Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

ESTUDO SOBRE OS MECANISMOS ENVOLVIDOS NA ATIVIDADE ANTINOCICEPTIVA DAS PURINAS: O PAPEL DOS DERIVADOS DA GUANINA.

TESE DE DOUTORADO

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PARTE I

Onde é descrita uma introdução e os objetivos são traçados.

PREFÁCIO

Dentro do abrangente campo de pesquisa em neurociências, e mais especificamente dentro da área de dor, a presente tese de doutorado versa sobre o papel dos derivados da guanina na transmissão da dor.

A tese é dividida em três partes. A primeira delas é destinada a uma breve revisão a cerca dos conhecimentos necessários para o entendimento da segunda parte, onde são apresentados os resultados obtidos através de artigos científicos. Esta segunda parte da tese pode ainda ser dividida em três conjuntos de resultados: o primeiro refere-se a uma revisão crítica sobre os dados referentes ao papel dos derivados da guanina no sistema nervoso central; o segundo conjunto abordando os resultados obtidos através de procedimentos experimentais com animais realizados dentro do Departamento de Bioquímica da UFRGS; e um terceiro conjunto de resultados que foi obtido através de estudos experimentais com humanos, avaliados em três hospitais universitários brasileiros. Por fim, na terceira parte da tese os resultados obtidos são um pouco mais explorados e discutidos, desta vez de forma um pouco mais informal do que a exigida pelos padrões atuais adotados na preparação de manuscritos científicos. Espero que esta terceira parte também ajude na integração dos resultados e na geração de perspectivas para futuras pesquisas.

De uma maneira geral, acredito que ainda temos muito a avançar na descoberta de novos fármacos no tratamento da dor. Creio que esta tese demonstra, ainda que de maneira incipiente, uma tentativa de propor novos fármacos ou mesmo novos alvos para desenvolvimentos de alternativas terapêuticas mais eficazes no tratamento da dor.

LISTA DE ABREVIATURAS

AMPA – ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropionato

ADA – adenosine deaminase

ADP – adenosina-5'-difosfato

AMP – adenosina-5'-monofosfato

AMPc – adenosina-3',5'-monofosfato-cíclico

ATP – adenosina-5'-trifosfato

CGRP – peptídeo relacionado ao gene da calcitonina

GABA – ácido gama-aminobutírico

GDNF – fator neurotrófico derivado da glia

GMP – guanosina-5'-monofosfato

GMPc – guanosina 3',5'-monofosfato-cíclico

GDP – guanosina-5'-difosfato

GTP – guanosina-5'-trifosfato

i.c.v. – intracerebroventricular

i.p. – intraperitoneal

i.t. – intratecal

KA - Cainato

LCR – líquido cefalorraquidiano

MAPK – proteína cinase ativada por mitógeno

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

NMDA – N-metil-D-aspartato

NGF – fator de crescimento neural

NO – óxido nítrico

PAF – fator de ativação plaquetário

PKA – proteína cinase A

PKC – proteína cinase C

SNA – sistema nervoso autônomo

SNC – sistema nervoso central

SNP – sistema nervoso periférico

TNF-α – fator de necrose tumoral alfa

RESUMO

(SCHMIDT AP – ESTUDO SOBRE OS MECANISMOS ENVOLVIDOS NA AÇÃO ANTINOCICEPTIVA DAS PURINAS: O PAPEL DOS DERIVADOS DA GUANINA) – Os objetivos da presente tese de doutorado foram os de investigar o papel dos derivados da guanina na nocicepção e compreender os mecanismos responsáveis pelos efeitos extracelulares desses compostos. Os resultados desta tese estão divididos em três partes, de acordo com sua natureza, em revisão da literatura (parte um), experimental (parte dois) e clínica (parte três). Para o desenvolvimento da primeira parte, uma extensa revisão da literatura em relação ao papel extracelular das purinas foi realizada e um novo sistema guanosinérgico foi proposto. Na segunda parte desta tese, procedimentos experimentais em animais demonstraram que a guanosina é antinociceptiva em diversos modelos de dor. A administração sistêmica de guanosina causou um aumento significativo nos níveis de guanosina no líquido cefalorraquiano (LCR), demonstrando que este composto penetra ativamente no sistema nervoso central. Diversos testes comportamentais e neuroquímicos demonstraram o baixo potencial de toxicidade apresentado pela guanosina. A seguir, os mecanismos de ação envolvidos nos efeitos antinociceptivos da guanosina foram investigados através de métodos neuroquímicos e farmacológicos. Guanosina foi escolhida porque tem demonstrado interagir com o sistema glutamatérgico – sabidamente envolvido na transmissão da dor – por estimular a recaptação de glutamato. Guanosina atenuou o comportamento doloroso induzido pela injeção intratecal de glutamato e seus agonistas (AMPA, cainato, trans-ACPD) e hiperalgesia induzida por altas doses de MK-801, um efeito provavelmente relacionado à diminuição das concentrações de glutamato e aspartato no LCR. Guanosina também foi testada em um método para avaliar a captação de glutamato em fatias de cérebro e medula. Nossos resultados demonstraram que a guanosina não alterou a captação basal de glutamato. Entretanto, a guanosina atenuou os efeitos da dor sobre a captação de glutamato. Baseado nestes dados, não é possível estabelecer uma relação causal entre os dados da captação e o comportamento doloroso. Entretanto, podemos argumentar que os efeitos da guanosina sobre a captação de glutamato foram provavelmente produzidos pela modulação do estímulo doloroso e não correspondem a um mecanismo de ação responsável por estas alterações. Também demonstramos que o alopurinol, um derivado das purinas e inibidor da xantina oxidase normalmente utilizado para o tratamento de hiperuricemia, também produziu efeitos antinociceptivos em camundongos. Este efeito está provavelmente relacionado ao acúmulo de adenosina e guanosina no meio extracelular. Apesar da utilização clínica das purinas ser demasiadamente precoce, podemos inferir que a utilização de alopurinol poderia ser uma excelente estratégia para testar nossas hipóteses, pois demonstra a vantagem de estar aprovado para uso comercial, apresentar boa tolerabilidade e baixo custo. A terceira parte desta tese demonstra trabalhos sobre o sistema purinérgico em humanos. Estes estudos investigaram a correlação entre a concentração de purinas no LCR e dor aguda ou crônica em humanos. Os resultados demonstram que adenosina, guanosina e inosina correlacionaram-se significativamente com a dor em humanos. Os resultados apresentados nesta tese ratificam o papel das purinas nos mecanismos de transmissão da dor e um novo papel para a guanosina é proposto: a modulação da dor. Guanosina e outras purinas são alvos para desenvolvimento de novos fármacos e podem ser úteis no tratamento de dor relacionada a hiperestimulação do sistema glutamatérgico.

ABSTRACT

(SCHMIDT AP – MECHANISMS INVOLVED IN THE ANTINOCICEPTIVE ACTION OF PURINES: THE ROLE OF GUANINE-BASED PURINES) – The main aims of this work were the investigation of the role of guanine-based purines on nociception and the understanding of the mechanism of action leading to extracellular effects of these compounds. The results obtained are presented in three distinct parts based on their nature, namely, literature review (part one), experimental (part two) and clinical (part three). For the development of the first part, an extensive review of the literature regarding the extracellular roles for the purinergic system was performed and a guanine-based purinergic system was proposed. In the second part, experimental procedures in animals demonstrated that guanosine is antinociceptive in several acute or chronic pain models in rodents. Systemic administration of guanosine significantly increased cerebrospinal fluid (CSF) guanosine levels, showing that this compound actively penetrates in the central nervous system. Several behavioral tasks and neurochemical approaches demonstrated that guanosine presents minimal toxic effects. After that, the mechanisms of action underlying the antinociceptive effects of guanosine were investigated, by using pharmacological and neurochemical means. Guanosine was chosen because it has been shown to interact with the glutamatergic system – which is known to be involved in the mechanisms of pain transmission – by promoting astrocytic glutamate reuptake. Guanosine attenuated nociceptive behavior induced by intrathecal administration of glutamate and its receptor agonists (AMPA, kainate, trans-ACPD) and hyperalgesia induced by high-dose MK-801, an effect that seems to be related with the reduction of glutamate and aspartate accumulation in the CSF. Guanosine was tested in a model of glutamate uptake by brain and spinal cord slices from rats and mice. Our results demonstrated that guanosine did not alter basal glutamate uptake at both brain and spinal cord. However, guanosine attenuated effects on glutamate uptake induced by pain behavior in animals. It is not possible to establish whether the changes in the spinal cord glutamate uptake were responsible for nociceptive behavior or it caused the changes. However, we may argue that the changes in the glutamate uptake induced guanosine were probably produced by modulation of nociceptive stimuli rather than an underlying mechanism of action. In the second part of this work, we demonstrated that allopurinol, a purine derivative and xanthine oxidase inhibitor commonly used to treat hyperuricemia, also produced consistent antinociception in mice. This effect was probably related to the accumulation of extracellular adenosine and guanosine. It was therefore concluded that, although is early to propose the use of purines in a clinical setting, allopurinol could be an excellent alternative to test this hypothesis since shows the advantage of be already approved for commercial use, presenting well tolerability and very low cost. The third part of this work presents some results derived from the human models of pain. These studies investigated the correlation between the CSF concentration of purines and acute or chronic pain in humans. Our results show that adenosine, guanosine and inosine were significantly correlated with pain in humans. Altogether these results further demonstrate the role of purines in pain mechanisms and provide a new role for guanosine: pain modulation. Guanosine and other purines presents as a new target for future drug development and might be useful for treat pain related to overstimulation of the glutamatergic system.

APRESENTAÇÃO

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

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

Detalhes técnicos mais precisos sobre a metodologia empregada em cada trabalho podem ser encontrados nos artigos científicos correspondentes.

I.1. INTRODUÇÃO

I.1.a. Dor

A exposição da pele ou outros órgãos ao dano ou estímulos nocivos induz uma sensação desagradável chamada dor. A dor continua sendo uma das grandes preocupações da humanidade. O homem sempre procurou esclarecer as razões que justificassem a ocorrência da dor e propor procedimentos destinados ao seu controle. A dor é componente fundamental da homeostase e seu propósito inicial é alertar sobre estímulos que podem provocar lesão tecidual (estímulos nocivos ou potencialmente nocivos), permitindo que mecanismos de defesa ou fuga sejam adotados [Millan, 1999; Julius e Basbaum, 2001].

Ao contrário desses propósitos claramente protetores, a dor pode se tornar persistente ou crônica em determinadas situações, onde o organismo não é capaz de produzir resolução da lesão ou quando são estabelecidos mecanismos adaptativos inadequados [D'Mello e Dickenson, 2008]. Estas alterações neuroplásticas podem ocorrer em diversas situações como, por exemplo, após lesão ou disfunção nervosa (dor neuropática) [Zhuo, 2007]. Nestes quadros, o processamento sensorial está alterado e usualmente os pacientes apresentam dor de característica persistente, associada a hiperalgesia (percepção exagerada de estímulos dolorosos) e alodinia (estímulos inócuos gerando resposta dolorosa) [Millan, 1999]. Este tipo de dor apresenta-se como um grande desafio na atualidade pois ocasiona intenso sofrimento, queda da qualidade de vida, diminuição da capacidade funcional e é de difícil tratamento [Zimmermann, 2001]. Os custos dos quadros dolorosos agudos e principalmente crônicos são elevados. Este custo é direto (gastos em saúde) e indireto (faltas ao trabalho, licenças médicas, etc). Vários quadros dolorosos crônicos são refratários aos tratamentos disponíveis na atualidade. Neste

contexto, o entendimento da fisiopatologia da dor é fundamental na busca de novas alternativas terapêuticas.

De acordo com a Associação Internacional para o Estudo da Dor (IASP), dor é definida como uma experiência sensorial e emocional desagradável associada com lesão tecidual real ou potencial, ou descrita em termos de tais danos [Loeser e Melzack, 1999]. Esta definição ratifica o caráter multifatorial da dor, envolvendo componentes sensoriais, cognitivos e emocionais [Julius e Basbaum, 2001]. Conseqüentemente, a transmissão da dor apresenta extensa modulação ao longo do sistema nervoso, principalmente no cérebro [Stucky et al., 2001]. Considerando estes aspectos, podemos definir o componente sensorial da dor como nocicepção, isto é, a sensação determinada pela estimulação de receptores presentes nas fibras aferentes primárias [Millan, 1999]. Enquanto a dor envolve a percepção e interpretação de estímulos nocivos, a nocicepção corresponde às manifestações neurofisiológicas e neuroquímicas geradas pelo estímulo nocivo. Portanto, podemos inferir que nos modelos animais a dor é avaliada indiretamente através de respostas comportamentais, constituindo-se efetivamente em modelos de nocicepção [Millan, 1999].

Apesar da IASP ter definido a dor como uma sensação subjetiva relacionada com uma lesão tecidual, há evidências que essa associação possa não ocorrer. Diversas síndromes dolorosas como por exemplo a migrânea e a dor pélvica crônica parecem existir sem lesão tecidual detectável pelos métodos diagnósticos disponíveis na prática clínica atual, favorecendo a hipótese de que podem ocorrer alterações neurofuncionais restritas ao âmbito biomolecular [Millan, 1999]. Neste contexto, o avanço no conhecimento da neuroanatomia das vias de condução, da neurofarmacologia e da fisiopatologia da dor possibilita o desenvolvimento de pesquisas visando a novas modalidades de tratamento, principalmente para os quadros mais refratários.

A dor também é normalmente classificada em tipos: eventual, aguda e crônica. Dor eventual ou transitória é a dor transmitida pelos nociceptores periféricos e não está relacionada a um estímulo nocivo ou lesão significativos (exemplo: uma vacina, venóclise, etc). Dor aguda está relacionada a algum estímulo nocivo com substancial lesão tecidual (queimadura, incisão cirúrgica, etc). Neste tipo de dor, nociceptores, conecções nervosas no sistema nervoso central (SNC) e sistema nervoso autônomo (SNA) já estão envolvidos. Dor crônica trata-se da dor que persiste mesmo após o processo de cicatrização do organismo ou, muitas vezes, nem está relacionado a processo nocivo detectado (fibromialgia, neuralgia pós-herpética, etc) [Millan, 1999].

Entretanto, não é apenas a duração que caracteriza o tipo de dor, mas também a capacidade do organismo de reparar o sítio de lesão e o processamento neural responsável pela transmissão dolorosa [Hill, 2001]. A dor apresenta diversos componentes, desde a sensibilização da periferia pelo estímulo nocivo (nocicepção) até alterações neuroplásticas adaptativas, tais como sensibilização central, alterações na capacidade modulatória de neurônios inibitórios na medula espinhal, reorganização do circuito neuronal e vias nervosas, entre outras alterações [Millan, 1999; Hill, 2001; Zimmermann, 2001]. Estas peculiaridades da transmissão dolorosa geram comportamentos dolorosos diversos que podem ser, por exemplo, de retirada no caso de um estímulo agudo ou de posição antálgica em quadros dolorosos crônicos. Alguns quadros dolorosos podem desencadear sofrimento manifestado das mais diversas formas [Apkarian et al., 2008].

Quanto a sua origem, a dor pode ser classificada em quatro tipos principais: i. Dor nociceptiva: originada da estimulação excessiva dos nociceptores localizados na pele e alguns outros órgãos; ii. Dor neurogênica: desencadeada por dano do tecido neuronal na periferia ou no SNC; iii. Dor neuropática: ocasionada por disfunção ou lesão nervosa; iv.

Dor psicogênica: não causada por fonte somática detectável e pode refletir forte influência de fatores psicológicos [Millan, 1999].

A ocorrência de dor, especialmente crônica, é crescente, talvez devido aos novos hábitos de vida, da maior longevidade, do prolongamento da sobrevida de doentes portadores de afecções letais, das modificações ambientais, e, principalmente, do maior diagnóstico e reconhecimento dos quadros dolorosos [Tunks et al., 2008]. No Brasil e em outros países, 10% a 50% dos indivíduos procuram clínicas médicas devido ao sintoma dor [Rocha et al., 2007; Nampiaparampil, 2008; Tunks et al., 2008]. A dor está presente em mais de 70% dos pacientes que buscam os consultórios brasileiros por motivos diversos, sendo a razão de consultas médicas em um terço dos casos [Rocha et al., 2007]. Esses argumentos enfatizam a importância da busca de elementos que permitam uma melhor abordagem da dor aguda e crônica. Considerando que a dor é um evento altamente complexo, provavelmente nunca teremos um tratamento único e ideal para todos os pacientes e afecções clínicas. Portanto, a busca de novas terapêuticas adjuvantes deve ser incessante.

I.1.b. Mecanismos de transmissão dolorosa

A primeira grande publicação sobre os mecanismos de transmissão dolorosa data de 1965, com a publicação da famosa "teoria dos portões" publicada por Melzack e Wall que procura explicar como ocorre a diferenciação entre estímulos sensoriais nocivos (dor) e não-nocivos (tato) [Melzack e Wall, 1965]. Desde a publicação desta teoria, grande atenção dos pesquisadores em área básica experimental foi concedida a novas descobertas nos mecanismos de transmissão da dor, que hoje se encontram muito mais complexos do que a

teoria inicial. A fundação de órgãos específicos para estudar e divulgar o tema (IASP) e a criação de periódicos específicos para divulgar o conhecimento na área contribuíram de forma significativa para o estado atual do conhecimento. O tema dor tem sido cada vez mais discutido na formação médica. Portanto, o conhecimento dos novos avanços obtidos sobre o conhecimento dos mecanismos de transmissão dolorosa é fundamental no desenvolvimento de novas terapêuticas.

O primeiro passo na seqüência dos eventos que originam o fenômeno doloroso é a transformação dos estímulos agressivos em potenciais de ação que, das fibras nervosas periféricas, são transferidos para o sistema nervoso central [Zhang e Bao, 2006]. Os receptores específicos para a dor estão localizados nas terminações de fibras nervosas Aδ e C [Fitzgerald, 2005] e, quando ativados, sofrem alterações na sua membrana, permitindo a deflagração de potenciais de ação. As terminações nervosas das fibras nociceptivas Aδ e C (nociceptores) são capazes de traduzir um estímulo nocivo (de natureza térmica, química ou mecânica) em estímulo elétrico que será transmitido até o sistema nervoso central através da medula espinhal e interpretado no córtex cerebral como dor [Wood, 2004]. As fibras Aδ são mielinizadas e as fibras C não são mielinizadas e possuem a capacidade de transmitir estímulos dolorosos em diferentes velocidades. As fibras Aδ (2 a 6 mm de diâmetro), em função da presença da bainha de mielina, transmitem o estímulo doloroso de forma rápida (12 a 30 $m.s^{-1}$), enquanto as fibras C (0,4 a 1,2 mm de diâmetro) são responsáveis pela transmissão lenta da dor (0,5 a 2 $m.s^{-1}$). Ambas são classificadas em subtipos $A\delta_1$, $A\delta_2$, C_1 e C_2 [Schaible, 2006]. Também é relevante citar as fibras Aβ, caracterizadas por serem mais espessas (mais de 10 mm de diâmetro), mielinizadas e de condução rápida (30 a 100 $m.s^{-1}$). Estas fibras normalmente não participam da transmissão da dor. Entretanto, caso ocorram

alterações neuroplásticas, estas fibras podem auxiliar no processo de amplificação da dor em nível medular [Millan, 1999; D'Mello e Dickenson, 2008].

A sensibilização dos nociceptores se deve a diversos estímulos, tais como mudança de temperatura, lesão tecidual direta, hipóxia e isquemia, entre outros. Existem alguns nociceptores que permanecem silenciosos durante estímulos de menor intensidade, mas são ativados em situações específicas como na inflamação, amplificando a resposta dolorosa [Julius e Basbaum, 2001]. A maioria das fibras C são polimodais, isto é, são ativadas por estímulos mecânicos, térmicos ou químicos. Algumas fibras do tipo C são insensíveis a estímulos mecânicos, mas respondem a estímulos térmicos. Essas fibras são classificadas de acordo com a presença de peptídeos e a localização dos terminais sinápticos no corno dorsal da medula espinhal em fibras C peptidérgicas e não-peptidérgicas. As fibras C não-peptidérgicas expressam receptores purinérgicos do tipo P_2X_3 e receptores para o fator neurotrófico derivado da glia (GDNF), apresentando terminais sinápticos localizados mais internamente na substância gelatinosa da medula espinhal (lâmina II). As fibras peptidérgicas contêm peptídeos como a substância P e o peptídeo relacionado ao gene da calcitonina (CGRP) e expressa receptores para o fator de crescimento neural (NGF), apresentando terminais sinápticos em regiões mais externas no corno dorsal da medula espinhal (lâmina I) [Millan, 1999]. As fibras Aδ respondem principalmente a estímulos nocivos de origem mecânica e térmica, e apresentam-se subdivididas de acordo com o limiar de temperatura para a transmissão do estímulo através das vias ascendentes [Millan, 1999].

I.1.c. Mecanismos periféricos envolvidos na transmissão da dor

Os nociceptores podem sensibilizados pela ação de substâncias químicas, denominadas algogênicas, presentes no ambiente tissular: acetilcolina, bradicinina, histamina, serotonina, leucotrieno, substância P, fator de ativação plaquetário (PAF), íons potássio e hidrogênio, prostaglandinas, tromboxanos, interleucinas, fator de necrose tumoral (TNF-α), fator de crescimento neural (NGF) e monofosfato cíclico de adenosina (AMPc) [Wood, 2004]. Quando o estímulo provoca a lesão tecidual, há o desencadeamento de processo inflamatório seguido de reparação. As células lesadas também liberam diversas enzimas de seu interior, que no ambiente extracelular degradam ácidos graxos e formam cininas, tais como a bradicinina. Esta provoca intensa dilatação arteriolar e aumento da permeabilidade capilar, contribuindo para a propagação da reação inflamatória [Millan, 1999; Huang et al., 2006]. Associado a este processo, fosfolipase A desencadeia a liberação de ácido araquidônico na membrana celular. Este é metabolizado através de sistemas enzimáticos, produzindo: i. prostaglandinas, tromboxanos, e prostaciclinas (cicloxigenase); ii. leucotrienos e lipoxinas (lipoxigenase); iii. os produtos da via da epoxigenase (citocromo P-450). Essas substâncias promovem a diminuição do limiar de excitabilidade dos nociceptores [Huang et al., 2006].

A substância P e a neurocinina A produzem vasodilatação e aumento da permeabilidade vascular, contribuindo também para a geração e manutenção do processo inflamatório. A bradicinina, a prostaglandina E_2, o NGF e as interleucinas também exercem papel fundamental nos mecanismos de sensibilização periférica à dor. A prostaglandina e a bradicinina causam alterações em receptores específicos ($TRPV_1$) acoplados a canais iônicos ligante-dependente via ativação do AMPc, e das proteínas cinases A (PKA) e C

(PKC), reduzindo o tempo pós-hiperpolarização da membrana neural, causando redução do limiar para disparo da fibra nervosa. O NGF e outras neurotrofinas aumentam a síntese, o transporte axonal anterógrado e quantidade de substância P e CGRP nas fibras C e reduzem a atividade do ácido gama-aminobutírico (GABA) nas terminações nervosas periféricas e centrais [Hill, 2001; Julius e Basbaum, 2001]. As neurotrofinas provocam mudanças nos receptores vanilóides (VR_1) de fibras Aδ acoplados a canais iônicos ligante-dependente e acionam as proteínas cinases ativadas por mitógenos (MAPK) que podem fosforilar o AMPc e iniciar a transcrição genética responsável por alterações fenotípicas, as quais contribuem para a amplificação da eficácia sináptica [Hill, 2001; Julius e Basbaum, 2001]. A persistência da agressão causa modificações no sistema nervoso periférico e sensibilização de fibras nervosas, com conseqüente ativação de nociceptores silentes, hiperalgesia e aumento dos níveis de AMPc e cálcio nos nociceptores. Esse fenômeno ocorre por ação dos mediadores inflamatórios e conseqüente atividade espontânea dos neurônios, aumento da resposta a estímulos supraliminares e diminuição do limiar de ativação dos nociceptores [Huang et al., 2006]. Após a liberação dos mais diversos mediadores químicos da lesão, os nociceptores desenvolvem descargas espontâneas, tornando-se capazes de responder de maneira intensa a estímulos nociceptivos e não-nociceptivos [Huang et al., 2006].

