatérgico (Souza e Ramirez, 1991; Rubin, et al., 1996; Malcon, et al., 1997; Roesler, et al., 2000; Schmidt, et al., 2000), seu mecanismo de ação ainda não foi totalmente elucidado. Como a guanosina é um pobre deslocador de ligantes de glutamato, parece apresentar um antagonismo direto de glutamato (Tasca e Souza, 2000). Roesler, et al. (2000), usando o protocolo de administração por via intraperitoneal, já havia demonstrado que a guanosina tem efeito amnésico.

Considerando que, *in vitro*, a guanosina estimula a liberação de adenosina em cultura de astrócitos (Rathbone, et al, 1999), e que ambos são liberados em condições isquêmicas (Ciccarelli, et al., 2001), não é possível descartar uma relevante contribuição da adenosina no efeito anticonulsivante da guanosina, porém várias linhas de evidências neuroquímicas (Ciccarelli, et al., 1999; Frizzo, et al., 2001; Zimmermann, 1996) e comportamentais sugerem que a guanosina atua independente da adenosina. Também em estudos *in vitro*, a guanosina previne danos isquêmicos (Frizzo, et al., 2002), e a excitotoxicidade induzida por NMDA (Ciccarelli, et al., 2001).

In vivo, tanto a administração aguda como crônica de guanosina preveniu convulsões e a toxicidade induzida por ácido quinolínico e α-DTX, drogas utilizadas para provocar hiper estimulação do sistema glutamatérgico, reproduzindo excitotoxicidade *in vivo* (Baron, et al., 1989; Lara, et al., 2001; Schmidt, et al., 2000; Vinadé, et al. 2003).

Tanto cultura de neurônios como de astrócitos são capazes de liberar guanosina em situação basal ou tóxica (Ciccarelli, et al., 1999; 2001) e a guanosina estimula significantemente a captação de glutamato em cultura de astrócitos (Frizzo, et al., 2001) e em fatias de cérebro de ratos (Frizzo, et al., 2002), um processo fisiológico que previne a toxicidade glutamatérgica. Assim, nós sugerimos que os efeitos *in vivo* encontrados neste estudo estão relacionados a uma ação estimulatória da guanosina sobre a captação de glutamato (Frizzo, et al., 2002; Souza e Ramirez, 1991) e não a um antagonismo direto de receptores glutamatérgicos.

Ambos os nucleosídeos, guanosina e adenosina atuam na modulação do sistema glutamatérgico. O glutamato estimula a liberação de adenosina, a qual atua através dos receptores A_1 pré-sinápticos inibindo a liberação de glutamato (feedback negativo) (Brundege e Dunwiddie, 1997). Similarmente o kainato estimula a liberação de guanosina, que apresenta um efeito modulatório na captação de glutamato (Dobolyi, et al., 2000). Portanto, a adenosina pode estar contribuindo para os efeitos comportamentais e neuroquímicos da guanosina, uma vez que a guanosina estimula a liberação de adenosina em cultura de astrócitos e ambos são liberados em condições excitotóxicas (Ciccarelli, et al., 1999; 2001). Por outro lado, a cafeína, que é um antagonista de adenosina, não inibe o efeito anticonvulsivante da administração aguda de guanosina em convulsões induzidas por ácido quinolínico, sugerindo que os receptores de adenosina A1 e A2a não estão envolvidos nesse efeito anticonvulsivante (Lara, et al., 2001). A recente identificação de um sítio de ligação de alta afinidade para a guanosina em membranas de cérebro de ratos (Traversa, et al., 2002) reforça a possibilidade de que a guanosina atue independentemente da adenosina.

Em outros trabalhos, foi demonstrado que outros derivados da guanina, como GMP e GTP, também possuem ação anticonvulsivante (Baron, et al., 1989; Lara, et al., 2001; Schmidt, et al., 2000), além de exercer efeitos tróficos (Rathbone, et al, 1999; Zimmermann, 1996) e neuroprotetor (Lara, et al., 2001; Schmidt, et al., 2000; Rathbone, et al., 1999; Rubin, et al., 1996). Entretanto, esses derivados são hidrolisados á guanosina por ação das ectonucleotidases (Zimmermann, 1996).