Portanto, a agressão tecidual resulta na liberação de diversos mediadores químicos que culminam em alterações na permeabilidade vascular, no fluxo sangüíneo local e produção dos sinais clássicos inflamatórios de rubor, calor, dor, tumor e impotência funcional. Tem início o processo de sensibilização periférica com conseqüente exacerbação da resposta ao estímulo doloroso (veja Figura 1). Os mediadores periféricos levam a

despolarização da membrana neural por tempo prolongado, com conseqüente aumento da condutividade dos canais de sódio e cálcio e redução do influxo de potássio e cloro para o meio intracelular. Em situações onde este evento seja prolongado, alterações neuroplásticas em nível central podem ocorrer, desencadeando os mecanismos de sensibilização central (Figuras 2 e 3) [Apkarian, 2008].

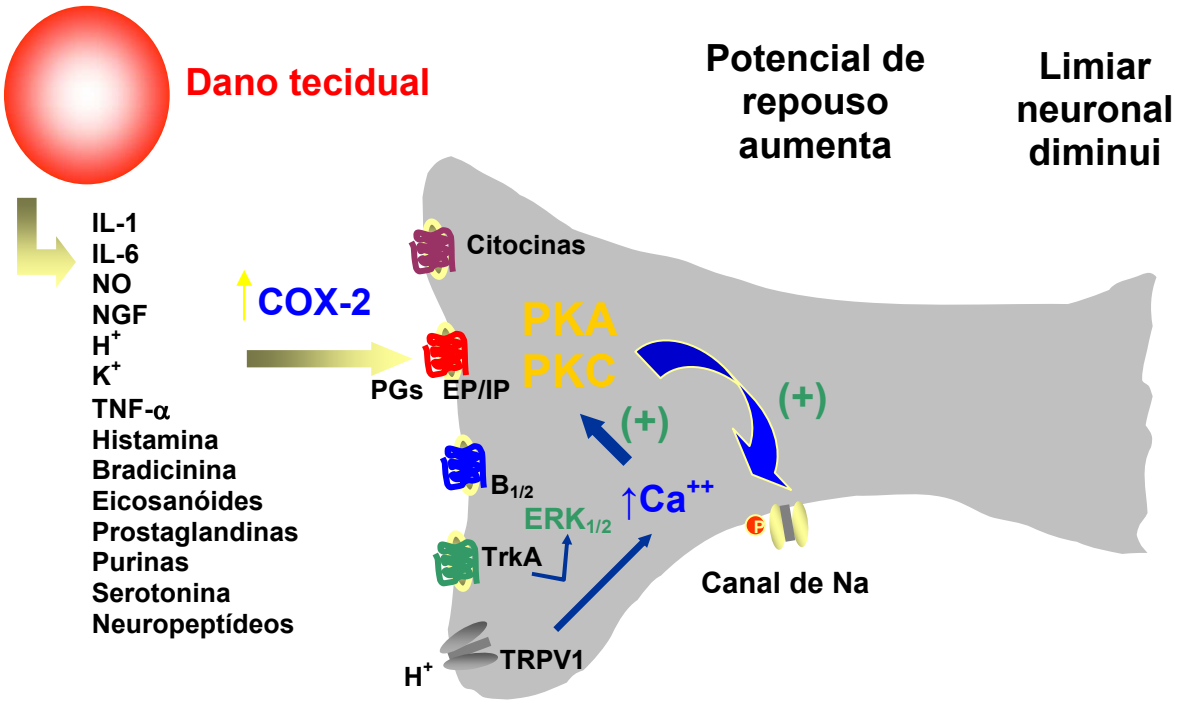

Figura 1: Representação esquemática dos mecanismos de sensibilização periférica à dor. O estímulo doloroso começa na periferia geralmente através de uma lesão tecidual. Esta injúria promove liberação de diversos fatores sensibilizantes provenientes da lesão tecidual como os citados acima o que culmina com diminuição do limiar dos nociceptores periféricos e ativação de canais de sódio na periferia. IL= interleucinas; NO = óxido nítrico; TNF-α = fator de necrose tumoral alfa; NGF = fator de crescimento neural; PKA = proteína cinase A; PKC = proteína cinase C; PGs = prostaglandinas; $B_{1/2}$ = receptores de bradicinina tipo 1 e 2; TrkA = receptor de tirosina cinase A ; $TRPV_1$ = canais voltagem-dependentes de

receptores transitórios; EP/IP = receptores de prostanóides; $ERK_{1/2}$ = cinases reguladas por sinais extracelulares.

I.1.d. Processamento central da dor

A lesão periférica e a conseqüente liberação de diversos medidores algogênicos na periferia desencadeiam um processo de transmissão do estimulo doloroso até níveis mais centrais [Millan, 1999]. A transmissão dos estímulos nocivos através da medula espinhal não é um processo passivo. Os circuitos intramedulares têm a capacidade de alterar o estímulo e a conseqüente resposta dolorosa. A interação entre esses circuitos medulares determinará as mensagens que atingirão o córtex cerebral [Zhang e Bao, 2006]. Estudos clínicos e experimentais têm demonstrado que estímulos nocivos provocam alterações no sistema nervoso central, modificando os mecanismos desencadeados pelos estímulos aferentes. A estimulação persistente de nociceptores provoca dor espontânea, redução do limiar de sensibilidade, hiperalgesia e alodinia. A hiperalgesia pode ser classificada como primária e secundária. A hiperalgesia primária é conceituada como sendo o aumento da resposta ao estímulo doloroso no local da lesão, enquanto a hiperalgesia secundária é aquela que se estende para áreas adjacentes. A presença de todos esses elementos sugere que a sensibilização periférica não é o único fenômeno responsável por todas essas mudanças e que deve haver envolvimento do SNC neste processo [Zhuo, 2007].

Inicialmente, os impulsos nociceptivos são transmitidos através de aferentes primários até o corno dorsal da medula espinhal, área primária de recebimento da maioria das informações sensoriais [D'Mello e Dickenson, 2008]. O corno dorsal da medula espinhal é dividido morfologicamente e funcionalmente em lâminas com base em sua

citoarquitetura, sendo que cada lâmina se caracteriza por receber diferentes tipos de informações [Millan, 1999]. As fibras aferentes primárias do tipo Aδ e C realizam suas sinapses primárias nas lâminas mais superficiais. A partir da integração dos impulsos nervosos no corno dorsal da medula espinhal, diferentes projeções a territórios superiores como estruturas subcorticais e corticais ocorrem [Fitzgerald, 2005].

As vias ascendentes de transmissão da dor podem ser monossinápticas e polissinápticas [Millan, 1999]. As vias monossinápticas projetam-se diretamente a estruturas superiores e incluem diversos feixes, tais como o espinotalâmico e espinoreticular. As vias polissinápticas apresentam uma estação de retransmissão a neurônios de segunda ordem até atingirem estruturas mais superiores e incluem feixes como o paleoespinotalâmico e o espinocervical. Muitas destas vias ascendentes fazem conexões com neurônios de terceira ordem em nível talâmico, projetando-se após até estruturas corticais [Fitzgerald, 2005].

Considerando a complexidade das vias nervosas envolvidas na transmissão da dor, é importante ressaltar o papel fundamental de mecanismos inibitórios endógenos presentes em nosso organismo. Além da existência de mediadores químicos endógenos de caráter antinociceptivo amplamente distribuídos em nosso organismo, diversas estruturas espinhais e supra-espinhais descendentes modulam fortemente a resposta dolorosa [Zhang e Bao, 2006; Yoshimura e Furue, 2006]. Esta modulação se dá através de vias descendentes provenientes de diversas estruturas do SNC (tálamo, tronco cerebral, hipotálamo, córtex, núcleo magno da rafe, substância cinzenta periaquedutal, entre outras estruturas superiores) [Yoshimura e Furue, 2006]. As vias descendentes modulam a resposta dolorosa através de ações sobre as vias aferentes primárias e sobre interneurônios inibitórios e excitatórios em

nível medular. Estas vias são provenientes principalmente do tronco cerebral até a medula espinhal e são predominantemente de caráter noradrenérgico e serotoninérgico [Yoshimura e Furue, 2006]. Estudos eletrofisiológicos demonstraram diversas características inibitórias das vias noradrenérgicas e serotoninérgicas: i. hiperpolarizam diretamente os neurônios situados no corno dorsal da medula; ii. inibem a liberação de glutamato de fibras aferentes Aδ e C; iii. aumentam a liberação de GABA e glicina proveniente de interneurônios inibitórios na medula espinhal. Apesar de efeitos excitatórios terem sido descritos e relacionados a estas vias, o efeito predominante é anti-nociceptivo, demonstrando, portanto, o papel fundamental destas vias de transmissão nervosa descendente no controle endógeno da dor (Figura 2) [Yoshimura e Furue, 2006].

Entretanto, caso o estímulo periférico seja suficientemente sustentado ou intenso, ou em determinadas ocasiões, ocorrer uma disfunção intrínseca das vias de transmissão dolorosa, fenômenos neuroplásticos reversíveis ou até irreversíveis podem ocorrer em nível central [Zhuo, 2007]. Neste caso, começam a ocorrer mecanismos de sensibilização central, que podem, em muitas situações, desencadear síndromes dolorosas crônicas altamente refratárias (Figuras 3 e 4) [Tunks et al., 2008].

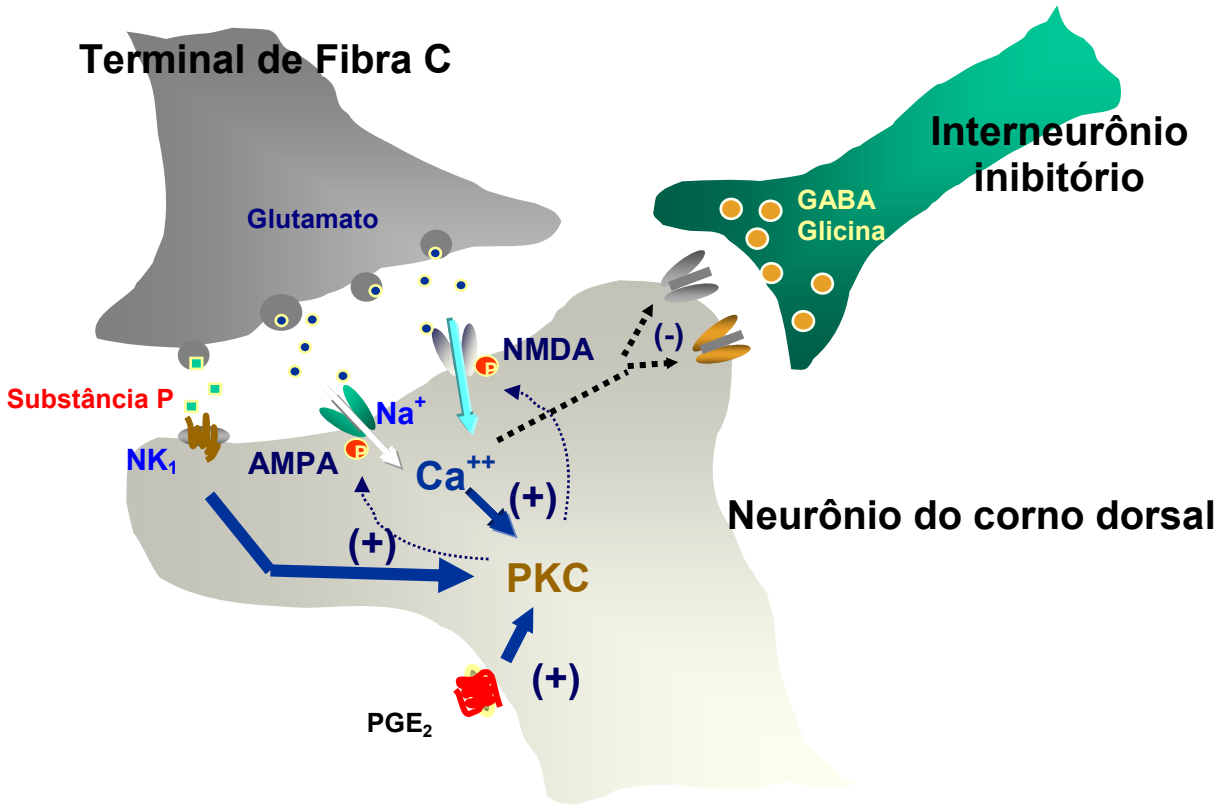

Figura 2: Representação esquemática dos mecanismos de processamento da dor no corno dorsal da medula espinhal. Observe neste esquema o início da transmissão do processo doloroso em nível medular. Após ativação suficientemente intensa e persistente de receptores AMPA, inicia-se a ativação de receptores NMDA com aumento do influxo de íons cálcio. No processamento central da dor e no início do processo de sensibilização central, os interneurônios inibitórios com liberação dos neurotransmissores GABA e glicina ainda permanecem ativos e modulam negativamente a dor. PKC = proteína cinase C; PGE_2 = prostaglandina E_2; NK_1 = receptor de neurocininas do tipo 1; GABA = ácido gama-aminobutírico; AMPA = ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropionato; NMDA = receptor N-metil-D-aspartato.

I.1.e. Mecanismos de sensibilização central

A sensibilização central implica em alterações dos impulsos periféricos, com adaptações positivas ou negativas. Ocorre redução do limiar ou aumento da resposta aos impulsos aferentes, descargas persistentes após estímulos repetidos e ampliação dos campos receptivos de neurônios do corno dorsal. Impulsos repetidos em fibras C amplificam sinais sensoriais em neurônios espinhais, enviando mensagens para o encéfalo [Zhuo, 2007]. Após a agressão tecidual, há liberação de neurotransmissores, como substância P, somatotastina, CGRP, neurocinina A, glutamato e aspartato. Essas substâncias estão relacionadas com a ativação de potenciais pós-sinápticos excitatórios [Millan, 1999]. Estímulos freqüentes dos aferentes geram a soma dos potenciais de ação e conseqüente despolarização pós-sináptica cumulativa. O aumento do cálcio tem como conseqüência a ativação da enzima óxido nítrico sintase e a estimulação da transcrição de proto-oncogenes. Estes são genes localizados no SNC e estão envolvidos na formação de dinorfinas e encefalinas. As encefalinas têm ação antinociceptiva e estão envolvidas no processo de redução da neuroplasticidade e hiperalgesia. Entretanto, as dinorfinas têm um efeito mais complexo, já que possuem ação algogênica e antinociceptiva dependendo da situação. Estudos recentes têm sugerido que a ativação do c-fos e c-jun promove a transcrição de RNAm responsável pela síntese de proteínas fundamentais, as quais estão envolvidas na alteração da expressão fenotípica e, conseqüentemente, na perpetuação da hipersensibilidade neuronal [Delander et al., 1997; Jongen et al., 2005].

A sensibilização do corno dorsal da medula espinhal pode ser de diferentes modalidades, envolvendo mecanismos homossinápticos ou heterossinápticos. Os mecanismos homossinápticos significam que o estímulo e o condicionante estão associados

ao mesmo aferente. Isto pode ser exemplificado durante o fenômeno de "wind up", que ocorre quando estímulos contínuos, por tempo prolongado e de baixa freqüência (inferior a 5 Hz) provenientes de fibras aferentes do tipo C provocam amplificação da resposta de células específicas do corno dorsal da medula espinhal [Herrero et al., 2000]. Este fenômeno estimula a liberação de neurotransmissores excitatórios no corno dorsal da medula espinhal e produz a despolarização relacionada com a remoção do bloqueio voltagem-dependente exercido pelo magnésio nos receptores NMDA. Ocorre, então, aumento da condutividade ao cálcio e a resposta à dor, a cada estímulo repetido e de mesma intensidade. Na sensibilização heterossináptica, o estímulo e o condicionante se relacionam com diferentes aferentes como, por exemplo, no aumento da eficácia sináptica de mecanorreceptores devido a impulsos nocivos. Portanto, a sensibilização sináptica central clássica é causada por uma seqüência sincronizada de estímulos periféricos nociceptivos repetidos por uma única estimulação nociceptiva assíncrona, aumentando a resposta de aferentes de fibras Aδ e C (potencialização homossináptica) e de aferentes de fibras Aβ não-estimulados (potencialização heterossináptica). Este mecanismo ocorre por aumento da eficácia sináptica ou como conseqüência da liberação excessiva de neurotransmissores excitatórios, como aminoácidos, peptídeos e de neurotrofinas no corno dorsal da medula espinhal [Millan, 1999].

Os neuropeptídeos como a substância P e o CGRP ligam-se aos receptores para neurocininas do tipo NK_1 e NK_2, enquanto as neurotrofinas possuem como receptores as tirocinases tipo A e B (trkA, trkB) [Hill e Oliver, 2007]. Após a liberação de aminoácidos excitatórios, peptídeos e neurotrofinas e sua interação com receptores específicos, há a ativação de segundos mensageiros, do tipo AMPc, PKA, PKC, fosfatidil-inositol, fosfolipase C, fosfolipase A_2. Após ativação suficientemente intensa e persistente dos

receptores glutamatérgicos do tipo AMPA, inicia-se a ativação de receptores NMDA. Isto promove a abertura de canais de cálcio, aumentando o influxo de íons cálcio e a produção de prostaglandinas e óxido nítrico. Estes migram do intracelular em direção à fenda sináptica e estimulam a liberação de glutamato, aspartato, substância P e CGRP, contribuindo para a ampliação do processo álgico [Birklein e Schmelz, 2008].

Os mecanismos que contribuem para o aumento da eficácia da transmissão sináptica podem ser decorrentes da fosforilação dos receptores de membrana e das alterações no tempo de abertura dos canais iônicos, ou da formação e do transporte de substâncias excitatórias do interior da célula para a fenda sináptica. Além disso, no corno dorsal da medula espinhal, as proteínas cinases ativadas por mitógenos (MAPK) modulam a fosforilação dos receptores NMDA e AMPA, amplificando a resposta nociceptiva. A facilitação de longo termo envolve a ativação de fatores de transcrição e alterações na transcrição. Os fatores de transcrição modulam a relação entre o complexo receptor-neuromediador e as alterações na expressão gênica [Zhuo, 2007]. Durante o estabelecimento do fenômeno de sensibilização central na dor neuropática, através do que chamamos de excitotoxicidade (toxicidade mediada pelo glutamato), ocorre morte de neurônios inibitórios. Isto contribui de forma significativa para a manutenção do quadro doloroso em longo prazo (Figuras 3 e 4) [Zhuo, 2007].

I.1.f. Mediadores químicos envolvidos na dor

A ação direta ou indireta de mediadores químicos é responsável pela multiplicidade e complexidade de eventos que ocorrem durante a transmissão dolorosa periférica e central [Fürst, 1999; Millan, 1999]. Os principais mediadores químicos envolvidos nestes

processos são: prostaglandinas, leucotrienos, aminoácidos excitatórios (glutamato e aspartato), purinas (ATP), noradrenalina, serotonina, dopamina, óxido nítrico, cininas, taquicininas, substância P, CGRP, galanina, colecistocinina, peptídeo vasoativo intestinal, citocinas, fatores tróficos neurais, entre outros [Millan, 1999].

Dentre os principais mediadores químicos relacionados à transmissão e manutenção da dor, abordaremos apenas os mais relevantes e envolvidos diretamente na realização desta tese. São eles: glutamato, óxido nítrico, substância P e purinas. Considerando que a presente tese enfatiza revisar a influência do sistema purinérgico nos mecanismos de transmissão dolorosa, as substâncias derivadas deste sistema (purinas) serão abordadas em item à parte posteriormente.

- Glutamato:

Os aminoácidos excitatórios, principalmente o glutamato, estão envolvidos em excitação neural rápida, em plasticidade neural, em processos de aprendizado e memória, são capazes de interagir com outros sistemas de neurotransmissores, apresentam sensibilidade a agentes externos, assim como também estão envolvidos em processos de neurotoxicidade (excitotoxicidade) [Danbolt, 2001; Carozzi et al., 2008]. O efeito excitatório do glutamato no SNC foi descrito inicialmente no início da década de 1960 [Curtis e Watkins, 1960; Krnjevic e Phillis, 1963], desencadeando pesquisas intensas no sentido de caracterizar esta substância como um neurotransmissor fundamental.

Na década de 1980, o glutamato adquiriu status de principal neurotransmissor excitatório no SNC de humanos e mamíferos em geral [McLennan e Liu, 1982]. Baseados em estudos de biologia molecular e eletrofisiologia, de especificidade para agonistas e antagonistas e de acoplamento com sistemas de segundos mensageiros, receptores

glutamatérgicos podem ser classificados em dois grandes grupos: ionotrópicos e metabotrópicos. Receptores ionotrópicos são canais iônicos modulados por agonistas (NMDA, AMPA, cainato); receptores metabotrópicos são acoplados a sistemas de segundos mensageiros através de proteínas G [Danbolt, 2001; Bleakman et al., 2006]. Descrições completas dos receptores envolvidos com a transmissão glutamatérgica estão amplamente divulgadas na literatura [Ozawa et al., 1998; Bleakman et al., 2006; Neugebauer, 2007].

A ativação de receptores glutamatérgicos metabotrópicos e ionotrópicos por glutamato, aspartato ou agonistas dos receptores, podem modular a atividade de várias enzimas (adenilato ciclase, guanilato ciclase, fosfolipase C) e fluxos iônicos transmembrana. A modulação destes efetores altera os níveis de segundos mensageiros específicos (AMPc, diglicerídios e fosfo-inositóis) e variações de níveis iônicos intracelulares, que especificam respostas celulares à ativação de receptores glutamatérgicos [Bleakman et al., 2006; Neugebauer, 2007].

Além de suas ações fisiológicas, glutamato pode ser um potente agente neurotóxico. Em determinados insultos agudos ao SNC (como isquemia, hipoglicemia, traumatismo craniano, ação de drogas) ou em doenças crônicas neurodegenerativas (Huntington, Alzheimer, Parkinson, epilepsia), glutamato liberado de maneira não modulada para o espaço extracelular pode exercer efeitos neurotóxicos, com graves e irreversíveis alterações estruturais, neuroquímicas e comportamentais [Parsons et al., 2005].

Os primeiros antagonistas glutamatérgicos descritos eram competitivos e seletivos para o receptor NMDA: α-amino adipato, d-amino-2-fosfono-ácido valérico (1-APV) e 4-(3-fosfonopropil)piperazina-2-ácido carboxílico (CPP) [Hicks et al., 1978]. Posteriormente,

os antagonistas não-competitivos para o receptor NMDA cetamina, fenciclidina e MK-801 foram descritos [Anis et al., 1983; Wong et al., 1986]. Sítios moduladores do receptor NMDA como a glicina e poliamina também foram descritos posteriormente [Ransom e Stec, 1988]. Desde então, muitos antagonistas seletivos dos diferentes receptores de glutamato e maior conhecimento a respeito de suas funções foram desenvolvidos [Parsons et al., 2005; Bleakman et al., 2006; Neugebauer, 2007].

Glutamato tem demonstrado papel essencial na transmissão de estímulos dolorosos [Bleakman et al., 2006]. Novas evidências farmacológicas, eletrofisiológicas, e comportamentais têm surgido embasando a hipótese de que os receptores glutamatérgicos apresentam papel fundamental nas vias de dor e que modulação destes receptores pode ser terapêutica efetiva para tratamento de quadros dolorosos crônicos [Bleakman et al., 2006].

Os receptores AMPA e NMDA para glutamato têm sido implicados na geração de estados centrais de hipersensibilidade e potenciam a transmissão de estímulos nocivos [Millan, 1999]. Estudos têm demonstrado que os receptores glutamatérgicos estão criticamente envolvidos na transmissão nociceptiva aferente primária, tanto no desenvolvimento quanto na manutenção da nocicepção [Coggeshall e Carlton, 1997; Bleakman et al., 2006]. Resumidamente, o estímulo nocivo ocorre na periferia e desencadeia um potencial de ação transmitido até sinapses centrais no corno dorsal da medula espinhal. Este potencial de ação desencadeia a liberação de neurotransmissores excitatórios, primariamente o glutamato e secundariamente o aspartato, conhecidos como aminoácidos excitatórios. Em muitas sinapses, a liberação de glutamato é conjunta com a liberação de outros neuromoduladores como substância P e neurocininas. A freqüência e a duração dos estímulos conduzidos até a medula espinhal determinam fenômenos de nocicepção com finalidade biológica (protetora) ou sua transformação em estados

patológicos (dor persistente ou crônica). A ação do glutamato em receptores AMPA no corno dorsal desencadeia a formação de potenciais excitatórios pós-sinápticos rápidos, possibilitando uma corrente iônica para dentro da célula de curta duração. Em algumas sinapses, principalmente relacionadas a fibras de fino calibre, o glutamato desencadeia suas ações através de três componentes. Inicialmente observamos ação sobre os receptores AMPA, com reposta rápida (milissegundos) seguida de ativação do receptor NMDA (cerca de 5 segundos). O último componente, insensível a antagonistas NMDA, é prolongado e parece ser mediado por taquicininas como substância P e neurocinina A. O receptor NMDA está associado a canal iônico e normalmente encontra-se bloqueado pelo íon magnésio, ou seja, quando o glutamato liga-se a este receptor a corrente iônica resultante é pequena. Entretanto, se ocorrer estímulo prolongado e/ou repetitivo, há remoção do íon magnésio do interior do receptor, permitindo a entrada maciça de sódio e de cálcio para a célula, produzindo despolarização da célula pós-sináptica e estimulação de mensageiros secundários, do que resulta a amplificação e o prolongamento da resposta ao impulso doloroso, fenômenos de "*wind up*" e mecanismos de sensibilização central com estabelecimento, em alguns casos, de dor crônica patológica [Millan, 1999; Herrero et al., 2000; Julius e Basbaum, 2001; Stucky et al., 2001; Zhuo, 2007; D'Mello e Dickenson, 2008].

Neste contexto, substâncias capazes de bloquear os receptores glutamatérgicos ionotrópicos e metabotrópicos apresentam importante efeito antinociceptivo em diferentes espécies de mamíferos, inclusive em humanos [Wiech et al., 2004]. Antagonistas dos receptores de glutamato como cetamina e MK-801 podem atenuar estados dolorosos relacionados à reação inflamatória, dano tecidual agudo, isquemia ou dano nervoso [Wiech et al., 2004]. Entretanto, a grande limitação para o seu uso clínico são os efeitos adversos

intoleráveis que muitos destes fármacos acarretam como sedação, disforia, alucinações, distúrbios motores, entre outros [Gardoni e Di Luca, 2006]. Portanto, a busca por novos fármacos que modulem a atividade glutamatérgica anormal e apresente menor perfil de efeitos adversos é constante.

- Óxido nítrico

O óxido nítrico (NO) é um importante mensageiro biológico, amplamente distribuído em nosso organismo, que está envolvido na transmissão sináptica no SNC e SNP [Millan, 1999]. O NO é sintetizado enzimaticamente a partir do aminoácido L-arginina através de três isoformas da óxido nítrico sintase (NOS): a neuronal (nNOS), a endotelial (eNOS) e a induzida (iNOS). As formas endotelial e neuronal são constitutivas, enquanto a induzida se expressa nas células do sistema imune e responde a vários tipos de estímulos. Após sua síntese, o NO ativa a enzima guanilato ciclase que converte a guanosina-5'-trifosfato (GTP) em guanosina-3'-5'-monofosfato cíclico (GMPc). Este, por sua vez, age como mensageiro secundário, ativando proteínas cinase, canais iônicos e fosfodiesterases [Millan, 1999].

O NO é uma molécula gasosa pequena, altamente reativa, e que passa através de membranas celulares com facilidade. O NO é considerado um transmissor retrógrado, isto é, a ativação de algum receptor (NMDA, por exemplo) no neurônio pós-sináptico induz a produção de NO, que se difunde rapidamente e entra no neurônio pré-sináptico para modular sua atividade [Levy e Zochodne, 2004].

O NO pode ser liberado após estimulação direta das fibras aferentes primárias e auxilia na sensibilização dos nociceptores e aumenta a excitabilidade das fibras nervosas após um estímulo doloroso [Aley et al., 1998]. Entretanto, o NO apresenta um

comportamento complexo na transmissão da dor, pois pode provocar efeitos antagônicos [Millan, 1999]. Por exemplo, quando utilizamos ativadores ou inibidores como ferramentas farmacológicas para investigar a via L-argnina-NO-GMPc, o NO pode agir como pró-nociceptivo ou antinociceptivo no SNC ou SNP [Millan, 1999]. Esta dualidade pode estar relacionada a diversos fatores como o tipo de modelo de dor utilizado, as espécies animais envolvidas, as drogas, regimes e dosagens utilizadas, entre outros fatores [Millan, 1999]. Em geral, apesar das controvérsias envolvendo esta substância, o óxido nítrico provoca hiperalgesia e está implicado na sensibilização central, fortalecendo a ação de prostaglandinas em diversos modelos de dor, principalmente com característica neuropática [Millan, 1999; Zhuo, 2007].

- Substância P

A substância P é um neuropeptídeo amplamente distribuído no organismo, onde atua como neurotransmissor e neuromodulador. A substância P desempenha papel fundamental na transmissão dolorosa no SNC e SNP através de ações em seus receptores específicos, denominados receptores para neurocininas (NK_1, NK_2 e NK_3) [Wiley, 2008].

No sistema nervoso, a substância P é sintetizada pelos neurônios no gânglio da raiz dorsal e liberado nas fibras do tipo C em resposta a agressões ou a estímulos suficientemente intensos sob os nervos periféricos [Yaksh et al., 1999]. A substância P age principalmente no receptor NK_1 localizado predominantemente na lâmina I do corno dorsal da medula espinhal [Yaksh et al., 1999; Wiley, 2008].

A substância P, além de sua função fundamental na transmissão da dor aguda, interage com outros sistemas de neurotransmissão como o glutamatérgico [De Biasi e Rustioni, 1988; Skilling et al., 1993; Siebel et al., 2004]. A interação entre substância P e

glutamato em receptores do tipo NMDA em nível pré-sináptico nas terminações nervosas do tipo C facilitam e prolongam a transmissão da dor [De Biasi e Rustioni, 1988]. Antagonistas seletivos do receptor NK_1 bloqueiam hiperalgesia e alodinia, sugerindo que a substância P e seus receptores apresentam papel relevante na geração e manutenção de quadros dolorosos crônicos [Siebel et al., 2004].

I.1.g. Dor patológica: dor crônica neuropática

A dor neuropática é definida como dor decorrente de lesão ou disfunção do nervo e de modo mais amplo, como conseqüência de lesão ou doença do sistema somestésico [Zimmermann, 2001]. É uma síndrome complexa, com mecanismos biológicos pouco esclarecidos, envolvendo diversos medidores químicos e imunes [Campbell e Meyer, 2006]. A dor neuropática ainda é um desafio para os pesquisadores clínicos e experimentais, pois sua etiologia é heterogênea e pode ser ocasionada por um insulto primário ao sistema nervoso central ou periférico [Zimmermann, 2001]. Entender a neurobiologia da dor neuropática é um passo para melhoria dos resultados no tratamento dessa síndrome. Essa compreensão poderá resultar na elaboração de fármacos que visem a alvos específicos e que proporcionem respostas eficazes.

Apesar dos mecanismos desencadeadores da dor neuropática ainda não estarem completamente esclarecidos, o desenvolvimento de dor crônica após lesão nervosa ocorre através de alterações medulares, tais como excitabilidade aumentada, vias inibitórias com atividade reduzida, neuroplasticidade com mudança fenotípica na organização celular. Para que este processo se estabeleça, é necessária a participação de diversos mediadores químicos de forma semelhante ao que ocorre na dor inflamatória. A produção e liberação

destes mediadores desencadeiam uma cascata de eventos que culmina com a manutenção do potencial de ação. Por tratar-se de um evento crônico, uma das principais características desta doença é a ocorrência de eventos neuroplásticos relacionados a alterações gênicas de receptores, neurotransmissores e neuromoduladores, canais iônicos, proteínas intracelulares, entre outras [Woolf e Thompson, 1999; Willis, 2001; Binns et al., 2005; Zhuo, 2007].

Após ativação suficientemente intensa e persistente de receptores AMPA, inicia-se a ativação de receptores NMDA com aumento do influxo de íons cálcio. No início do processo de sensibilização central, os interneurônios inibitórios, com a liberação dos neurotransmissores GABA e glicina, ainda permanecem ativos e modulam negativamente a dor. Durante o estabelecimento do fenômeno de sensibilização central, através do que chamamos de excitotoxicidade (toxicidade mediada pelo glutamato), ocorre morte de neurônios inibitórios. Isto contribui de forma significativa para a manutenção do quadro doloroso em longo prazo. Após este processo inicial, sinapses aberrantes ocorrem na medula (neuroplasticidade e memória de dor). Estas sinapses, somadas a perda de neurônios inibitórios, desencadeiam a amplificação do processo doloroso que se torna, em muitas ocasiões, espontânea [Gold, 2000; Zhuo, 2007]. Neste momento, tratar a dor que resulta deste evento torna-se tarefa difícil e muitos casos são refratários aos tratamentos existentes na atualidade.

As células da glia interagem com os neurônios promovendo manutenção da homeostase, regulando as concentrações de neurotransmissores, de íons e o pH do meio extracelular [Danbolt, 2001]. Na dor neuropática, a microglia parece desempenhar papel fundamental no início da lesão e os astrócitos na manutenção [Watkins et al., 2001; Watkins e Maier, 2003]. A microglia é ativada por vários neuromediadores tipo ATP, bradicinina, substância P, fractalcina, receptor Toll-like tipo 4 (TLR_4) [Liao e Chen, 2001].

O ATP ativa receptores $P_2X_{2/3}$, P_2X_3 e P_2X_4 no sistema nervoso central; a fractalcina é uma quimiocina expressa na superfície dos neurônios medulares que ativa o receptor CX_3CR_1 na microglia e os receptores TLR_4 reconhecem moléculas de estruturas variadas liberadas durante a lesão nervosa [Liao e Chen, 2001]. Entretanto, ainda é desconhecido como a dor neuropática se desenvolve após a ativação da microglia. É provável que as células da glia liberem vários neurotransmissores excitatórios, como a prostaglandina, o óxido nítrico, as citocinas e as quimiocinas, ativando diretamente o aferente sensitivo. Também pode haver propagação de ondas de cálcio por entre as junções neurais, facilitando a liberação de neuromediadores excitatórios [Spataro et al., 2004; Ueda, 2006]. A resposta imune também apresenta papel fundamental durante o desenvolvimento da dor neuropática. As células de Schwann interagem com as células tipo T expressando moléculas de histocompatibilidade MHC classe II. As células de Schwann secretam citocinas (IL-6, IL-1, TNF-α), fatores neurotróficos (NGF), prostaglandinas E_2 e ATP [Liu et al., 2000; Moalem et al., 2004]. Também expressam canais iônicos e receptores para glutamato e citocinas, contribuindo na gênese da dor neuropática (Figuras 3 e 4) [Ueda, 2006].

Há diversos modelos animais de dor neuropática descritos na literatura. A maioria destes modelos foi desenvolvida em ratos a partir de lesões periféricas traumáticas, metabólicas ou tóxicas: ligadura do nervo espinhal, ligadura parcial do nervo ciático, lesão constritiva crônica do nervo ciático, lesão limitada do nervo tibial e fibular, lesão do plexo braquial, entre outros modelos de lesão nervosa direta ou indireta [Le Bars et al., 2001]. Outros métodos incluem a injeção intraperitoneal de estreptozocina para mimetizar neuropatia diabética ou de quimioterápicos (paclitaxel ou vincristina) para mimetizar neuropatia induzida por quimioterapia. Os modelos para dor central utilizam a contusão

(trauma utilizando a força do impacto com deslocamento tissular), ou lesões isquêmicas por compressão lenta por meio de pinçamento ou da insuflação com balonetes [Le Bars et al., 2001]. Métodos citotóxicos empregam a injeção de análogos de glutamato ou de substâncias que permitem a lesão de locais específicos do SNC. As técnicas descritas visam a provocar hiperalgesia mecânica e térmica e apresentam boa correlação com as alterações fenotípicas (clínicas e neuroquímicas) desenvolvidas em humanos [Le Bars et al., 2001; Campbell e Meyer, 2006].

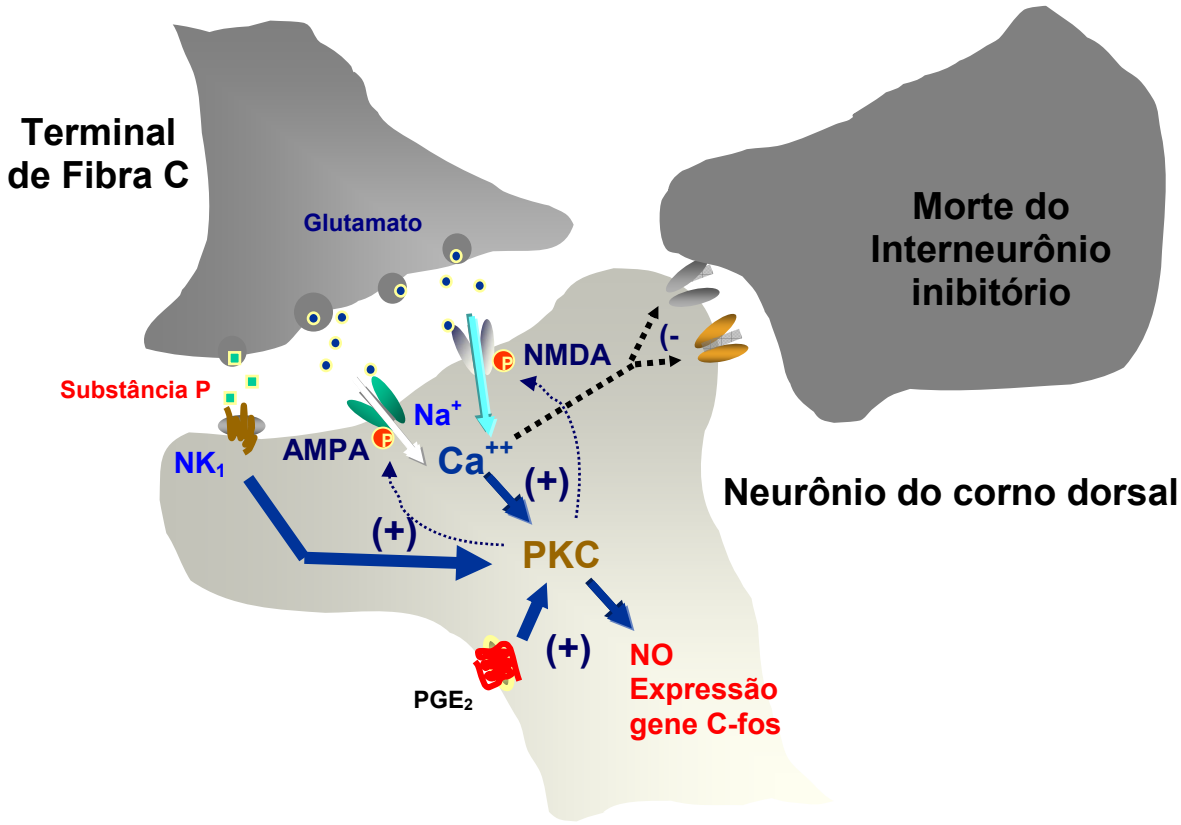

Figura 3: Representação esquemática dos mecanismos de sensibilização central no corno dorsal da medula espinhal. Observe neste esquema que durante o estabelecimento do fenômeno de sensibilização central, através do que chamamos de excitotoxicidade

(toxicidade mediada pelo glutamato), ocorre morte de neurônios inibitórios. Isto contribui de forma significativa para a manutenção do quadro doloroso em longo prazo. PKC = proteína cinase C; PGE_2 = prostaglandina E_2; NK_1 = receptor de neurocininas do tipo 1; GABA = ácido gama-aminobutírico; AMPA = ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropionato; NMDA = receptor N-metil-D-aspartato; NO = óxido nítrico.

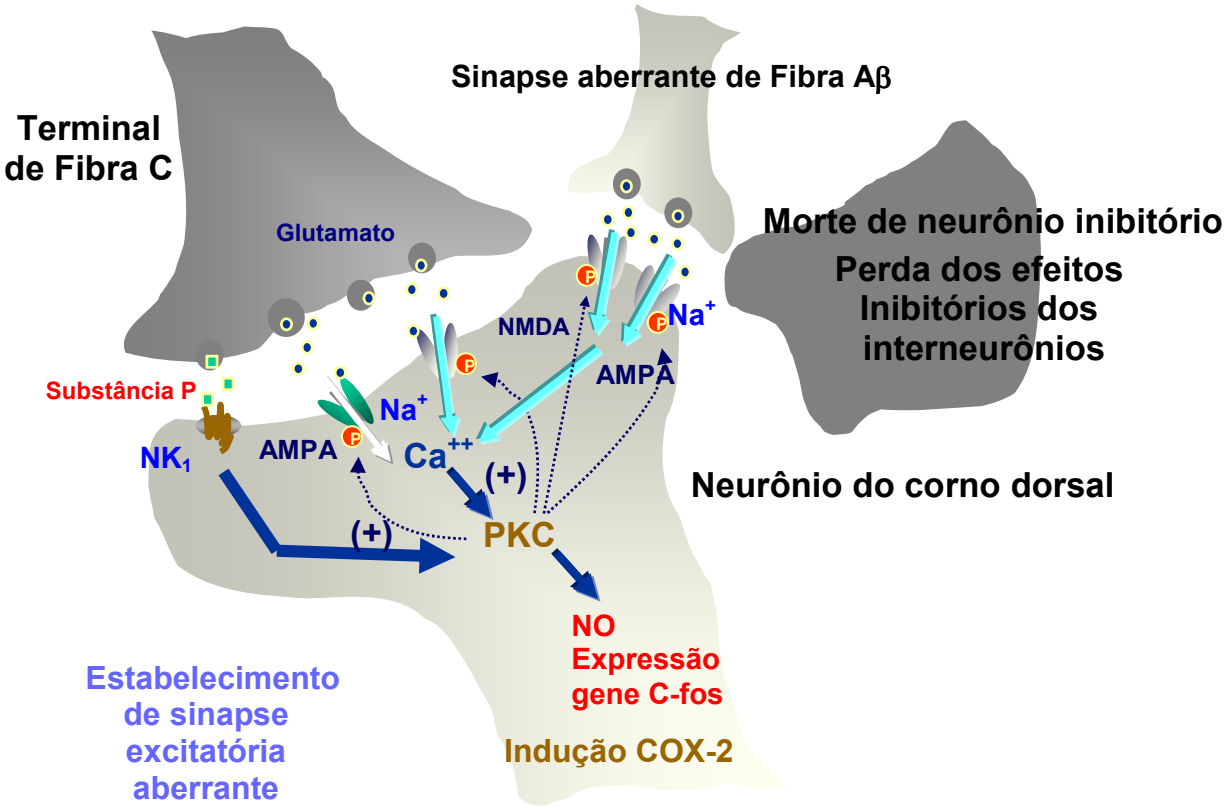

Figura 4: Representação esquemática dos mecanismos neuroplásticos no corno dorsal da medula espinhal responsáveis pela dor crônica patológica. Acima observe que sinapses aberrantes ocorrem na medula (neuroplasticidade e memória de dor). Estas sinapses, somada a perda de neurônios inibitórios, desencadeia a amplificação do processo doloroso.

Neste momento, tratar a dor que resulta deste evento torna-se tarefa difícil e muitos casos são refratários aos tratamentos existentes na atualidade. PKC = proteína cinase C; PGE_2 = prostaglandina E_2; NK_1 = receptor de neurocininas do tipo 1; GABA = ácido gama-aminobutírico; AMPA = ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropionato; NMDA = receptor N-metil-D-aspartato; NO = óxido nítrico; COX-2 = enzima ciclooxigenase do tipo 2.

I.1.h. O Sistema Purinérgico

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

As purinas podem ser classificadas em derivados da adenina (ATP, ADP, AMP, adenosina, adenina) e derivados da guanina (GTP, GDP, GMP, guanosina e guanina). Ainda compõem as purinas os metabólitos diretos dos derivados da adenina e da guanina: inosina, xantina, hipoxantina e ácido úrico. Os derivados da adenina, principalmente o nucleotídeo ATP e o nucleosídeo adenosina são considerados os principais efetores do sistema purinérgico em nível extracelular [Ralevic e Burnstock, 1998]. O papel do ATP

como neurotransmissor, em nível central e periférico, está amplamente estabelecido [Ralevic e Burnstock, 1998]. ATP é armazenado e liberado de terminais pré-sinápticos e age em receptores do tipo P_2, como descrito detalhadamente em diversas revisões [Ralevic e Burnstock, 1998; Burnstock, 2007]. Os efeitos neuromodulatórios da adenosina também estão amplamente reconhecidos e caracterizados, assim como seus substratos [Brundege e Dunwiddie, 1997]. Adenosina e ATP agem não só como neurotransmissores e neuromoduladores, mas como fatores tróficos em diversos processos plásticos desenvolvidos no SNC e SNP como aprendizado e memória, proliferação neural, neuroproteção endógena contra estímulos nocivos e regulação da morte celular programada (apoptose celular) [Rathbone et al., 1999; Ciccarelli et al., 2001]. Adicionalmente, as purinas, principalmente a adenosina, são importantes moduladoras da atividade sináptica no sistema nervoso central, interagindo com vários sistemas, como glutamatérgico, dopaminérgico, serotoninérgico e colinérgico [Schmidt et al., 2007; Rathbone et al., 2008].