Os resultados apresentados nesta tese e os já publicados pelo grupo do Prof. Dr. Diogo Souza, *in vivo* e *in vitro*, evidenciam um antagonismo da atividade do sistema glutamatérgico pela guanosina, tanto em condições excitotóxicas (por exemplo, convulsões) como

fisiológicas (por exemplo, memória). Também é possível sugerir que tal antagonismo envolve uma estimulação, induzida pela guanosina, da captação de glutamato por astrócitos.

A guanosina é um composto naturalmente endógeno, como a adenosina, e que mostra um amplo espectro de atividades biológicas. Adicionalmente, outros dados a respeito da eficácia da guanosina em outros modelos animais de excitotoxidade glutamatérgica, seu mecanismo de ação, toxicidade e biodisponibilidade, assim como o papel das GBP e nucleotidases são atualmente investigados em nosso laboratório.

3.2. Tratamento agudo – camundongos e ratos

O glutamato tem um papel chave no fenômeno de plasticidade do SNC, incluindo aprendizado e memória. Por conseguinte, a inibição dos receptores glutamatérgicos NMDA e AMPA é amnésico para a tarefa de esquiva inibitória (Izquierdo e Medina, 1997). Apesar da importância fisiológica do glutamato, a hiper estimulação do sistema glutamatérgico tem sido envolvida em várias doenças cerebrais, crônicas ou agudas, como epilepsia, acidente vascular cerebral e doenças neurodegenerativas como Alzheimer, por exemplo, (Lipton e Rosemberg, 1994; Ngo, et al., 2001); conseqüentemente, a inibição da excessiva atividade glutamatérgica é uma estratégia usada para combater essas doenças (Lipton e Rosemberg, 1994).

Neste sentido, tem sido demonstrado que a guanosina protege células neurais de hypoxia/hipoglicemia *in vitro* (Frizzo et al, 2002). *In* vivo, estudos de neuroproteção utilizando os derivados da guanina contra a neurotoxicidade glutamatérgica mostram que GMP antagoniza a perda de células neurais em ratos, induzidas com ácido quinolínico (Malcon, et al., 1997) e que guanosina e GMP atuam como anticonvulsivante em camundongos, em modelos que utilizam ácido quinolínico ou α-dendrotoxina (Lara, et al., 2001; Schmidt, et al. 2000). Neste estudo, estendendo resultados prévios (Rubin, et al., 1996; Roeseler, et al., 2000; Vinadé et al, 2003), nós mostramos que a guanosina administrada via oral, agudamente, provoca amnésia na tarefa de esquiva inibitória, tanto em ratos como em camundongos, sem alterar a atividade locomotora ou a ansiedade dos animais.

A adenosina modula a atividade glutamatérgica e prejudica a formação de memória na tarefa de esquiva inibitória para ratos e camundongos (Normile et al, 1994; Pereira et al, 2002; Tchekalarova et al, 2002). Visto que a guanosina estimula a liberação de adenosina em cultura de astrócitos (Rathbone, et al., 1999) e ambas são liberadas sob condições isquêmicas excitotóxicas (Cicarelli et al, 2001), não é possível excluir uma contribuição da adenosina ao

efeito amnésico observado. Neste estudo, somente adenosina na dose de 100 mg/kg, apresentou efeito amnésico na tarefa de esquiva inibitória, este efeito foi prevenido pela cafeína, um antagonista de receptor de adenosina. No entanto, a cafeína não foi capaz de suprir o efeito amnésico da guanosina, e isso fortalece as evidências prévias de que ambos os nucleosídeos atuam por mecanismos diferentes.

Em resumo, esses resultados sugerem que a guanosina por via oral modula, independentemente da adenosina, a atividade do sistema glutamatérgico, prejudicando a memória da tarefa de esquiva inibitória.

Conjuntamente, esses resultados obtidos com camundongos e ratos representam um bom indicativo para o uso oral da guanosina, pois sendo um composto endógeno, ela pode ser bem tolerada para uso humano com propósitos terapêuticos.

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