I.1.i. O papel do sistema purinérgico na transmissão dolorosa

Adenosina e ATP exercem múltiplas influências na transmissão dolorosa em sítios periféricos e centrais [Sawynok, 1998; Sawynok et al., 1999; Sawynok e Liu, 2003]. Efeitos antinociceptivos da adenosina estão relacionados à inibição intrínseca de neurônios pelo aumento da condutância ao K^+ e inibição pré-sináptica dos terminais nervosos sensoriais, diminuindo a liberação de substância P e glutamato [Sawynok e Liu, 2003]. Adenosina, através de sua ação agonista em receptores A_1, atenua produção de óxido nítrico mediado pelo receptor glutamatérgico NMDA e está diretamente relacionada a analgesia opióide [Sawynok e Liu, 2003].

O ATP é um neurotransmissor clássico, mas também é liberado por células não-neuronais e tecido lesado. Age em receptores purinérgicos específicos (P_2). Os receptores P_2 podem ser subdivididos em P_2X e P_2Y que são acoplados, respectivamente, à proteína G e aos canais iônicos [Burnstock, 2007]. Em modelos experimentais de dor neuropática, há redução (após axotomia ou ligadura parcial do nervo) ou aumento (lesão constritiva crônica) de receptores P_2X_3; contudo, mesmo na redução, há aumento da sensibilidade desses receptores [Jarvis et al., 2002]. O bloqueio de receptores P_2X_3 atenua a alodinia térmica e mecânica em ratos [Jarvis et al., 2002]. Os receptores P_2X_4 também aumentam sua expressão na microglia após a lesão de nervo e o bloqueio farmacológico do P_2X_4 reverte a alodinia [Tsuda et al., 2003]. Os receptores P_2X_7 estão presentes nas células T e macrófagos. Ratos que não expressam este receptor são resistentes ao desenvolvimento de dor neuropática [Chessell et al., 2005]. Por outro lado, os receptores P_2Y_1 aumentam em 70% após lesão do nervo ciáticos em ratos, e também podem estar relacionados ao desenvolvimento de quadros dolorosos [Xiao et al., 2002].

Entretanto, fármacos que interagem diretamente com os receptores purinérgicos ainda não estão adequadamente disponíveis para uso clínico. Uma abordagem interessante para avaliar o potencial do sistema purinérgico na modulação da dor seria utilizarmos fármacos que modulam a atividade da adenosina e que já se encontram disponíveis para uso clínico, como o alopurinol. O alopurinol é um inibidor da xantina oxidase, enzima responsável pela conversão de xantina em ácido úrico, passo final na degradação de purinas (que inclui a adenosina) [Day et al., 2007]. Com isso, acumulam-se hipoxantina e xantina e principalmente seus respectivos substratos, adenosina e guanosina, e há redução do produto final ácido úrico, sendo por isso usado para o tratamento da hiperuricemia [Day et al., 2007]. Portanto, o alopurinol trata-se de um fármaco comercialmente distribuído, com

perfil de segurança comprovado, e que pode ser um novo alvo de tratamento adjuvante a pacientes com dor crônica, pois causa aumento nos níveis plasmáticos e liquóricos de guanosina e adenosina, importantes neuromoduladores com potencial antinociceptivo [Marro et al., 2006]. Esta foi uma das abordagens adotadas na tese para demonstrar a capacidade do sistema purinérgico em modular a transmissão dolorosa e será descrita em detalhes posteriormente.

I.1.j. Efeitos das purinas derivadas da guanina sobre o SNC

Apesar dos derivados do ATP e da adenosina serem considerados os principais efetores do sistema purinérgico em nível extracelular, mais recentemente os derivados da guanina, principalmente os nucleotídeos GTP e GMP e o nucleosídeo guanosina, têm demonstrado diversos efeitos biológicos extracelulares, incluindo efeitos tróficos em células neurais [Ciccarelli et al., 2001; Rathbone et al., 2008] e antagonismo do sistema glutamatérgico [Baron et al., 1989; Souza e Ramirez, 1991; Burgos et al., 1998; Schmidt et al., 2007]. Nos últimos anos, vem sendo construído um corpo de evidências caracterizando uma atividade antiglutamatérgica dos derivados da guanina, a qual não é diretamente relacionada à modulação de proteínas-G que estes compostos sabidamente exercem [Baron et al., 1989; Souza e Ramirez, 1991].

Os derivados da guanina inibem a ligação de glutamato e seus análogos [Baron et al., 1989], previnem respostas celulares aos aminoácidos excitatórios [Souza e Ramirez, 1991], apresentam efeitos neuroprotetores em cultura de neurônios submetidos à hipóxia e causam aumento da captação de glutamato em culturas de astrócitos e fatiais cerebrais [Frizzo et al., 2001;2002;2003;2005]. Também apresentam efeitos anticonvulsivantes contra agonistas

glutamatérgicos em roedores [Schmidt et al., 2000]. Em particular, foi demonstrado que a guanosina extracelular é capaz de prevenir as convulsões induzidas por compostos que causam hiperestimulação do sistema glutamatérgico, como o ácido quinolínico e o composto α-dendrotoxina [Lara et al., 2001; Schmidt et al., 2000; Vinade et al., 2003]. A guanosina ainda demonstrou possuir efeito neuroprotetor em eventos convulsivos e hipóxicos em animais adultos e jovens [Lara et al., 2001; Frizzo et al., 2002; Soares et al., 2004, Oliveira et al., 2004] e efeitos amnésicos em diversos testes de memória, como a esquiva inibitória [Roesler et al., 2000; Vinadé et al., 2003;2004;2005].

Os diversos efeitos dos derivados da guanina, em especial do nucleosídeo guanosina, parecem ser mediados pela estimulação do transporte do glutamato em células gliais (astrócitos), conforme observado em fatias de cérebro e em cultura de astrócitos [Frizzo et al., 2001;2002;2003;2005]. Baseado nesta propriedade antiglutamatérgica da guanosina, e no exposto nas seções anteriores a respeito do papel do sistema glutamatérgico na transmissão e manutenção da dor, esta tese estudou a ação dos derivados da guanina, em especial da guanosina, sobre diversos modelos de dor em animais.

I.2. OBJETIVOS E ESTRUTURA DA TESE

Os trabalhos realizados na presente tese de doutorado foram divididos em três conjuntos: uma revisão, os experimentais com animais e os trabalhos com humanos. Os objetivos gerais e específicos de cada uma destas partes estão expostos a seguir. A importância e o embasamento destes objetivos podem ser encontrados no capítulo de introdução desta tese.

I.2.a. ESTUDO DE REVISÃO SOBRE AS PURINAS DERIVADAS DA GUANINA

Objetivo geral: propor um sistema purinérgico especificamente relacionado aos derivados da guanina com relevância fisiológica e fisiopatológica.

Objetivos específicos:

- revisar sucintamente o papel do sistema purinérgico no SNC.
- revisar sucintamente os efeitos dos derivados da adenina no SNC.
- revisar e discutir os efeitos neuroprotetores dos derivados da guanina.
- revisar e discutir sobre o mecanismo de ação dos derivados da guanina.

I.2.a. TRABALHOS EXPERIMENTAIS COM MODELOS ANIMAIS

Objetivo geral: investigar os potenciais efeitos antinociceptivos dos derivados da guanina em modelos animais de dor.

Objetivos específicos:

- investigar os mecanismos envolvidos na atividade antinociceptiva dos derivados da guanina.

- investigar a presença e a função dos integrantes do sistema purinérgico no líquido cefalorraquidiano de animais.

- investigar a interação entre os sistemas purinérgico e glutamatérgico na transmissão da dor.

- investigar os efeitos antinociceptivos de análogos de purinas em animais.

- investigar a capacidade de modulação *in vivo* do sistema purinérgico endógeno em animais pela dor e pelo exercício físico.

- propor um novo modelo para avaliação da captação de glutamato em fatias de medula espinhal.

I.2.b. TRABALHOS EXPERIMENTAIS COM MODELOS HUMANOS

Objetivo geral: investigar a correlação entre os níveis de purinas no SNC e os níveis de dor em humanos.

Objetivos específicos:

- avaliar a presença de integrantes do sistema purinérgico no líquido cefalorraquidiano de humanos.

- investigar a modulação *in vivo* dos integrantes do sistema purinérgico no líquido cefalorraquidiano de humanos com dor aguda ou crônica.

PARTE II

Onde os resultados são apresentados.

II.1. ESTUDO DE REVISÃO SOBRE AS PURINAS DERIVADAS DA GUANINA

II.1.a. Proposal of a guanine-based purinergic system in the mammalian central nervous system

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Proposal of a guanine-based purinergic system in the mammalian central nervous system

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Abstract

Guanine-based purines have been traditionally studied as modulators of intracellular processes, mainly G-protein activity. However, they also exert several extracellular effects not related to G proteins, including modulation of glutamatergic activity, trophic effects on neural cells, and behavioral effects. In this article, the putative roles of guanine-based purines on the nervous system are reviewed, and we propose a specific guanine-based purinergic system in addition to the well-characterized adenine-based purinergic system. Current evidence suggest that guanine-based purines modulate glutamatergic parameters, such as glutamate uptake by astrocytes and synaptic vesicles, seizures induced by glutamatergic agents, response to ischemia and excitotoxicity, and are able to affect learning, memory and anxiety. Additionally, guanine-based purines have important trophic functions affecting the development, structure, or maintenance of neural cells. Although studies addressing the mechanism of action (receptors and second messenger systems) of guanine-based purines are still insufficient, these findings point to the guanine-based purines (nucleotides and guanosine) as potential new targets for neuroprotection and neuromodulation.

Keywords: Guanine-based purines; Adenine-based purines; Guanosine; Purinergic neurotransmission; Neuroprotection; Glutamatergic excitotoxicity

Abbreviations: ADA, adenosine deaminase; ADP, adenosine 5′-diphoshate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; cAMP, adenosine 3′,5′ cyclic monophosphate; cGMP, guanosine 3′,5′ cyclic monophosphate; CNS, central nervous system; CSF, cerebrospinal fluid; DNA, deoxyribonucleic acid; ERK, extracellular regulated kinases; GDP, guanosine 5′-diphosphate; GMP, guanosine 5′-monophosphate; GTP, guanosine 5′-triphosphate; KA, kainic acid; MK-801, dizocilpine; NGF, neural growth factor; NMDA, *N*-methyl-D-aspartate.

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1. Introduction

The purinergic system usually relates to the adenine-based purines, including the nucleotides adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), and adenosine 5′-monophoshate (AMP) and the nucleoside adenosine. Adenine-based purines exert several biological roles, including the pivotal role on energy metabolism. However, guanine-based purines, namely the nucleotides guanosine 5′-triphosphate (GTP), guanosine 5′-diphosphate (GDP), and guanosine 5′-monophosphate (GMP) and the nucleoside guanosine can also be considered part of the purinergic system. Finally, the metabolites xanthine, hypoxanthine, uric acid, the nucleoside inosine, as well as receptors, transporters, and enzymes complete the purinergic system.

Traditionally, guanine-based purines have been studied as modulators of intracellular processes, especially regarding the activity of G proteins for signal transduction. Nonetheless, guanine-based purines have been shown to exert extracellular effects not related to their direct modulation of G proteins, including in vitro (Baron et al., 1989; Burgos et al., 1998, 2000a, 2000b, 2000c; Paz et al., 1994; Ramos et al., 1997; Souza & Ramirez, 1991; Tasca et al., 2005) and in vivo (Lara et al., 2001; Schmidt et al., 2000) modulation of the glutamatergic activity, behavioral effects (Roesler et al., 2000; Vinadé et al., 2005), and trophic effects on neural cells (Ciccarelli et al., 2001).

In this article, the putative roles of guanine-based purines in the nervous system are reviewed, with emphasis on their extracellular effects with potential role in neuroprotection. Similarly to the well-characterized adenine-based purinergic system, we propose a specific guanine-based purinergic system with relevant physiological and pathological implications.

1.1. Historical overview of the purinergic system

Purine bases, such as adenine and guanine, and their corresponding nucleosides and nucleotides are ubiquitous molecules found within and outside the cells of animals and plants. Among their several important biological roles, purine bases and their pyrimidine counterparts (thymine, cytosine, and uracil) are the building blocks of DNA and RNA. Purine nucleotides, mainly ATP, are involved in biochemical pathways and energy transfer within the cell. Moreover, cyclic nucleotides, such as adenosine 3′,5′-cylic monophosphate (cAMP) and guanosine 3′,5′-cylic monophosphate (cGMP) act as intracellular second messenger molecules during signal transduction (Barnstable et al., 2004; Bourne et al., 1990).

The molecule of ATP was discovered in 1929 (Fiske & SubbaRow, 1929; Lohman, 1929), and shortly the role of ATP as a universal source of chemical energy in biological systems was fully appreciated (Lippman, 1941). Although purines had been traditionally viewed as having mostly intracellular roles, it was discovered that extracellular adenosine is released by the heart during ischemia, triggers negative chronotropic effect on the heart, mediates dilatation of coronary vessels, and inhibits intestinal smooth muscle (Drury & Szent-Györgyi, 1929). It also became apparent that ATP was responsible for many purine-mediated physiological reactions (Drury, 1936).

Thirty years later, it was observed that ATP could be released from nerves upon stimulation (Holton, 1959). Through a firefly luminescence method for ATP detection, electrical stimulation of the rabbit great auricular nerve resulted in a transient elevation of extracellular ATP, establishing a foundation for the theory of purinergic neurotransmission.

In 1970, the first direct evidence that ATP may act as a transmitter in “nonadrenergic, noncholinergic” nerves in the gut and bladder was presented (Burnstock et al., 1970), and the concept of “purinergic nerves” and “purinergic neurotransmission” was introduced (Burnstock, 1972). Subsequently, Burnstock developed the concept of purinergic transmission in the peripheral nervous system, demonstrating that ATP fully conforms to the criteria for the definition of a neurotransmitter: (i) ATP is synthesized and stored in presynaptic terminals; (ii) ATP is released upon nerve stimulation; (iii) extracellular ATP can be rapidly degraded by coenzymes; and (iv) pharmacological agents that inhibited the effects of endogenous ATP also suppressed the effects of nerve stimulation. Finally, Burnstock postulated that ATP might be coreleased with other neurotransmitters, such as noradrenaline or acetylcholine (Burnstock, 1972).

Resistance to this concept remained for many years because ATP was recognized firstly for its intracellular roles in many biochemical processes, such as intracellular energy source, linking various metabolic cycles, and the intuitive feeling was that such a ubiquitous and simple compound was unlikely to be an extracellular messenger, although powerful extracellular enzymes involved in its breakdown had already been characterized (Burnstock, 2006a, 2006b).

The concept was eventually accepted and expanded, as purines are also important extracellular messengers to non-neuronal cells (Burnstock & Knight, 2004). Over the last 30 years, the roles of adenine-based purines, mainly the nucleoside adenosine and the nucleotide ATP, as neurotransmitters and neuromodulators in the central and peripheral nervous systems have been extensively elucidated (Burnstock, 2006a, 2006b). For this reason, the nucleotide ATP and the

nucleoside adenosine are usually considered the main effectors of the purinergic system (Ralevic & Burnstock, 1998).

Perhaps a similar story has been taking place with the extracellular roles of guanine-based purines, which may have been overshadowed by their well-known modulation of G protein-mediated signal transduction and by the abundance of information on adenine-based purines.

We will briefly review the adenine-based purinergic system in the nervous system, as it has been an obvious model for this putative guanine-based purinergic system.

1.2. Adenine-based purines

The role of ATP as an excitatory neurotransmitter, both centrally and in the periphery, is now well documented and accepted (Burnstock, 2006a, 2006b; Ralevic & Burnstock, 1998). ATP is stored in and released from neuronal presynaptic terminals, acting via specific P_2 receptors, described in detail elsewhere (Abbracchio & Burnstock, 1994; Ralevic & Burnstock, 1998; Burnstock, 2007).

The neuromodulatory effects and sources of adenosine have been well characterized. Extracellular adenosine is enzymatically formed from extracellular nucleotides or comes from the release of intracellular adenosine (Brundege & Dunwiddie, 1997). Intracellular adenosine is formed from the cleavage of *S*-adenosylhomocysteine by *S*-adenosylhomocysteine hydrolase or from the metabolization of 5′-AMP by an intracellular 5′-nucleotidase and can diffuse through bidirectional nucleoside transporters to the extracellular space. Released ATP and cAMP are sources for the production of AMP via ectonucleotidases or ectophosphodiesterase, respectively, and finally, an extracellular ecto-5′-nucleotidase hydrolyses AMP to adenosine (see Fig. 1). Extracellular adenosine can be taken up through nucleoside transporters and phosphorylated to AMP by adenosine kinase or deaminated to inosine by adenosine deaminase (ADA). These processes are mostly intracellular, but ADA is also associated with cell membranes (Brundege & Dunwiddie, 1997; Ralevic & Burnstock, 1998).

Brain extracellular adenosine and ATP act not only as neurotransmitters and neuromodulators, but also as trophic factors involved in plastic processes, such as memory and learning, collateral sprouting of nerve processes, neuroprotection against noxious stimuli, and regulation of cell number through induction of apoptosis (programmed cell death; Ciccarelli et al., 1999a, 2001).

1.3. Receptors for adenine-based purines

Signaling via extracellular adenine-based purines is very complex (see Fig. 2). An extensive review of the purinergic receptors is found elsewhere (Burnstock, 2007; Zimmermann, 2006a, 2006b). In 1978, 2 types of purinoceptors, identified as P_1 (for adenosine) and P_2 (for ATP/ADP) were proposed (Burnstock, 1978). Simultaneously, 2 subtypes of the P_1 receptors were firstly recognized (Londos et al., 1980; Van Calker et al., 1979), but it was not until 1985 that a proposal suggesting a pharmacological basis for distinguishing 2 types of P_2 receptors (P_2X and P_2Y) was made (Burnstock & Kennedy, 1985). Later, 2 further P_2 receptor subtypes were proposed, P_2T on platelets and P_2Z on macrophages, and receptors that

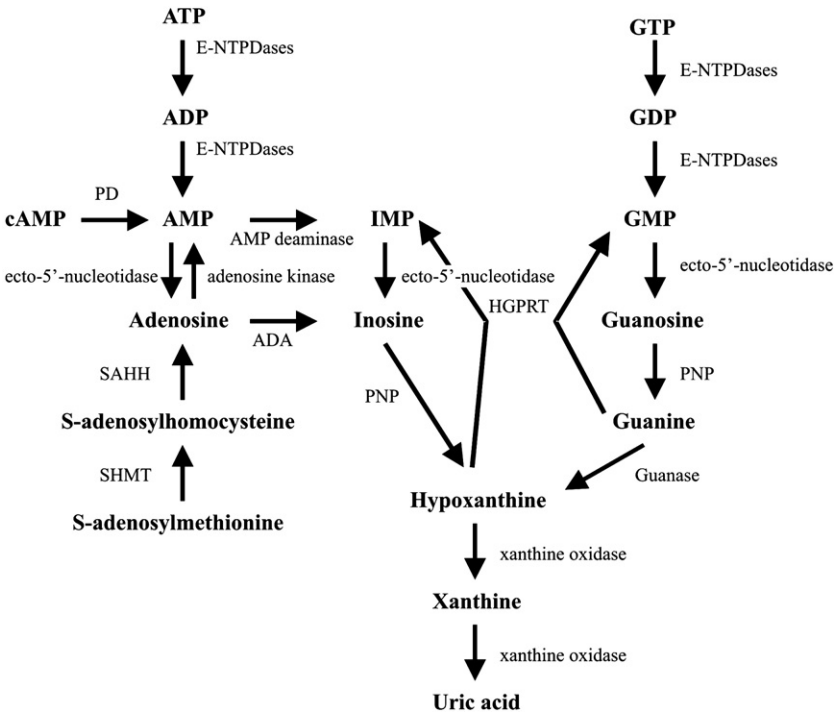

Fig. 1. Schematic model of the sources of extracellular adenine- and guanine-based purines. E-NTPDases, ectonucleotide-diphosphohydrolase; ADA, adenosine deaminase; SHMT, serine hydroxymethyltransferase; SAHH, *S*-adenosylhomocysteine hydrolase; PD, ectophosphodiesterase; PNP, purine nucleoside phosphorylase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase.

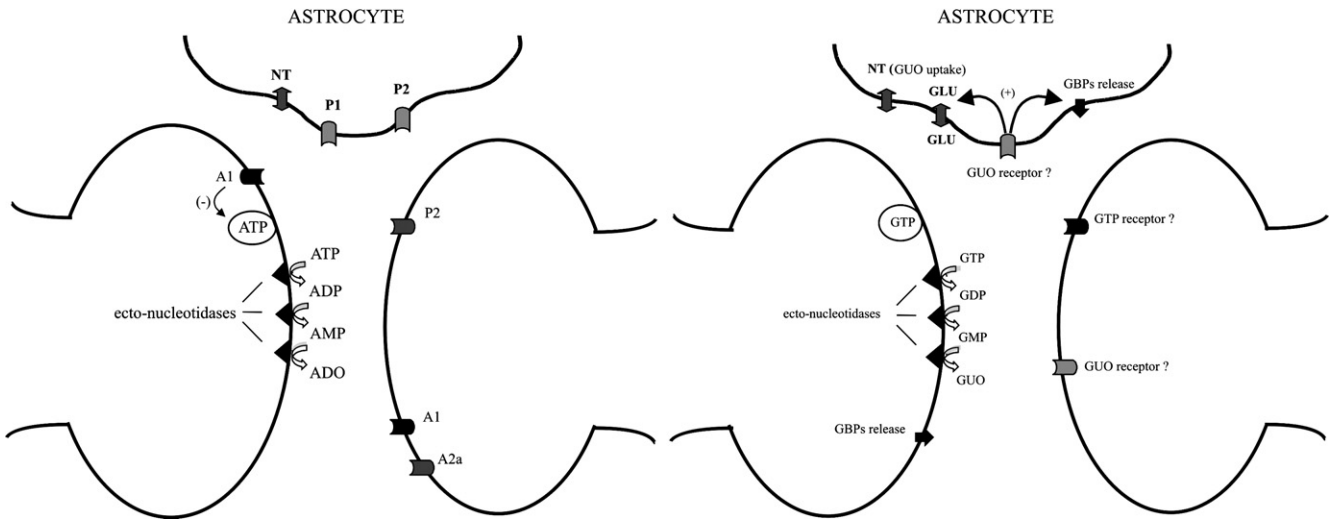

Fig. 2. Schematic representation of synapses for (**A**) adenine- and (**B**) guanine-based purinergic systems. Modulation of the glutamatergic system: NT, nucleoside transporters; GUO, guanosine; GLU, glutamate. GBP, guanine-based purines.

responded to pyrimidines and to purines were named P_2U receptors (Gordon, 1986). In 1993, the first G protein-coupled P_2 receptors were cloned (Lustig et al., 1993; Webb et al., 1993), and 1 year later 2 ion-gated receptors were also cloned (Brake et al., 1994; Valera et al., 1994).

Concerning ATP receptors, on the basis of molecular structure and transduction mechanisms, it was proposed that P_2 should belong to 2 major families: a P_2X family of ligand-gated ion channel receptors and a P_2Y family of G protein-coupled receptors (Abbracchio & Burnstock, 1994). This nomenclature has been widely accepted, and currently 7 P_2X subtypes and 8 P_2Y receptor subtypes are recognized, including receptors that are sensitive to pyrimidines as well as purines (Burnstock, 2007). P_2X receptors mediate the flow of Ca^{2+}, Na^{+}, and K^{+}, whereas P_2Y metabotropic receptors, via G proteins, activate second messenger systems, such as phospholipase C (PLC) and phospholipase A_2 (PLA_2). ADP and pyrimidine nucleotides (UTP and UDP) also activate some subtypes of P_2 receptors.

Concerning adenosine receptors, to date 4 different adenosine P_1 receptor subtypes, which are classified as A_1, A_{2A}, A_{2B} and A_3 have been cloned and characterized (Brundege & Dunwiddie, 1997; Cunha, 2005; Fredholm et al., 2001, 2005; Ralevic & Burnstock, 1998). A_1 and A_3 receptors inhibit, whereas A_{2a} and A_{2b} receptors stimulate, adenylate cyclase. Both A_1 and A_3 receptors also increase inositol-3-phosphate (IP_3) formation. The human A_{2B} receptor has also been found to regulate PLC activity (Burnstock, 2007). Adenosine A_1 receptors are widely distributed in the central nervous system (CNS) and have been shown to decrease neuronal excitability and synaptic activity and to inhibit the release of several neurotransmitters, such as glutamate, dopamine, serotonin, noradrenaline, and acetylcholine. A_{2A} receptors are concentrated in dopamine-rich areas, modulating dopaminergic activity, but are also present in the hippocampus and cerebral cortex. A_{2B} receptors are less well characterized and have been suggested to interact with inflammatory mediators. Similarly, A_3 receptors have also been related to inflammation, especially in lungs (see Burnstock, 2007, for review). A number of P_1 subtype-selective agonists and antagonists have been identified. All of the known P_1 receptor agonists are closely related to adenosine in structure. Methylxanthines, such as caffeine and theophylline, are the classical nonselective A_1–A_2 adenosine antagonists.

2. Guanine-based purinergic system

2.1. Historical overview

In 1971, the first evidence for a more complex class of signaling pathway emerged, establishing that the sensor and intracellular effector are separate proteins that communicate through proteins called guanine nucleotide-dependent regulatory proteins, GTP binding proteins, or G proteins (Rodbell et al., 1971). G proteins alternate between inactive GDP-bound and active GTP-bound forms. Activation is catalyzed by receptors and deactivation by an intrinsic property of G proteins, its GTPase activity. G proteins couple cell surface receptors to cellular effectors, modulating cell responses to external stimuli: the interaction of agonists with their receptors triggers the binding of GTP to G proteins, forming an active complex G protein/GTP, which simultaneously modulates the activity of effector systems and decreases the agonist binding to specific receptors (Gudermann et al., 1997; Johnston & Siderovski, 2007; Taylor, 1990).

Recently, guanine-based purines, including the nucleotides GTP, GDP, and GMP, and the nucleoside guanosine have also been shown to exert extracellular effects, based upon studies that could be subdivided into 3 approaches: (i) inhibitory effects on the activity of the glutamatergic system in physiological and pathological conditions; (ii) effects on memory and behavior; (iii) trophic effects on neural cells. Before reviewing these aspects, we will summarize the findings regarding the characterization of the main constituents of the guanine-based purinergic system.

2.2. Metabolism, storage, and release of guanine-based purines

GTP may be stored in synaptic vesicles (Santos et al., 2006; Zimmermann, 1996) and indirect evidence indicated that guanosine could be released from synaptosomes (Fredholm & Vernet, 1979). Cultured astrocytes may release guanine-based purines (Ciccarelli et al., 1999b), a process that increased after hypoxia/hypoglycemia. Of note, the release of guanine-based purines was much greater than that of their adenine-based counterparts (Ciccarelli et al., 1999b). In cultured astrocytes, inhibition of ecto-5′-nucleotidase activity significantly reduced accumulation of extracellular guanosine, indicating that, like extracellular adenosine, it is to some extent derived from the extracellular metabolism of guanine nucleotides (Caciagli et al., 2000).

The presence of adenine- and guanine-based purines and their metabolites in human and animal cerebrospinal fluid (CSF) has been described (Castro-Gago et al., 1992; Regner et al., 1997). The enzymes involved in extracellular nucleotide hydrolysis include membrane-bound ectonucleotidases, ecto-nucleotidases released from membranes, and the naturally occurring soluble nucleotidases (see Fig. 1). These enzymes, in association with ecto-5′-nucleotidase, hydrolyze extracellular nucleotides in a stepwise fashion down to nucleosides and are crucial for physiological modulation of CNS functions, as well as for the purine-dependent neuroprotective activities against brain insults (Sebastião et al., 1999). These soluble nucleotidases are also present and active in rat CSF (Portela et al., 2002), wherein they hydrolyze all guanine and adenine nucleotides with the following order of catalytic efficiency: GDP>ADP=ATP=GTP>AMP=GMP. Interestingly, at high concentrations, GDP hydrolysis rate is greater than that of ADP, perhaps favoring the accumulation of GMP and consequently guanosine. In fact, these enzymes can be released to the extracellular space (CSF) from choroid plexus, endothelial cells or even microglia (Zimmermann, 2001, 2006a, 2007b) and play an important regulatory role of the purinergic system under physiological and pathological conditions.

Astrocytes, the main source of cerebral purines (Ciccarelli et al., 1999b), are involved in multiple brain functions in physiological conditions, participating in neuronal development, synaptic activity, homeostatic control of the extracellular environment, and also in processes related to brain injuries, by arresting and repairing further brain damage (Chen & Swanson, 2003). Astrocytes, as well as neurons, are also responsible for both nucleoside metabolism and uptake of adenosine and guanosine (Parkinson et al., 2005). Uptake of purine and pyrimidine nucleosides by astrocytes is also important for nucleic acid synthesis and synthesis of AMP, ADP, and ATP from adenosine and GTP from guanosine (Rathbone et al., 1999b). A recent study (Peng et al., 2005) identified 2 equilibrative nucleoside transporters in astrocytes (ENT_1 and ENT_2), together with the concentrative nucleoside transporter (CNT_2) responsible for nucleoside uptake (see Fig. 2).

In regard to guanosine bioavailability, animals treated with oral guanosine presented a 2-fold increase in CSF concentration of guanosine as compared to vehicle group (Vinadé et al., 2005). We also observed that i.p. administration of GMP in anticonvulsant doses produced a 3-fold increase of cerebrospinal fluid levels of guanosine in rats, not affecting GMP levels (Soares et al., 2004).

2.3. Modulation of the glutamatergic system: Neuroprotective effects of guanine-based purines

It has been classically demonstrated that by acting via G proteins, GTP is able to simultaneously inhibit binding of neurotransmitters (and their agonists) to metabotropic receptors and modulate adenylate cyclase activity (Gudermann et al., 1997). However, we demonstrated that the effects of guanine nucleotides on kainic acid (a glutamatergic ligand to receptors not coupled to G proteins) binding site and on adenylate cyclase activity could be dissociated (Souza & Ramirez, 1991). In lysed membrane preparations (G proteins and receptors exposed to the incubation medium) from chicken brain, the guanine nucleotides GMP (which does not bind to G proteins), GDP and GTP were able to inhibit the binding of kainic acid with the same efficiency, whereas only GTP was able to stimulate adenylate cyclase. However, in vesicular preparations (G proteins not exposed to the incubation medium), all guanine nucleotides were still able to inhibit binding of kainic acid, whereas GTP lost the ability to stimulate adenylate cyclase activity. These findings strongly suggested that the inhibition of kainic acid binding by guanine nucleotides was not dependent on a G protein-mediated system. This result corroborated studies from other groups, which had previously shown that the inhibitory effects of guanine nucleotides on the binding of glutamate or ionotropic glutamatergic ligands presented several inconsistencies, when compared with studies on receptors known to be coupled to their second messengers through a G protein (Baron et al., 1989; Butcher et al., 1986; Hood et al., 1990; Monahan et al., 1988; Paas et al. 1996; Sharif & Roberts, 1981). Subsequent studies from our group supported the hypothesis that guanine nucleotides could antagonize the glutamatergic transmission by acting at extracellular sites located on the membrane surface. By using a poorly hydrolyzed GTP analogue (GMP-PNP) we were able to observe some distinctions between 2 groups of binding sites for guanine-based purines, such as stability to washing procedures of the intracellular (G proteins) but not of the extracellular (receptors) [^{3}H]GMP-PNP binding. Dealing separately with each of these 2 groups of sites, we were able to discriminate some properties of extracellular and intracellular guanine-based purine binding sites in rat CNS (Paz et al., 1994; Ramos et al., 1997; Rotta et al., 2004; Rubin et al., 1997). This receptor antagonism was shown to be competitive in moderate to high micromolar concentrations of guanine nucleotides (Baron et al., 1989). In all these studies, the nucleoside guanosine had no (or very little) effect on the binding of glutamate and analogs to glutamate receptors (Porciuncula et al., 2002; Souza & Ramirez, 1991).

Searching for a relevance of the inhibitory action of extracellular guanine nucleotides on glutamate binding, our

group and Ramirez's group investigated their putative effects on neural cell responses to glutamate and/or analogs (Aleu et al., 1999; Burgos et al., 1998; Burgos et al., 2000a, 2000b, 2000c; Burgos et al., 2003; Paz et al., 1994; Regner et al., 1998; Rubin et al., 1997; Tasca et al., 1995; Tasca et al., 1998; Tasca et al., 1999; Tasca & Souza, 2000). It was observed that guanine nucleotides inhibited glutamate-stimulated GFAP (astrocytic protein) phosphorylation (Tasca et al., 1995), glutamate (and analogs)-induced increase in intracellular cAMP levels (Tasca et al., 1998), glutamate-induced luminescence (Regner et al., 1998), kainate-stimulated LDH release (Burgos et al., 1998), kainate-activated currents (Aleu et al., 1999; Burgos et al., 2003), and kainate-stimulated increase in Ca^{2+} influx (Burgos et al., 2000a, 2000b, 2000c). Since most excitatory synapses in the CNS have glutamate as neurotransmitter, which participates in several physiological and pathological processes, the potential modulatory action of guanine nucleotides on the glutamatergic neurotransmission claimed attention to new investigations on their extracellular effects.

Guanine nucleotides administered intracerebroventricularly had long been shown (Baron et al., 1989) to prevent seizures induced by quinolinic acid, a toxin that overstimulates the glutamatergic neurotransmission (Stone, 2001). This effect was compatible with the antagonistic properties of guanine nucleotides on glutamate receptors being studied in our group. However, after further exploring the interaction of guanine nucleotides with glutamate, we observed that intraperitonial administration of not only GMP, but also guanosine (that does not inhibit the binding of glutamatergic ligands), was able to prevent seizures induced by quinolinic acid (Schmidt et al., 2000). Later we showed that oral administration of guanosine was also effective in this model (Lara et al., 2001). Additional studies also provided evidence that guanosine and GMP administered intracerebroventricularly (i.c.v.), intraperitonially or orally protected against seizures induced by the glutamatergic agents quinolinic acid, kainate and α-dendrotoxin in adult and young rodents (Oliveira et al., 2004; Schmidt et al., 2005; Soares et al., 2004; Vinadé et al., 2003; Vinadé et al., 2005). Acute i.c.v. administration of the nucleotides GTP and GDP was also protective against seizures induced by quinolinic acid in mice (Schmidt et al., 2005). Chronically, GMP had also been shown to be neuroprotective against quinolinic acid-induced striatal neuronal cell death in rats (Malcon et al., 1997).

Guanine-based purines, mainly GMP and guanosine, have usually presented similar neuroprotective profile in several in vivo and in vitro protocols (Lara et al., 2001; Schmidt et al., 2000; Schmidt et al., 2005; Vinadé et al., 2003; Vinade et al., 2005). However, most effects of nucleotides (mainly GMP) seemed to be due to their conversion to guanosine. Specifically for seizures, an acute i.c.v. administration of the ecto-5′-nucleotidase (enzyme that converts GMP to guanosine) inhibitor α,β-methyleneadenosine 5′-diphosphate (AOPCP) prevented the anticonvulsant effects of GMP against quinolinic acid in rats, without affecting the effect of guanosine (Soares et al., 2004). Moreover, we demonstrated that anticonvulsant effects of i.c.v. GTP and GDP seemed to be mediated by their conversion to guanosine, since their poorly hydrolysable analogs GTPγS, GppNHp, and GDPβS were not capable of preventing seizures induced by quinolinic acid in mice (Schmidt et al., 2005).

Taken together, as guanosine does not exert glutamate receptor antagonism, and guanine-purines seemed to be effective after conversion to guanosine, the hypothesis of direct receptor interaction as the mechanism of neuroprotective action of guanine-based purines was weakened, although this issue (direct interaction with glutamatergic receptors) deserves further investigation (Mendieta et al., 2001, 2005).

2.4. Astrocytic glutamate uptake and guanine-based purines

Astrocytic glutamate uptake is a crucial process for the maintenance of extracellular glutamate concentrations below toxic levels in physiological conditions and under brain stress, thus supporting synapse homeostasis (glutamate–glutamine cycle; Anderson & Swanson, 2000; Chen & Swanson, 2003; Matute et al., 2006; Schousboe & Waagepetersen, 2005). In the search for other mechanisms of action of extracellular guanine-based purines than antagonism of glutamatergic receptors, we found that guanosine was able to increase the glutamate uptake by cultured astrocytes and brain slices (Frizzo et al., 2001, 2002, 2003). In basal or physiological conditions, the effects of guanosine on glutamate uptake in brain slices seemed to be age (more in young animals)- and structure (more in cortex)-dependent but, in excitotoxic conditions, guanosine was more broadly involved in modulating glutamate uptake (Gottfried et al., 2002; Frizzo et al., 2005; Thomazi et al., 2004). In cultures of primary astrocyte from cortices of 1-day-old Wistar rats and in adult rat brain cortical slices, guanosine was shown to increase the sodium-dependent uptake of glutamate in a dose-dependent manner (Frizzo et al., 2001). Depending on the preparation, the minimum effective concentration of guanosine was 100 nM to 1 μM. The maximal stimulation of uptake by guanosine was around 80% over control values. Importantly, adenosine affected neither the basal uptake nor the stimulatory effect of guanosine. Theophylline, a nonspecific P_1 (A_1/A_{2A}) adenosine receptor antagonist, stimulated basal uptake of glutamate without affecting the stimulatory effect of guanosine. Finally, dipyridamole, a nucleoside transport inhibitor, also stimulated basal uptake, and this stimulatory effect was additive with that of guanosine. Thus, these data suggest that the guanosine stimulatory effect on astrocytic uptake of glutamate is exerted from the extracellular side and is independent of adenosine and its receptors (Frizzo et al., 2001).

GMP and GTP mimicked the stimulatory effect of guanosine on glutamate uptake by astrocytic cells in culture (Frizzo et al., 2003). However, a significant additive effect on uptake was not observed with the simultaneous addition of guanosine, GMP and GTP to the culture medium, compared with the effect of each compound alone. These data were consistent with the possibility that only one compound was mediating the stimulatory effect on uptake or the 3 compounds were metabolically

interconvertible with each other. Importantly, a poorly hydrolysable analogue of GTP (GMP-PNP) was not able to stimulate the uptake of glutamate by cultured astrocytes and the effect of GMP was abolished when cultures were pretreated with AOPCP. Finally, guanosine failed to affect the astrocytic uptake of GABA. Therefore, guanosine seems to be mediator of the stimulatory effect of guanine-based purines on the astrocytic uptake of glutamate, and this process was independent of adenosine and relatively specific for glutamate (Frizzo et al., 2003). As astrocytic uptake of glutamate is the most important mechanism for terminating its actions within the synapse, the stimulation of uptake by guanosine may be a relevant process in regulating glutamatergic neurotransmission, especially under excitotoxic conditions (Chen & Swanson, 2003; Duan et al., 1999; Matute et al., 2006; Schousboe & Waagepetersen, 2005).

Oral administration of guanosine prevented the decrease of glutamate uptake by brain slices of rats submitted to quinolinic acid-induced seizures (Oliveira et al., 2004; Vinadé et al., 2005). Additionally, a recent study demonstrated that guanosine prevented the decrease of glutamate uptake by hippocampal slices of neonatal rats exposed to a hypoxic–ischemic insult in vivo (Moretto et al., 2005). Moreover, we demonstrated that in vitro and in vivo quinolinic acid stimulated synaptosomal glutamate release and inhibited glutamate uptake by astrocytes, which could induce an increase in extracellular glutamate levels and consequently seizure behavior (Tavares et al., 2000, 2002). However, this neurochemical effect was prevented by in vivo pretreatment with systemic guanosine or GMP (Tavares et al., 2005). Quinolinic acid also stimulates glutamate uptake by synaptic vesicles, an effect prevented by glutamate antagonists and the guanine-based purines guanosine and GMP (unpublished results).

Interestingly, we found that GTP, GDP, GMP, and guanosine inhibited glutamate uptake by synaptic vesicles in vitro (Tasca et al., 2004), pointing to an intracellular interaction between guanine-based purines and the glutamatergic neurotransmission. The physiological significance of this intracellular effect remains to be clarified.

2.5. In vitro neuroprotection by guanine-based purines

Several studies have indicated that guanosine may be a neuroprotective endogenous compound released under excitotoxic conditions, preventing further toxicity to neurons. Both neuronal and astrocytic cell cultures are able to release guanosine and adenosine under basal or toxic (ischemic) conditions (Ciccarelli et al., 1999b; Ciccarelli et al., 2001; Dobolyi et al., 2000) and kainate stimulates the release of guanosine (Dobolyi et al., 2000). Interestingly, 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 (Caciagli et al., 2000).

In slices submitted to glucose deprivation, GMP prevented LDH leakage and the loss of cell viability induced by glutamate (Molz et al., 2005). In slices submitted to ischemic conditions, GMP partially prevented the decrease in cell viability induced by glucose and oxygen deprivation and the addition of kainate (Oliveira et al., 2002). However, in these studies the possible role of guanosine in mediating the effects of GMP was not addressed.

2.6. Behavioral effects of guanine-based purines

It has been well demonstrated for several glutamate antagonists, mainly NMDA receptor antagonists, that they may induce amnesia and severe locomotor deficits in animals (Chen & Lipton, 2006). It is well documented that glutamate plays a key role on memory mechanisms (Izquierdo et al., 2006), and previous studies demonstrated that GMP was able to reverse the facilitatory effect of posttraining intrahippocampal glutamate administration on inhibitory avoidance task performance in rats (Rubin et al., 1996). Further studies demonstrated that GMP and guanosine are capable to modulate memory processes since pretraining administration of both guanine-based purines impaired retention of inhibitory avoidance responses in rats (Roesler et al., 2000). Treated animals, when retrained 1 week later, showed normal learning ability and guanosine administration immediately after training or pretest had no effect (Roesler et al., 2000). The guanine-based purine effects on memory were reproduced with anticonvulsant doses after acute/chronic intraperitonial/oral administration and adenosine-receptor antagonists failed to prevent these effects (Vinadé et al., 2003, 2004). Furthermore, the amnesic effect related to the pretreatment with GMP also depended on its conversion to guanosine (Saute et al., 2006). These findings suggest an amnesic effect of guanosine on inhibitory avoidance in rodents, in a pattern compatible with inhibition of glutamatergic activity and independent of adenosine A_1 and A_{2A} receptors.

NMDA receptor antagonists induce locomotor effects caused at least in part by a paradoxical increase in glutamate release (Adams & Moghaddam, 2001). Interestingly, guanosine produced a ~60% attenuation of hyperlocomotion induced by dizocilpine (MK-801, a NMDA-receptor antagonist), whereas it did not affect the hyperlocomotion induced by the indirect dopamine agonist amphetamine or by the nonselective adenosine-receptor antagonist caffeine (Tort et al., 2004). However, most studies indicate that guanosine per se did not affect spontaneous locomotion rodents after systemic administration (Lara et al., 2001; Tort et al., 2004; Vinadé et al., 2003). Additionally, no obvious motor disturbance or sedative effects were observed since acute or chronic administration of guanine-based purines did not alter rotarod and open field performance, as evidenced with other glutamate antagonists such as MK-801 (Lara et al., 2001; Vinadé et al., 2003).

The contribution of adenosine A_1 and A_{2a} receptors to the effects of guanosine has also been ruled out in behavioral studies. The adenosine antagonist caffeine failed to inhibit the anticonvulsant effect of an acute orally administration of guanosine on quinolinic acid-induced seizures in mice (Lara et al., 2001) or the amnesic effect of guanosine in rats (Roesler et al., 2000; Vinade et al., 2004).

2.7. Neurotrophic effects of guanine-based purines

In addition to their effects on neurotransmission, guanine-based purines also have important trophic functions, affecting the development, structure or maintenance of neural cells, as observed by Rathbone's group (Rathbone et al., 1999b). Some trophic effects of purines are mediated via purinergic cell surface receptors, whereas others require uptake of purines by the target cells (Rathbone et al., 1999b). Both extracellular guanosine and GTP, apparently through different mechanisms (i) have mitogenic effects promoting astroblast growth (Kim et al., 1991), (ii) are potent stimulators of in vitro axonal growth and proliferation of a wide range of cell types (Rathbone et al., 1992a, 1992b), (iii) can exert trophic effects on the nervous system (Rathbone et al., 1998, 1999b), including stimulation of astrocyte proliferation (Ciccarelli et al., 2000; Kim et al., 1991), synthesis and release of trophic factors such as immunoreactive nerve growth factor from astrocyte cultures (Caciagli et al., 2000; Middlemiss et al., 1995), and (iv) can enhance the differentiation of PC12 cells and hippocampal neurons in vitro (Gysbers & Rathbone, 1992, 1996). The role of GTP as a trophic mediator received strong support from data confirming that specific binding sites for GTP are present on the plasma membrane of neuronal-like PC12 cells (Gysbers & Rathbone, 1996; Gysbers et al., 2000; Guarnieri et al., 2004) and C2C12 mouse skeletal muscle cells (Pietrangelo et al., 2002) and that GTP is stored in synaptic vesicles (Santos et al., 2006; Zimmermann, 1996). The expression of GTP specific binding sites is directly correlated with the effects of this nucleotide in promoting neuronal differentiation (Gysbers et al., 2000). Extracellular GTP enhances the neuritogenic effects of nerve growth factor on PC12 cells, significantly increasing the proportion of cells that have neuritis (Gysbers & Rathbone, 1996; Gysbers et al., 2000; Guarnieri et al., 2004). Although some extracellular effects of GTP might be related to its conversion to guanosine, other findings indicate that a different mechanism of action between them may be present, as in the case of neurite outgrowth stimulation. For example, GTP transduction mechanisms in PC12 cells probably involve intracellular calcium mobilization and enhancement of NGF-induced extracellular regulated kinases (ERK). This mobilization, due to the activation of voltage-sensitive and ryanodine-sensitive calcium channels, as well as pertussis toxin-sensitive purinoceptors, modulated Ca^{2+}-activated K^+ channels not involved in activation of ERK (Guarnieri et al., 2004).

Guanosine has also been shown to stimulate the output of adenine-based purines from astrocytes and triggered these cells to proliferate and to produce large amounts of neuroprotective factors (Ciccarelli et al., 2001).

2.8. Receptors and second messenger systems associated with guanine-based purines

Astrocytes are important targets for purines since they express several P_1 and P_2 types receptor subtypes (Burnstock, 2006b; Ciccarelli et al., 2001; Neary et al., 1996). ATP (via P_2 receptors) and adenosine (via A_2 receptors) stimulate astrocyte proliferation, and adenosine (via A_1 and/or A_3 receptors) inhibits astrocyte proliferation, thus controlling the excessive reactive astrogliosis triggered by P_2 receptors. The activation of A_1 receptors also stimulates astrocytes to produce trophic factors, such as NGF, S100β protein, and transforming growth factor beta, which contribute to protect neurons against injuries. However, evidence for putative receptors or specific binding sites for either guanosine or GTP in astrocytes (Ciccarelli et al., 2001) or other cell types (Vuorinen et al., 1992) has been lacking, despite several documented biological effects of these compounds. Neither guanosine nor GTP binds with high-affinity to adenine-based purine receptors (Muller & Scior, 1993), suggesting that guanine-based purines had distinct cellular targets from adenine-based purines.

Some of the trophic actions of guanine-based purines may be indirect, occurring as a result of stimulating the synthesis and release of trophic factors and/or enhancing the effects of these specific trophic factors. Another possibility is that some actions of guanosine could be mediated intracellularly after its uptake. However, with respect to a specific neurotrophic role for guanosine, its extracellular levels remained elevated for up to a week after focal brain injury (Uemura et al., 1991). Additionally, many trophic effects of guanine-based purines were not affected by the nucleoside uptake inhibitors, such as NBTI or dipyridamole (Gysbers & Rathbone, 1992), indicating that they are triggered extracellularly. Guanosine also stimulated the release of adenine-based purines from astrocytes, which may, in turn, be responsible for some other effects of guanosine (Ciccarelli et al., 2000). Possibly, guanine-based purines would increase extracellular levels of adenine-based purines by interfering with their uptake and metabolism as well as by stimulating their release. For example, the ability of guanine-based purines to stimulate proliferation of rat brain microglia in a concentration-dependent manner appears to be mediated by specific purinergic receptors that recognize adenine-based purines (Ciccarelli et al., 2000). But this explanation is also incomplete, since many of the effects of guanine-based purines persist in the presence of P_1 and/or P_2 purine receptor antagonists (Frizzo et al., 2001; Gysbers & Rathbone, 1992; Tasca & Souza, 2000).

An alternative hypothesis is that there are distinct receptors for guanine-based purines. Moreover, several of the effects of guanosine may be mediated through G protein-dependent signaling pathways involving cyclic nucleotides or MAP kinase pathway (Caciagli et al., 2000; Gysbers & Rathbone, 1996), raising the possibility that some of the effects of guanine-based purines, particularly guanosine, involve activation of cell-surface receptors.

As commented above, there is data supporting the possible existence of specific binding sites for GTP in PC12 cells (Gysbers et al., 2000). In regard to guanosine, Traversa et al. showed that a specific binding site for guanosine was detected on membrane preparations from rat brain (Traversa et al., 2002, 2003). Kinetics of guanosine binding to membranes was described (Traversa et al., 2002) as a single high-affinity binding site for guanosine with a K_D of 95.4±11.9 nM and B_{max} of 0.57±0.03 pmol mg^{-1} protein. The order of potency

in displacing guanosine was: guanosine = 6-thio-guanosine > inosine > 6-thio-guanine > guanine. Other naturally occurring purines, such as adenosine, hypoxanthine, xanthine, caffeine, theophylline, GDP, GMP, and ATP were unable to significantly displace the radiolabelled guanosine. Thus, this binding site is distinct from the well-characterized receptors for adenine-based purines. The addition of GTP produced a small concentration-dependent decrease in guanosine binding, which could suggest that this site is linked to a G protein. These findings are consistent with the existence of a novel cell membrane receptor site(s), specific for guanosine. Through its specific binding site, guanosine may promote its extracellular effect by activating MAP kinase cascade in astrocytes (Traversa et al., 2002). However, genetic and biochemical characterization of this specific membrane site for guanosine, its related second messengers and mechanisms involved in guanosine signal transduction remain to be clarified in future research.

3. Potential physiological and pharmacological implications of a new guanine-based purinergic system

The neuromodulatory effects of guanosine on the glutamatergic system are potentially relevant. Glutamate is the main excitatory neurotransmitter in mammalian CNS and is essential for its normal functions (Ozawa et al., 1998; Sheng & Hoogenraad, 2006). Glutamate acts via ionotropic (ligand-gated ion channel; NMDA, KA, or AMPA receptors) or metabotropic (coupled to G proteins) receptors, modulating several plastic brain processes, such as learning and memory, pain, ontogeny, and development, and several brain responses to external stimuli (Beart & O'Shea, 2007 ; Izquierdo et al., 2006; Parsons et al., 1998; Segovia et al., 2001).

However, overstimulation of the glutamatergic system (by exogenous or endogenous stimuli), which occurs when extracellular glutamate levels increase over the physiological range, is involved in various acute and chronic brain diseases (excitotoxicity), including neurodegenerative diseases, traumatic brain injury, cerebral ischemia, and seizures (Allen et al., 2004; Castellano et al., 2001; Lipton & Rosemberg, 1994; Maragakis & Rothstein, 2004; Meldrum, 1994). It is now clearly shown that glutamatergic excitotoxicity is prevented by astrocytic glutamate uptake, a process responsible for maintaining the extracellular glutamate levels below toxic levels (Anderson & Swanson, 2000; Chen & Swanson, 2003; Hertz, 2006; Matute et al., 2006; Schousboe & Waagepetersen, 2005). Since adenosine decreases glutamate release and guanosine increases glutamate uptake (and persists for longer periods of time extracellularly), both purine nucleosides may act in concert to reduce the impact of glutamate-induced excitability.

Glutamate undoubtedly plays a pivotal role on epilepsy and probably in other CNS diseases accompanied by seizures (Allen et al., 2004; Castellano et al., 2001; Lipton & Rosemberg, 1994; Maragakis & Rothstein, 2004; Meldrum, 1994). However, cellular and molecular mechanisms involved in the generation and maintenance of seizures and toxicity are not fully understood. Several studies demonstrated that guanine-based purines, mainly guanosine, are anticonvulsant against glutamatergic agents in animals as discussed earlier (Lara et al., 2001; Oliveira et al., 2004; Schmidt et al., 2000; Schmidt et al., 2005; Vinadé et al., 2003; Vinadé et al., 2005). Quinolinic acid is a NMDA agonist, but also stimulates glutamate release and inhibits its uptake (Tavares et al., 2002). We demonstrated that systemic (intraperitonial or oral) administration of GMP or guanosine dose-dependently protected against seizures induced by quinolinic acid in mice and rats (Lara et al., 2001; Schmidt et al., 2000; Tavares et al., 2005). This same effect was observed with all guanine-based purines when administered i.c.v. (Schmidt et al., 2005). These effects were reproducible even after chronic oral systemic exposure to guanosine or GMP, indicating that these substances are orally active in the long term (Vinadé et al., 2003, 2005). Guanosine also protected against quinolinic acid-induced seizures and prevented quinolinic acid-induced decrease on glutamate uptake by brain slices in young (12–14 days old) (Oliveira et al., 2004) or adult (Vinadé et al., 2005) rats, pointing that guanosine could be considered for epilepsy treatment and possibly other neurological disorders in adults and children. Additionally, when injected into the rat striatum, quinolinic acid causes dose-dependent widespread cell death. All cell types, including the NADPH-diaphorase-positive neurons appear to be sensitive to the toxin. The NADPH-diaphorase-positive cells are chronically destroyed by in vivo quinolinic acid injections, but this effect was blocked by the concomitant administration of GMP (Malcon et al., 1997), strengthening the notion that the guanine-based purinergic system may be a valuable target for the treatment of neurodegenerative disorders.

Guanine-based purines have recently been enrolled in the pathogenesis of the Lesch-Nyhan syndrome (Deutsch et al., 2005). Investigation of this disorder and the neurobiological consequences of the hypoxanthine phosphoribosyltransferase (HPRT) deficiency demonstrated the potential roles that guanine-based purines play in neurodevelopment and as neuromodulators and neurotransmitters. Conceivably, diminished reutilization of free guanine bases due to absent or negligent HPRT activity and relatively high guanase activity in the brain could lead to deficient pools of guanosine associated with glutamatergic synapses in Lesch-Nyhan syndrome. Nonetheless, if a guanosine deficiency were to exist in Lesch-Nyhan syndrome, the administration of guanosine itself or its analogs that could promote glutamate uptake might be a useful pharmacological strategy to be considered in the treatment of this disorder.

Apoptosis is implicated in the pathophysiology of Alzheimer's disease. Recently, it was shown that extracellular guanosine inhibited staurosporine-induced apoptosis in astrocytes (Di Iorio et al., 2004). Guanosine has also been shown to protect SH-SY5Y cells against beta-amyloid-induced apoptosis (Pettifer et al., 2004). More recently, guanosine was shown to dose-dependently inhibit the CD40-induced expression in mouse microglia cells, as well as functional CD40 signaling by suppressing IL-6 production (D'Alimonte et al., 2007). The antiapoptotic effects of guanosine seemed to be mediated

by activation of the PI3K/Akt/PKB and MAPK (ERK1/2 and p38) pathways (D'Alimonte et al., 2007). Additional evidence suggests that guanosine increases cholesterol efflux from astrocytes and rat glioma cells and increases the expression of apolipoprotein E (ApoE) in astrocytes (Ballerini et al., 2006). Given the emerging role of cholesterol balance in neuronal plasticity and in stabilization of synaptic transmission, and the fact that cell cholesterol depletion has recently been linked to a reduction in beta-amyloid formation, these findings point to a role of guanosine as a potential pharmacological tool in the modulation of cholesterol homeostasis in the brain. Altogether, these findings suggest a role for the guanine-based purinergic system as a potential drug target in the experimental therapy of neuroinflammatory/neurodegenerative diseases, particularly Alzheimer's disease (D'Alimonte et al., 2007; Pettifer et al., 2004).

Loeffler et al. investigated the influence of increased dopamine metabolism through administration of reserpine or levodopa on striatal concentrations of guanosine, guanine, and their metabolites. Interestingly, reserpine treatment decreased striatal guanosine by 41% and increased guanine by 50%, while levodopa decreased guanosine by 48% (Loeffler et al., 1998). Because of the neurotrophic properties of guanosine and guanine, changes in striatal concentrations of these purines secondary to increased dopamine turnover may have relevance for survival of remaining dopaminergic neurons in Parkinson's disease (Loeffler et al., 2000).

Recent findings suggest that guanine-based purines, especially guanosine, may also be a new target for trauma rehabilitation or CNS diseases related to demyelination. Administration of guanosine for 7 consecutive days improved locomotor function and spinal cord remyelination in rats submitted to a spinal cord injury model (Jiang et al., 2003). Guanosine-induced remyelination seemed to result from activation of endogenous oligodendrocyte lineage cells. These findings may have significant implications for chronic demyelinating diseases. Additionally, the trophic effects of GTP and guanosine may have physiological and therapeutic implications in sprouting and functional recovery after neuronal injury in the CNS, due to the high levels of nucleosides and nucleotides released from dead or injured cells (Ciccarelli et al., 2001). Therefore, guanine-based purines or their analogs may be useful in future research for treating not only epilepsy but also other neurodegenerative disorders such as Huntington's, Lesch-Nyhan's, Parkinson's, or Alzheimer's diseases and traumatic injuries.

Brain ischemia is responsible for significant morbidity and mortality and significant resources have been dedicated to developing neuroprotective strategies. Since adenine-based purines have been demonstrated to play a role in endogenous neurodegenerative and neuroprotective processes (Franke et al., 2006), guanine-based purines could also be further investigated for possible therapeutic manipulations as well as adenine-based purines. Using primary cultures of astrocytes prepared from the cerebral hemispheres, it was shown that under basal conditions they spontaneously release guanine-based purines. Interestingly, the amount of guanine-based purines (mainly guanosine) released over a 3-hr period was greater than that of adenine-based purines (Ciccarelli et al., 1999b). Moreover, the exposure of these cultures to hypoxia/hypoglycemia resulted in sustained increase in the release of guanine- and adenine-based purines over basal values up to 90 min after the insult. Importantly, the release of purines was not related to an artifact of diminished cell viability (Ciccarelli et al., 1999b). These effects in an in vitro ischemia/stroke model are consistent with the hypothesis that these compounds, especially guanosine, may exert pivotal modulatory effects on synaptic transmission and more sustained trophic effects. The potential ability of exogenously administered guanine-based purines to provide an alternative source of energy to ATP has been suggested as an explanatory hypothesis for their neuroprotective effects in the context of oxidative stress and cell damage (Ciccarelli et al., 2001; Litsky et al., 1999). For example, after exposure to rotenone, an inhibitor of the mitochondrial respiratory chain, and the induction of chemical hypoxia, inosine and guanosine (250–500μM) were shown to preserve the viability of cultured astrocytes and neurons (Jurkowitz et al., 1998; Litsky et al., 1999). The ability of purine nucleosides (inosine and guanosine) to maintain cellular levels of ATP above a critical threshold under hypoxia may provide an explanation of the mechanism of their cell damage prevention. Indeed, the addition of a purine nucleoside phosphorylase inhibitor to the cultures, which would interfere with a pathway for the participation of purine nucleosides in the production of ATP under anaerobic conditions, attenuated their protective effect; this effect of purine nucleosides to preserve cell viability was most dramatic with neurons. The data also suggested that neuronal protection by purine nucleosides is either dependent on or enhanced by the presence of glia (Litsky et al., 1999).

Adenine-based purines have been suggested to play a role in psychiatric diseases, such as schizophrenia and bipolar disorder (Lara & Souza, 2000; Lara et al., 2006; Machado-Vieira et al., 2002). Since guanine- and adenine-based purines are closely related and share many in vitro and in vivo extracellular effects and pathways, it is not surprising that guanine-based purines also play a role in psychiatric diseases, as well as adenine-based purines. Recently, we showed that guanosine attenuates hyperlocomotion induced by dizocilpine (a pharmacological model of schizophrenia) in mice (Tort et al., 2004) and presented an anxiolytic profile after chronic oral treatment (Vinadé et al., 2003). These effects may be due to an increase in glutamate uptake by astrocytes promoted by guanosine, reducing the neurotransmitter levels at the synaptic cleft, leading to less activation of non-NMDA receptors, with subsequent less increase in the efflux of dopamine in the prefrontal cortex (Tort et al., 2004). These findings point to a potential antipsychotic property of guanosine, as this model could predict compounds that target psychotic symptoms that are not generally treated with typical antipsychotics (Adams & Moghaddam, 2001). Moreover, the neuroprotective and neurotrophic effects of guanosine may also be advantageous for the treatment of schizophrenia, which is associated with inadequate

Table 1
Summary of main extracellular effects of guanine-based purines

In vitro studies	
Guanine nucleotides	
Inhibit kainic acid binding	Souza & Ramirez, 1991
Inhibit glutamate binding and its analogs	Rubin et al., 1997
Prevent cell responses to excitatory amino acids	Burgos et al., 1998
Stimulate glutamate uptake by astrocytes	Frizzo et al., 2003
Stimulate the glutamate uptake by synaptic vesicles	Tasca et al., 2004
GMP prevents loss of cell viability induced by glutamate	Molz et al., 2005
GMP prevents loss of cell viability induced by hypoxia	Oliveira et al., 2002
GTP induces astrocyte proliferation	Ciccarelli et al., 2000
GTP induces synthesis and release of trophic factors	Middlemiss et al., 1995
GTP stimulates proliferation of a wide range of cell types	Rathbone et al., 1992a
Guanosine	
Increases glutamate uptake by astrocytes	Frizzo et al., 2001, 2002
Prevents the decrease of glutamate uptake induced by hypoxic-ischemic insult	Moretto et al., 2005
Protects brain slices exposed to hypoxia/hypoglycemia	Frizzo et al., 2002
Prevents NMDA-induced toxicity in neurons	Caciagli et al., 2000
Induces trophic effects on neural cells (astrocytes)	Rathbone et al., 1999a, 1999b
Prevents beta-amyloid-induced apoptosis	Pettifer et al., 2004
Inhibits staurosporine-induced apoptosis in astrocytes	Di Iorio et al., 2004
Increases the expression of ApoE in astrocytes	Ballerini et al., 2006
In vivo studies	
Guanine nucleotides	
Prevent quinolinic acid-induced seizures in rodents	Schmidt et al., 2005
GMP prevents quinolinic acid-induced neural death	Malcon et al., 1997
GMP prevents quinolinic acid-induced release of glutamate on synaptosomes	Tavares et al., 2005
GMP induces amnesia in rats	Saute et al., 2006
GMP reverses the facilitatory effect of glutamate on memory in rats	Rubin et al., 1996
Guanosine	
Prevents seizures induced by several glutamatergic agents in rodents	Lara et al., 2001
Chronic treatment is anxiolytic in mice	Vinadé et al., 2003
Induces amnesia in rats	Roesler et al., 2000
Attenuates hyperlocomotion induced by MK-801	Tort et al., 2004
Prevents in vivo decrease of astrocytic glutamate uptake induced by quinolinic acid	Vinadé et al., 2005
Improves locomotor function and remyelination in rats submitted to a spinal cord injury model	Jiang et al., 2003

neurodevelopment and increased brain loss after onset of the disorder (Lara et al., 2006).

Considering that purines, their metabolites, and the soluble nucleotidases responsible for their hydrolysis are detected in the human and animal CSF and blood serum (Castro-Gago et al., 1992; Oses et al., 2004a; Portela et al., 2002; Regner et al., 1997; Silva et al., 2004), their potential role on an "endogenous neuroprotection system," and the lack of precise and safe parameters to evaluate brain injury consequences, it is possible that these parameters may be new putative markers for CNS injury. We have demonstrated that PTZ-induced seizures promote an increase in CSF nucleotidases activity represented by further hydrolysis of GDP and ADP and an increase in concentration of guanosine and inosine (probably related to quick degradation of adenosine to inosine) 30 min after the insult (Oses et al., 2004b). Increases of GDP/ADP hydrolysis and levels of nucleosides guanosine/inosine after PTZ-induced seizures presented a somewhat similar profile to other well-known brain injury markers (S100β protein and neuron-specific enolase, NSE). This temporal similarity suggests that those compounds could become biochemical brain markers to evaluate neural injury.

Altogether, these findings point to the influence of guanine-based purines in the homeostasis of the glutamatergic system, modulating some glutamatergic parameters such as glutamate uptake by astrocytes and synaptic vesicles, seizures induced by glutamatergic agents, learning and memory, anxiety, ischemia, and excitotoxicity. Since guanosine is an endogenous

Table 2
Research agenda for new studies related to the guanine-based purinergic system

(1) Characterization of the guanosine and GTP receptors
(2) Identification of specific pharmacological tools to manipulate this system
(3) Elucidation of signal transduction mechanisms linked to guanosine and GTP receptors
(4) Correlation between some purinergic parameters (extracellular levels of nucleosides and nucleotidases activity) and CNS events
(5) Investigaton of the safety profile and toxicity of guanine-based purines after systemic and/or continuous administration
(6) Clinical trials in humans by using purine derivatives (guanosine, xantine oxidase inhibitors such as allopurinol, nucleoside transport inhibitors)

compound and seems to be well tolerated with no obvious CNS side effects except for memory impairment, it could be useful in the future to treat or prevent neurological diseases associated with overstimulation of the glutamatergic system. Additionally, these studies on the extracellular effects of guanine-based purines have strengthened the proposal for a specific guanine-based purinergic system in addition to the well-known adenine-based purinergic system.

4. Conclusions and perspectives for new guanine-based purine studies

This article reviews the evidence to hypothesize that a guanine-based purinergic system plays significant roles in the nervous system, providing new targets for neuroprotection and neuromodulation (Table 1). These guanine-based purines have been relatively neglected when compared to adenine-based purines and should be further investigated as a neurotransmission/neuromodulator system in terms of physiological, pharmacological, biochemical, and genetic parameters. Furthermore, the profile of guanine-based purines to modulate the glutamatergic system makes this system a very interesting object for discovery of new pharmacological options to treat diseases related to overstimulation of glutamatergic system (Table 2).

More specifically, to advance in guanine-based purine research, further work is necessary on the mechanisms of action of guanine-based purines, cloning of specific receptors, and characterization of second messengers related to their extracellular effects. Since the recent identification of a high-affinity binding site for guanosine in rat brain membranes (Traversa et al., 2002, 2003), a modest advance on guanosine receptor characterization and elucidation of its mechanism of action has emerged. New studies on guanosine binding are needed and should include a careful purification of cellular membranes by decreasing mitochondrial contamination. Although signal transduction mechanisms linked to guanosine receptor are also unknown, they may be linked via G proteins to the MAPK cascade since the ability of guanosine to enhance synthesis of trophic factors in astrocytes is associated with an increase in phosphorylation of ERK_1 and ERK_2 and is blocked by pretreatment with pertussis toxin (Caciagli et al., 2000). New studies regarding guanine-based purines receptors could further address this issue. Additional elucidation of the mechanism of action of guanosine and its membrane-binding site is under current investigation in our laboratory.

Guanine-based purines present several beneficial effects to CNS during excitotoxic conditions. However, little information about potential side effects of these compounds is available. Although no obvious disturbances regarding locomotion and behavior apart from amnesia were noted, specific studies about the safety profile of these compounds is pivotal for their future use in a clinical basis. These studies should include further investigation on absorption, metabolism, half-lives, storage, and elimination of these compounds (guanosine being the best candidate so far). Likewise, pharmacological determination of lethal doses and therapeutic index are also relevant and should be addressed in future studies.

Although it is early to propose the use of guanine-based purines for clinical research, an interesting approach to investigate their role clinically is the investigation of purine derivatives already used in humans. These studies should include clinical trials designed to address neuroprotection measures. It may be a reality for memory-enhancing agents in Alzheimer's or neurodegenerative diseases. Propentofylline and AIT-082 are purine derivatives that have been demonstrated to exert trophic effects in animals by increasing production of neurotrophic factors in brain and spinal cord (Rathbone et al., 1999a). Additionally, we have demonstrated that allopurinol, a xantine oxidase inhibitor, was an effective and well-tolerated adjuvant treatment for poorly responsive schizophrenia, refractory aggressive behavior, and mania (Brunstein et al., 2005; Lara et al., 2000; Lara et al., 2003; Machado-Vieira et al., 2001). These results were confirmed by an independent group (Akhondzadeh et al., 2005, 2006). Refractory epilepsy may also respond to allopurinol (Togha et al., 2007; Zagnoni et al., 1994). These effects may be due to an indirect increase in extracellular purine levels (adenosine and guanosine). Thus, allopurinol may be the first commercially available effective drug enhancing the effects of the purinergic systems for the treatment of human brain diseases. These findings, together with the evidence of purine modulation of CNS, indicate that new studies addressing xantine oxidase inhibitors in neuroprotection and psychiatric disorders could represent a fine approach to investigate the therapeutic potential of purine in a clinical basis.

In conclusion, the guanine-based purinergic system is yet to be fully characterized, but current evidence strongly suggests its functional roles in the mammalian nervous system, providing new targets and strategies for the treatment of brain diseases.

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II.2. RESULTADOS EXPERIMENTAIS COM MODELOS ANIMAIS

II.2.a. Intracerebroventricular Guanine-Based Purines Protect Against Seizures Induced by Quinolinic Acid in Mice

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Intracerebroventricular Guanine-Based Purines Protect Against Seizures Induced by Quinolinic Acid in Mice

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Acute and chronic administration of the nucleoside guanosine have been shown to prevent quinolinic acid (QA) and α-dendrotoxin-induced seizures, as well as to impair memory and anxiety in rats and mice. In this study, we investigated the effect of i.c.v. administration of guanine-based purines (GTP, GDP, GMP, and guanosine) against seizures induced by the NMDA agonist and glutamate releaser quinolinic acid in mice. We also aimed to study the effects of the poorly hydrolysable analogs of GTP (GppNHp and GTPγS) and GDP (GDPβS) in this seizure model. QA produced seizures in 100% of mice, an effect partially prevented by guanine-based purines. In contrast to GTP (480 nmol), GDP (320–640 nmol), GMP (320–480 nmol) and guanosine (300–400 nmol), the poorly hydrolysable analogs of GTP and GDP did not affect QA-induced seizures. Thus, the protective effects of guanine nucleotides seem to be due to their conversion to guanosine. Altogether, these findings suggest a potential role of guanine-based purines for treating diseases involving glutamatergic excitotoxicity.

KEY WORDS: Excitotoxicity; guanosine; guanine-based purines; seizures.

INTRODUCTION

Glutamate is the main excitatory neutransmitter in mammalian central nervous system (CNS), being essential for its normal functions (1). However, overstimulation of the glutamatergic system is involved in many acute and chronic brain diseases such as epilepsy (1–3). Quinolinic acid (QA), an over stimulator of the glutamatergic system, seems to be involved in the etiology of epilepsy (4,5) and has been proposed to be a useful seizure model in rodents (6,7).

Adenine-based purines (ABPs) and the nucleoside adenosine are considered the main effectors of the purinergic system (8). Glutamate and adenosine closely interact in modulating CNS functions. Glutamate stimulates the release of adenosine, which acts on presynaptic A_1 receptors inhibiting glutamate release (negative feedback) (9).

Similarly to ABPs, guanine-based purines (GBPs), namely the nucleotides GTP, GDP, GMP and the nucleoside guanosine, have also been shown to exert extracellular effects, presenting important neuromodulatory functions (not directly related to the modulation of G-proteins activity), such as trophic effects on neural cells (10) and antagonism of the glutamatergic system (11–14). *In vitro*, GBPs inhibit the binding of glutamate and analogs, prevent cell responses to excitatory amino acids and stimulate the uptake of extracellular glutamate by astrocytes cell cultures and brain slices, which is a process involved in neuroprotection (13–18). Guanosine protected brain slices exposed to hypoxia/hypoglycemia (19), and medium from astrocytes treated

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with guanosine prevented NMDA-induced toxicity in neurons (20). *In vivo*, acute or chronic administration of guanosine protected against seizures induced by glutamatergic agents in rodents (7,11,21–23). These behavior effects seem to be related to a stimulatory effect on glutamate uptake (15–17).

Extending previous reported effects of GBPs administration in mice and rats, the aim of this study was to evaluate the effects of i.c.v. administration of GTP, GDP, GMP or guanosine, and the poorly hydrolysable analogs of GTP (GTPγS or GppNHp) and GDP (GDPβS), against seizures induced in mice by i.c.v. quinolinic acid administration.

EXPERIMENTAL PROCEDURE

Chemicals. Guanosine (GUO), Guanosine 5′-monophosphate (GMP), Guanosine 5′-diphosphate (GDP), Guanosine 5′-triphosphate (GTP), guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS), guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), 5′-guanylylimidodiphosphate (GppNHp), 6,7-dinitroquinoxaline-2,3-dione (DNQX) and QA were purchased from Sigma Chemicals (St Louis, MO, USA). 5-methyl-10-11-dihydro-5H-dibenzo(a,b)cyclohepta-5-10-imine maleate (MK-801 or dizocilpine) was obtained from RBI-Research Biochemicals International (Natick, MA, USA). The anesthetic sodium thiopental was obtained from Cristália (Itapira, SP, Brazil). QA was dissolved in saline 0.9% and adjusted to pH 7.4. Guanosine was prepared in NaOH 10 μM (pH 7.4) and the concentration was limited to 100 mM by its poor water solubility. Guanine nucleotides were dissolved in saline 0.9%.

Animals. Male adult Swiss albino mice (20–30 g) were kept on a 12 h light/dark cycle (light on at 7:00 a.m.) at temperature of 22 ± 1°C, housed in plastic cages (five per cage) with tap water and commercial food *ad libitum*. Our institutional protocols for experiments with animals, designed to minimize suffering and limit the number of animals sacrificed, were followed throughout. All behavioral procedures were conducted between 1:00 and 5:00 p.m.

Surgical Procedure. Surgery and i.c.v. infusion techniques were adapted from Haley and McCormick (24). Animals were anesthetized with sodium thiopental (60 mg/kg, 10 ml/kg, i.p.). In an stereotaxic apparatus, the skin of the skull was removed and a 27 gauge 7 mm guide cannula was placed at 1 mm posterior to bregma, 1 mm right from the midline and 1 mm above the lateral brain ventricle. Through a 2 mm hole made at the cranial bone, the cannula was implanted 1.5 mm ventral to the superior surface of the skull, and fixed with jeweler acrylic cement. Experiments were performed 48 h after surgery.

Drugs Administration. A 30 gauge cannula was fitted into the guide cannula and connected by a polyethylene tube to a microseringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula aiming the lateral brain ventricle. Then, animals were pretreated with an i.c.v. infusion of vehicle (saline 0.9% or NaOH 10 μM), MK-801 (68 nmol), DNQX (68 nmol), Guanosine (up to 400 nmol), GMP (up to 1200 nmol), GDP (up to 800 nmol), GTP (up to 960 nmol), GDPβS (up to 640 nmol), GTPγS (320 nmol), GppNHp (up to 480 nmol). After 5 min, an i.c.v. infusion of either 4 μl of vehicle or QA (36,8 nmoles, the lowest dose causing seizures in all animals) was performed and behavior observed for 10 min.

Evaluation of Seizures and Toxicity. Mice were observed for 10 min in plexiglas chambers for the occurrence of tonic-clonic seizures lasting more than 5 s (25). Latency to start seizures, time of seizures and death were also evaluated. Animals not displaying seizures during these 10 min were considered protected. Methylene blue (4 μl) was injected through the cannula and animals without dye in the lateral brain ventricle were discarded.

Statistical Analysis. statistical analysis between groups was performed by the Fisher exact test for the occurrence of seizures and death. Latency to start seizures and time displaying seizures were evaluated by ANOVA plus Duncan test. All results with $P < 0.05$ were considered significant.

RESULTS

QA induced seizures in all animals, an effect which was completely prevented by i.c.v. administration (5 min before QA) of the NMDA antagonist MK-801 68 nmol ($P < 0.0001$), but not by the AMPA antagonist DNQX 68 nmol (70% of animals displaying seizures). Vehicle (saline 0.9% – i.c.v. administered) did not promote seizures (data not shown).

Guanosine (300–400 nmol) and GMP (320–480 nmol), administered 5 min before QA, dose-dependently protected against seizures induced by QA up to 60% (Figs. 1 and 2). Pretreatment with GDP (320 – 640 nmol) or GTP (480 nmol) produced similar effects, preventing up to 85% and 50% of QA-induced seizures, respectively (Fig. 3a,b). The poorly hydrolysable analogs GDPβS (320 – 640 nmol), GTPγS (320 nmol) or GppNHp (320 – 480 nmol) did not present any protective effects against QA-induced seizures. Latency to start seizure and time displaying seizures were not affected by any i.c.v. GBPs (data not shown).

Concerning the effect *per se* of GBPs, pretreatment with guanosine did not produce any seizure behavior. However, pretreatment with high doses of GMP (1200 nmol) and GDP (800 nmol) induced seizures *per se* in some animals (20% and 50% of animals, respectively).

DISCUSSION

Here, extending previous studies from our group, we investigated the anticonvulsant effect of

other GBPs than GMP and guanosine, namely the nucleotides GTP and GDP (and their poorly hydrolysable analogs), in an *in vivo* model of glutamatergic toxicity. It shows that acute i.c.v. administration of GTP, GDP, GMP or guanosine were protective against seizures induced in mice by the NMDA agonist and glutamate releaser QA.

Previously we showed that GMP also reverses the facilitatory effect of glutamate on inhibitory avoidance task in rats (26) and is neuroprotective against QA-induced toxicity in rats (27). Guanosine also presents amnesic and anxiolytic effects in rats and mice (23,28,29). Based on previous reports, these results seem to be mediated by modulation of glutamatergic system (15–17,23).

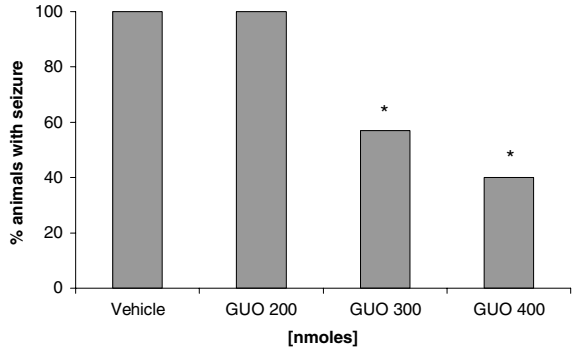

Fig. 1. Protection against the convulsant effect of QA by GUO (200 – 400 nmol). $n = 8$–10 animals per group. $* = P < 0.05$ (Fisher exact test), as compared with vehicle group.

We previously demonstrated that i.p. administration of GMP and i.p. or p.o. administration of guanosine prevent seizures induced by glutamatergic agents such as QA, kainate, or α-DTX in rodents (7,21–23). GMP and guanosine have already presented similar neuroprotective profile in other *in vivo* and *in vitro* protocols (16,27,30). Recently, we observed that an acute i.c.v. administration of the ecto-5′-nucleotidase inhibitor α,β-methyleneadenosine 5′-diphosphate (AOPCP) prevents the anticonvulsant effects of GMP against QA in rats, without affecting the guanosine effect (22). We also observed that i.p. administration of GMP, in anticonvulsant doses, produced a 3-fold increase of cerebrospinal fluid levels of guanosine in rats, not affecting GMP levels (22). GTP also presents trophic effects in the CNS (18) and is stored in synaptic vesicles (31). Recently, we have demonstrated that GTP, similar to guanosine and GMP, stimulates glutamate uptake *in vitro*, an effect not reproduced by GppNHp, a poorly hydrolizable analog of GTP (32). Since both GTP, GDP and GMP are hydrolyzed to guanosine by ecto-nucleotidases (31) and we recently observed that the AOPCP administration impairs the GMP anticonvulsant effects, we can speculate that guanine nucleotides actions involve their conversion to the nucleoside guanosine.

Guanosine was shown to prevent ischemic injury *in vitro* (19) and NMDA-induced excitotoxicity *in vitro* (10). Both neuronal and astrocytic cell cultures are able to release guanosine under basal and toxic conditions (10,33), and we recently found that guanosine, even at 1 μM, significantly stimu-

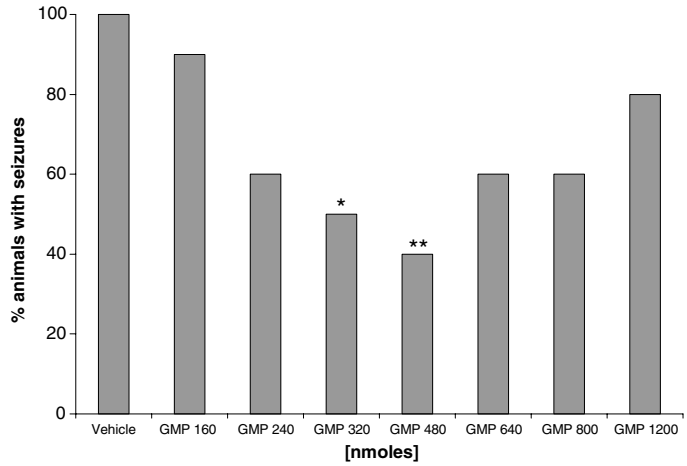

Fig. 2. Protection against the convulsant effect of QA by GMP (160 – 1200 nmol). $n = 10$ animals per group. $* = P < 0.05$ and $** = P < 0.01$ (Fisher exact test), as compared with vehicle group.

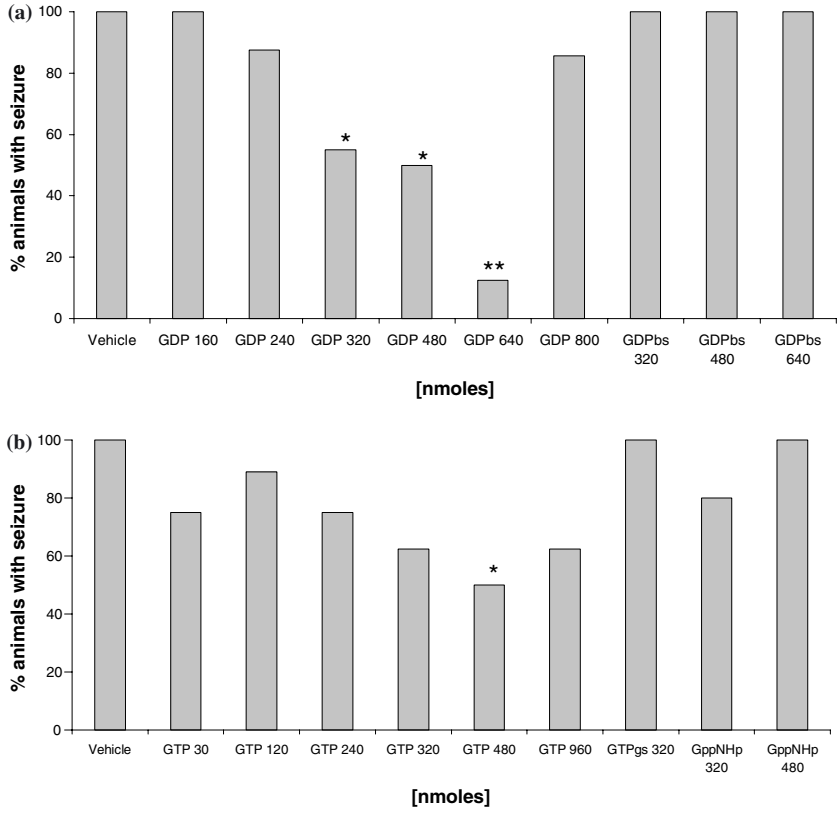

Fig. 3. (a) Protection against the convulsant effect of QA by GDP (160 – 800 nmol) or GDPβS (320 – 640 nmol). (b) Protection against the convulsant effect of QA by GTP (30 – 960 nmol), GTPγS (320 nmoles) or GppNHp (320 – 480 nmol). $n = 10$ animals per group. $*= P < 0.05$ and $**= P < 0.01$ (Fisher exact test), as compared with vehicle group.

lated glutamate uptake in cultured astrocytes (15–17), a physiological process preventing glutamate toxicity. Based upon the recent identification of a high affinity binding site for guanosine in rat brain membranes (34) and the evidence that guanosine is a poor displacer of glutamate ligands (35), is tempting to presume that the neuroprotective and anticonvulsant effects of GBPs involve the stimulatory action of guanosine on glutamate uptake (15–17), rather than a direct antagonism of glutamatergic receptors.

This study provides additional evidence of the anticonvulsant effects of guanine-based purines in mice, since the i.c.v. administration of the nucleotides GMP, GDP and GTP as well as guanosine protected against QA-induced seizures. Their anticonvulsant actions seem to be mediated, at least partially, by their conversion to guanosine, since the poorly hydrolizable analogs GDPβS, GTPγS or GppNHp had no effect. These effects may be due to guanosine-induced stimulation of glutamate uptake by astrocytes.

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II.2.b. Antinociceptive effects of intracerebroventricular administration of guanine-based purines in mice: Evidences for the mechanism of action

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Research Report

Antinociceptive effects of intracerebroventricular administration of guanine-based purines in mice: Evidences for the mechanism of action

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ABSTRACT

It is well known that adenine-based purines exert multiple effects on pain transmission. However, less attention has been given to the potential effects of guanine-based purines (GBPs) on pain transmission. The aim of this study was to investigate the effects of intracerebroventricular (i.c.v.) guanosine and GMP on mice pain models. Mice received an i.c.v. injection of vehicle (saline or 10 μM NaOH), guanosine (5 to 400 nmol), or GMP (240 to 960 nmol). Additional groups were also pre-treated with i.c.v. injection of the A_1/A_{2A} antagonist caffeine (15 nmol), the non-selective opioid antagonist naloxone (12.5 nmol), or the 5'-nucleotidase inhibitor AOPCP (1 nmol). Measurements of CSF purine levels and cortical glutamate uptake were performed after treatments. Guanosine and GMP produced dose-dependent antinociceptive effects. Neither caffeine nor naloxone affected guanosine antinociception. Pre-treatment with AOPCP completely prevented GMP antinociception, indicating that conversion of GMP to guanosine is required for its antinociceptive effects. Intracerebroventricular administration of guanosine and GMP induced, respectively, a 180- and 1800-fold increase on CSF guanosine levels. Guanosine was able to prevent the decrease on cortical glutamate uptake induced by intraplantar capsaicin. This study provides new evidence on the mechanism of action of GBPs, with guanosine and GMP presenting antinociceptive effects in mice. This effect seems to be independent of adenosine and opioid receptors; it is, however, at least partially associated with modulation of the glutamatergic system by guanosine.

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1. Introduction

It is well known that the extracellular adenine-based purines (ABPs), namely, the nucleotide ATP and the nucleoside adenosine, exert multiple effects on pain transmission (Sawynok, 1998; Sawynok and Liu, 2003). ATP can stimulate sensory nerve endings causing pain and a marked increase in the discharge from sensory neurons and is involved in the initiation of different types of nociception and pain (Burnstock and Wood, 1996). Adenosine presents antinociceptive effects by acting at spinal, supraspinal, and peripheral sites (Sawynok, 1998), plays a pivotal role in inflammatory and neuropathic pain (Dickenson et al., 2000), and mediates aspects of opioid-induced analgesia (Sawynok et al., 1989). Antinociceptive effects of adenosine are particularly related to the activation of adenosine A_1 receptors in the spinal cord where inhibition of intrinsic neurons by an increase in K^+ conductance and presynaptic inhibition of sensory nerve terminals and diminished release of substance P and glutamate contributes to its actions (Sawynok and Liu, 2003; Sollevi, 1997).

Although ATP and adenosine are usually considered the main effectors of the purinergic system (Burnstock, 2007; Cunha et al., 1998; Ralevic and Burnstock, 1998), extracellular guanine-based purines (GBPs), namely, the nucleotide GMP and the nucleoside guanosine also exert biological effects. Such action are unrelated to direct G-protein modulation and include trophic effects on neural cells (Ciccarelli, 2001) and modulation of glutamatergic activity (Schmidt et al., 2007). With respect to *in vitro* effects on the glutamatergic system, GBPs inhibit the binding of glutamate and analogs (Baron et al., 1989; Burgos et al., 1998), prevent cell responses to excitatory amino acids (Souza and Ramirez, 1991), present neuroprotective effects in cultured neurons submitted to hypoxia, and increase glutamate uptake in cultured astrocytes (Frizzo et al., 2001, 2002, 2003). *In vivo*, GBPs prevent glutamate-induced seizures in mice and rats (Lara et al., 2001; Schmidt et al., 2000, 2005; Soares et al., 2004; Vinadé et al., 2003, 2005). However, it remains unclear if extracellular GBPs can act as nociception modulators.

This study investigated the antinociceptive effects of intracerebroventricular (i.c.v.) administration guanosine and GMP in mice. By means of pharmacological and neurochemical approaches, we examined the mechanisms underlying GBP-induced supraspinal antinociception in mice.

2. Results

The results presented in Fig. 1 show that i.c.v. administration of guanosine produces antinociception against intraplantar

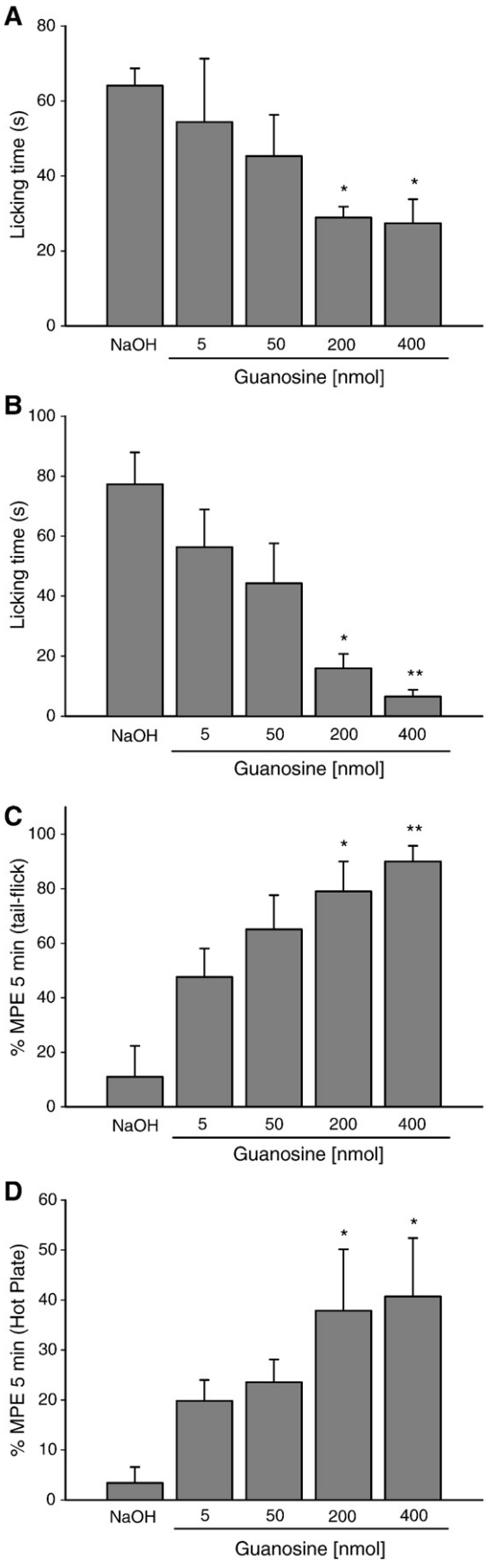

Fig. 1 – Effects of i.c.v. vehicle (10 μM NaOH), guanosine (5 to 400 nmol) against i.pl. capsaicin (A), i.pl. glutamate (B), tail-flick (C), and hot-plate (D) tests in mice. (A and B) Columns represent mean time spent licking the injected hindpaw and vertical bars represent SEM. (C and D) Columns represent mean percent of maximum possible effect (%MPE) and vertical bars represent SEM. N = 10–15 animals per group. *P < 0.05 and **P < 0.01 compared to vehicle (control), one-way ANOVA followed by Student–Newman–Keuls test.

(i.p.l.) capsaicin (1A) and i.pl. glutamate (1B)-induced pain as well as in the tail-flick (1C) and hot-plate (1D) tests. Neither i.c.v. saline nor 10 μM NaOH affected nociception as compared to control (sham) animals (data not shown). Mean ID_{50} values (95% confidence limits) for i.c.v. guanosine in the capsaicin and glutamate tests were 190 (127–284) and 125 (78–200) nmol, respectively; maximal inhibitions of 57±11% and 92±3%, respectively. Mean ID_{50} value (95% confidence limits) for i.c.v. GMP against capsaicin-induced pain was 554 (449–685) nmol, with maximal inhibition of 75±5%.

Fig. 2 shows that GMP caused a pronounced inhibition of the nociceptive response induced by i.pl. capsaicin (2A). AOPCP (1 nmol, i.c.v.) pre-administration completely prevented the antinociceptive effect of GMP (960 nmol) (2B). AOPCP *per se* did not affect nociception. I.c.v. saline did not affect nociception as compared to control (sham) animals (data not shown).

Fig. 3A shows that naloxone completely reversed morphine-induced antinociception against capsaicin-induced pain, without affecting antinociception induced by guanosine (Fig. 3A). As shown at Fig. 3B, i.c.v. adenosine (37.5 nmol) also produced antinociceptive effects in this model; pre-treatment with caffeine (15 nmol) prevented adenosine but not guanosine antinociceptive effects.

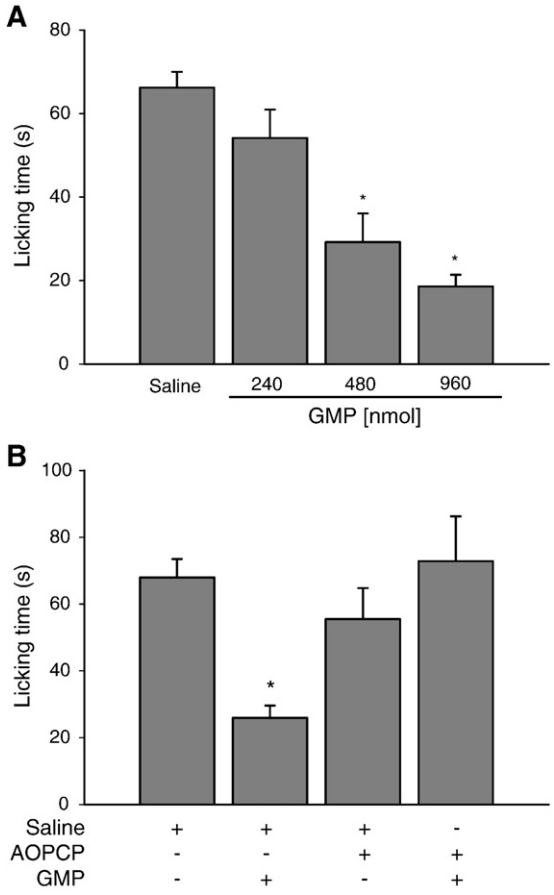

Fig. 2 – (A) Effects of i.c.v. vehicle (saline) or GMP (240 to 960 nmol) on capsaicin-induced pain. (B) Mice were treated with an i.c.v. injection of AOPCP (1 nmol) or vehicle (saline) 3 min before an i.c.v. injection of GMP (960 nmol) or vehicle (saline). After 5 min, animals received an i.pl. injection of capsaicin. Columns represent mean licking time and vertical bars represent SEM. N=10–12 animals per group. *P<0.01 compared to control groups, one-way ANOVA followed by Student–Newman–Keuls test.

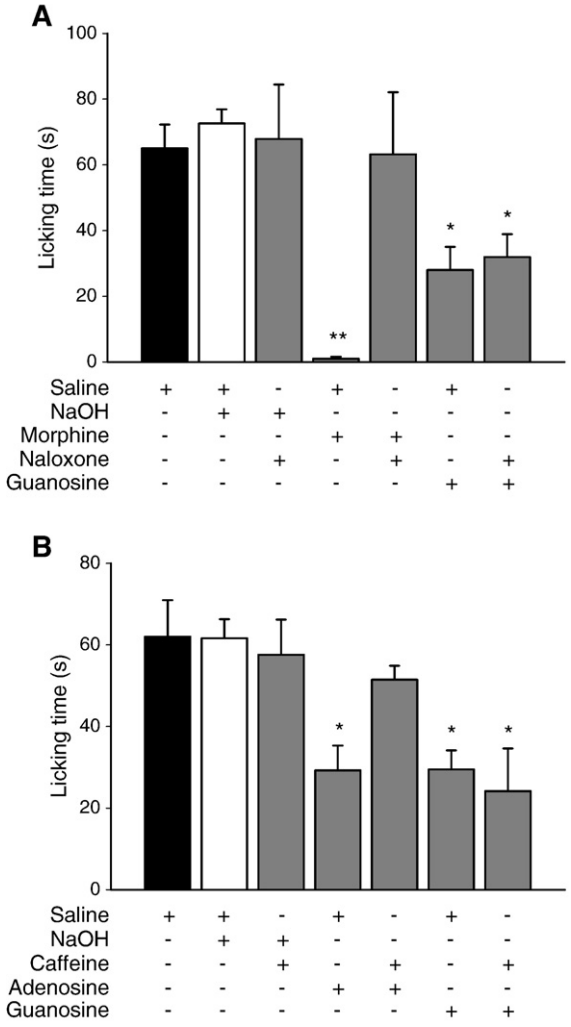

Fig. 3 – Effects of naloxone (12.5 nmol–A) or caffeine (15 nmol–B) pre-treatment on the antinociceptive effects of morphine (10 nmol), adenosine (37.5 nmol), or guanosine (400 nmol) against capsaicin-induced pain. Columns represent mean time spent licking the injected hindpaw and vertical bars represent SEM. N=10 animals per group. *P<0.05 and **P<0.001 compared to vehicle (control), one-way ANOVA followed by Student–Newman–Keuls test.

In the hole-board model, neither i.c.v. guanosine (400 nmol) nor GMP (960 nmol) affected latency to first head-dip and the number of head-dips, crossings, rearings, groomings, and defecations. Guanosine and GMP did not induce motor deficits or ataxia, as evaluated by the performance in the rotarod test. Additionally, neither guanosine nor GMP affected spontaneous locomotor activity as measured by activity cages as shown in Table 1.

CSF concentrations of uric acid, hypoxanthine, xanthine, guanosine, and GMP were significantly increased in mice treated with GMP in comparison with control (Table 2). I.c.v.

Table 1 – Effects of guanosine or GMP on the mice hole-board, rotarod, and spontaneous locomotor activity tests

Treatment	Saline	NaOH	Guanosine	GMP
Latency to head-dip (s)	7.3 (1.5)	6.8 (0.7)	10.6 (2.1)	10.6 (0.7)
Head-dips (n)	61.4 (3.9)	62.0 (6.6)	62.8 (9.7)	71.0 (4.9)
Squares crossed (n)	40.2 (6.4)	35.0 (5.7)	44.7 (5.3)	47.2 (7.7)
Rearings (n)	3.0 (1.1)	2.7 (1.1)	2.1 (0.8)	2.2 (0.7)
Groomings (n)	1.5 (0.3)	1.3 (0.5)	1.0 (0.4)	1.7 (0.4)
Fecal boli (n)	1.0 (0.4)	1.2 (0.5)	1.7 (0.5)	1.5 (0.4)
Latency to fall (s)	60.0 (0)	60.0 (0)	56.7 (2.3)	48.0 (6.0)
Crossings (n)	213.6 (22.6)	162.0 (31.1)	182.0 (40.1)	245.1 (52.0)

Guanosine (400 nmol), GMP (960 nmol), or vehicles (saline or 10 μM NaOH) were i.c.v. administered 5 min prior to the behavior measurements: latency to the first head-dip; head-dips; squares crossed; rearings; groomings; fecal boli; latency to fall (rotarod); number of crossings (spontaneous locomotor activity). Data are mean ± SEM. N = 8 animals per group; one-way ANOVA.

administration of guanosine did not affect GMP and hypoxanthine CSF levels in spite of causing a significant increase in guanosine, xanthine, and uric acid CSF levels. Administration of guanosine and GMP did not affect ATP, ADP, AMP, GTP, GDP, IMP, adenosine, and inosine CSF levels (data not shown).

Fig. 4 shows the effects of guanosine (i.c.v.) followed by capsaicin (i.pl.) on glutamate uptake by mice brain cortical slices. Capsaicin promoted a significant decrease in glutamate uptake, an effect prevented by pre-treatment with guanosine.

3. Discussion

Glutamate plays a crucial role in pain transmission mechanisms (Millan, 1999) and modulation of glutamate receptors may have therapeutic potential for several categories of pain (Herrero et al., 2000). However, a significant obstacle to the therapeutic use of glutamate-receptor antagonists in the management of pain is their potentially serious side effects. Therefore, the search for new agents which impact upon

Table 2 – Concentration of purines in mice CSF 5 min after an i.c.v. injection of vehicle (saline and 10 μM NaOH), guanosine, or GMP

Treatment	Vehicle	Guanosine	GMP
CSF purine concentration (μM)			
Uric acid	1.93 (0.33)	4.64 (0.75)**	3.10 (0.40)*
Hypoxanthine	1.14 (0.13)	0.78 (0.09)	7.12 (0.77)***
Xanthine	4.61 (0.24)	24.78 (0.81)***	116.1 (5.26)***
Guanosine	0.09 (0.01)	18.03 (2.88)***	188.3 (4.36)***
GMP	1.34 (0.15)	1.06 (0.08)	811.2 (93.3)***

Concentration of nucleotides (GMP), nucleosides (guanosine), and oxypurines (xantine, hypoxantine, and uric acid) in mice CSF. Guanosine (400 nmol), GMP (960 nmol), or vehicle (saline or 10 μM NaOH) were i.c.v. administered 5 min prior to CSF sampling. Data are mean (μM) ± SEM. N = 10–12 animals per group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to vehicle (control); one-way ANOVA followed by Student–Newman–Keuls test.

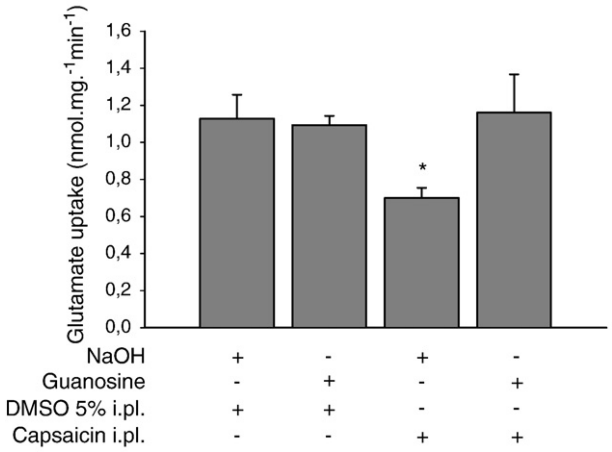

Fig. 4 – Effects of i.c.v. guanosine and i.pl. capsaicin on glutamate uptake by cortical slices from mice. Mice were treated with an i.c.v. injection of guanosine (400 nmol) or vehicle (10 μM NaOH); after 5 min, animals received an i.pl. injection of capsaicin or vehicle (DMSO 5%). After behavioral evaluation, the mice were sacrificed and the cortical slices processed for glutamate uptake assay. Data are mean ± SEM. N = 10 animals per group. *P < 0.05 compared to vehicle (control), one-way ANOVA followed by Student–Newman–Keuls test.

glutamatergic mechanisms remains pivotal in the understanding of pain and the search for innovative analgesics.

Recently, ABPs have been considered important targets for the development of new drugs for treating pain since the nucleoside adenosine and its analogs induce antinociceptive effects following both systemic and central administration (Fastbom et al., 1990; Fredholm, 1995; Mello et al., 1996; Sawynok, 1998; Sawynok and Liu, 2003). Notably, glutamate stimulates the release of adenosine, which acts on presynaptic A_1 receptors, inhibiting glutamate release (Brundege and Dunwiddie, 1997). Since the nucleosides guanosine and adenosine closely interact in modulating the glutamatergic system (Cunha, 2005; Dobolyi et al., 2000), GBPs, and especially guanosine, might well play a significant role in pain transmission and nociception.

In this study, we used anticonvulsant i.c.v. doses of GBPs (guanosine or GMP) to investigate their potential supraspinal antinociceptive effects. Guanosine produced dose-dependent antinociceptive effects in several different nociceptive tests. Guanosine induced-antinociception was consistent in the four pain models used in this study. Nevertheless, all of these analgesimetric procedures are essentially based on acute, short-lasting noxious stimuli. Because different mechanisms are responsible for acute or persistent pain, further experiments are needed to clarify the effects of guanosine on models of chronic persistent pain. As extracellular guanosine results from cellular release and hydrolysis of GMP by ecto-5'-nucleotidase activity (Ciccarelli et al., 1999), we also investigated the effects of GMP. GMP produced antinociceptive effects against capsaicin-induced pain; this effect was prevented by the 5'-nucleotidase inhibitor AOPCP, suggesting that GMP antinociception results from its conversion to guanosine.

Guanosine was previously suggested to be the mediator of GMP actions resulting in stimulation of brain glutamate uptake *in vitro* (Frizzo et al., 2003), anticonvulsant and amnesic effects (Saute et al., 2006; Soares et al., 2004; Schmidt et al., 2005), and the present observation extend this set of actions to nociception. Only guanosine was further used to investigate antinociception properties in the other pain models (tail-flick, hot-plate, and i.pl. glutamate) as well as for the putative mechanism of action. Importantly, a limitation of this study is the lack of availability of guanosine receptor antagonists, which would be useful to verify the requirement of guanosine receptors for the antinociceptive effects of GMP.

Previous studies have suggested involvement of adenosinergic systems in effects of guanosine, but different experimental approaches have yielded conflicting results. Guanosine stimulates the release of adenosine in cultured astrocyte, and both are released under excitotoxic conditions (Ciccarelli et al., 1999, 2001). Guanosine induces proliferative effects that are partially inhibited by adenosine deaminase and adenosine receptor antagonists (Ciccarelli et al., 2000), pointing to the involvement of adenosine on guanosine effects. In contrast, guanosine-induced enhancement of neurite outgrowth in PC12 cells was not affected by adenosine receptor antagonists (Gysbers and Rathbone, 1996) nor were the effect of guanosine on glutamate uptake (Frizzo et al., 2001), seizures induced by quinolinic acid (Lara et al., 2001), or impairment in the inhibitory avoidance task (Roesler et al., 2000; Vinadé et al., 2004). In the present study, since guanosine-induced antinociception was not prevented by caffeine, adenosine A_1 and A_{2A} receptors do not seem to be required. Moreover, i.c.v. guanosine or GMP (which would later produce guanosine) failed to increase adenosine levels in the CSF. Our data also shows that opioid mechanisms are unlikely to be involved in the guanosine-induced antinociception since naloxone does not affect antinociception by guanosine. Considering that a high affinity binding sites for guanosine have been reported in the rat brain (Traversa et al., 2002, 2003), this study reinforce the proposal that guanosine could act independently from the adenosinergic and opioid systems in inhibiting nociception. Notably, i.c.v. administration of guanosine or GMP induced a significant increase in CSF levels of oxypurines, mainly xanthine, which probably indicate an *in vivo* degradation. Although improbable, the production of oxypurines cannot be excluded to play a role in the antinociceptive effects of guanosine.

In vitro, guanosine has been shown to prevent ischemic injury (Frizzo et al., 2002) and NMDA-induced excitotoxicity (Ciccarelli et al., 2001). *In vivo*, acute and chronic administration of guanosine prevents seizures and toxicity induced by drugs that overstimulate the glutamatergic system (Baron et al., 1989; Malcon et al., 1997; Regner et al., 1998; Schmidt et al., 2000; Lara et al., 2001; Vinadé et al., 2003). Although the overall effects of guanosine seem related to attenuating glutamatergic overstimulation, its precise mechanism of action remains unclear. Guanosine is a poor displacer of glutamate ligands (Souza and Ramirez, 1991), so direct antagonism on glutamatergic receptors seems unlikely. Both neurons and astrocytes release guanosine under basal and toxic conditions (Ciccarelli et al., 1999, 2001), and guanosine significantly stimulates glutamate uptake thereby preventing glutamate toxicity (Chen and Swanson, 2003; Frizzo et al., 2001, 2002, 2003). In this study, *in vivo* i.c.v. administration of guanosine prevented the decrease of *in vitro* glutamate uptake induced by i.pl. capsaicin in mice brain slices without affecting basal glutamate uptake. Therefore, it is tempting to speculate that the *in vivo* antinociceptive effect of guanosine against capsaicin could result from its effect on glutamate removal from the synaptic cleft, leading to less activation of non-NMDA and NMDA receptors. These observations so not exclude the involvement of other neurochemical parameters in the guanosine effects.

Although it is now accepted that glutamate plays an essential role in pain transmission at the spinal cord (Herrero et al., 2000), there has been growing evidence that supraspinal glutamatergic transmission is also involved in nociception (Millan, 1999). Additionally, there is compelling evidence suggesting that excitatory amino acids (glutamate and aspartate) are released in the CNS in response to several peripheral noxious stimuli, including i.pl. administration of capsaicin (Ueda et al., 1993). Since the i.pl. capsaicin test involves peripheral and central mechanisms, and the nociceptive response is prevented by glutamate-receptor antagonists (Sakurada et al., 1998), it is not surprising that an i.pl. injection of capsaicin impaired glutamate uptake at a supraspinal level (parietal cortex). It is, however, difficult to evaluate if the guanosine-induced antinociception is related to supraspinal and/or spinal mechanisms, since we here show that i.c.v. guanosine or GMP leads to marked increases in CSF guanosine levels. Because CSF was obtained from *cisterna magna* 5 min after the i.c.v. administration of GMP or guanosine, a wide dispersion of GBPs throughout the CNS (brain and spinal cord) could have occurred. In order to test this proposal, data on the antinociceptive effects of spinal administration of guanosine and its effects on spinal cord glutamate uptake, currently under investigation, will be useful to further clarify this issue.

Regarding side effects, a significant point to be considered is that purines, mainly adenosine, may cause hypotension, sedation, and impaired motor function (Sawynok and Liu, 2003). It is possible that such alterations could have influenced the pain scores here observed. However, our results showed that neither i.c.v. guanosine nor GMP in the doses here used induced obvious behavioral disturbances (hole-board) and altered coordination (rotarod) or locomotion (activity cages) consistent with previously reported observations (Lara et al., 2001; Vinadé et al., 2003).

In summary, this is the first study showing an antinociceptive action by supraspinal administration of GBPs (guanosine and GMP). It provides new evidence on the role of extracellular GBPs, guanosine in particular, in the CNS. Because guanosine is an endogenous compound apparently well tolerated, it could eventually be developed as a drug useful for treating pain related to glutamatergic overstimulation.

4. Experimental procedures

4.1. *Animals*

Male adult Swiss albino mice (30–40 g) were kept on a 12-h light/dark cycle (light on at 7:00 am) at temperature of 22 ± 1 °C

housed in plastic cages (five per cage) with tap water and commercial food *ad libitum*. All behavioral procedures were conducted between 8:00 and 10:00 a.m. In all nociceptive behavioral experiments, the animals were acclimatized to the laboratory for at least 1 h before testing and used only once throughout the experiments. The ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983) and our institutional protocols for experiments with animals, designed to avoid suffering and limit the number of animals sacrificed, were followed throughout. The number of animals and the number of intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

4.2. *Drugs*

Capsaicin, guanosine-5'-monophosphate (GMP), guanosine, adenosine, caffeine, naloxone, glutamate, and α,β-methyleneadenosine 5'-diphosphate (AOPCP), morphine were purchased from Sigma Chemicals (St. Louis, MO, USA). The anesthetic sodium thiopental was obtained from Cristália (SP, Brazil). Guanosine was dissolved in 10 μM NaOH. The amount of NaOH caused no detectable effect. Capsaicin was diluted in DMSO (dimethyl sulfoxide, 5%). All other solutions were dissolved in saline (NaCl 0.9%) and buffered with 0.1 N NaOH or 0.1 N HCl to pH 7.4 when necessary. All other chemicals were of high grade quality.

4.3. *Surgical procedure*

Surgery and i.c.v. infusion techniques were according to Schmidt et al. (2000). Mice were anaesthetized with sodium thiopental (60 mg/kg, 10 ml/kg, i.p.). In an stereotaxic apparatus, the skin of the skull was removed and a 27-gauge 7-mm guide cannula was placed at 1 mm posterior to bregma, 1 mm right from the midline, and 1 mm above the lateral brain ventricle. Through a 2 mm at the cranial bone, the cannula was implanted 1.5 mm ventral to the superior surface of the skull and fixed with jeweler acrylic cement. Experiments were performed 48 h after surgery.

4.4. *Drug administration*

Experiments were performed according to Schmidt et al. (2000): 20 min before the experiment, animals were placed individually in acrylic boxes, which served as observation chambers; treatments followed immediately after this adaptation period. 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 lateral brain ventricle. Animals received the i.c.v. infusion (4 μl) of vehicle (saline or 10 μM NaOH), morphine (10 nmol), guanosine (5 to 400 nmol), GMP (240 to 960 nmol), or adenosine (37.5 nmol). Higher doses of guanosine were limited by its solubility. Other groups were additionally pre-treated (3 min) with a second i.c.v. injection of caffeine (15 nmol), naloxone (12.5 nmol), or AOPCP (1 nmol). Caffeine, adenosine, and AOPCP doses were adapted from Fredholm (1995), Ushijima et al. (1992), and Saute et al. (2006).

4.5. *Capsaicin-induced nociception*

The method is similar to that described by Sakurada et al. (1993). Five minutes after i.c.v. treatments, 20 μl of capsaicin (1.6 μg/paw) was injected intraplantarly (i.pl.) under the plantar skin of the right hindpaw (Hamilton microsyringe with a 26-gauge needle). Animals were observed individually for 5 min after capsaicin for the time spent in licking the injected paw, considered as indicative of nociception.

4.6. *Glutamate-induced nociception*

The procedure was similar to Beirith et al. (2002). Five minutes after the i.c.v. treatments, 20 μl of glutamate solution (10 μmol/paw prepared in saline) was injected i.pl. under the plantar skin of the right hindpaw. The mice were observed individually for 15 min following glutamate for the amount of time spent in licking the injected paw, considered indicative of nociception.

4.7. *Tail-flick*

Nociception was assessed with a tail-flick apparatus (Albrasch Electronic Equipments, Brazil), as described in detail elsewhere (D'Amour and Smith, 1941). A source of light was positioned below the tail, focused on a point 2.3 cm rostral to the tip of the tail and the time that the mouse took to withdraw its tail from the noxious stimulus was recorded. Deflection of the tail activates a photocell and automatically terminated the trial. The light intensity was adjusted in order to obtain baseline tail flick latency (TFL) of 3–4 s. A cutoff time of 10 s was employed in order to prevent tissue damage (a mouse that did not flick by 10 s was considered as fully analgesic). On day 1, the animals were habituated with the tail flick apparatus through three separate measures (data not shown). On day 2, baseline tail flick latency was measured for each mouse prior to the treatments. Animals displaying at least two TFL of 10 s on the baseline were excluded from the study. Immediately after the third TFL measurement, animals received i.c.v. treatments and 5 min thereafter were submitted to the tail flick apparatus for TFL measurement. Data are expressed as the mean percent of maximum possible effect (%MPE) ± SEM according to the following formula (Calcagnetti et al., 1990): %MPE: 100 × (postdrug latency − baseline latency)/(cutoff time − baseline latency).

4.8. *Hot-plate*

Response latencies were measure according to Eddy and Leimback (1953), with minor modification. Animals were placed into a glass cylinder of 24-cm diameter on the heated (55 ± 0.5 °C) Hot Plate apparatus (Ugo Basile, model-DS 37, Italy) surface. The time between placement of the animal on the hot-plate and the occurrence of licking of the hindpaws or jumping off the surface was recorded as response latency. On day 1, the animals were habituated with the turned off apparatus. On day 2, mice were tested and animals displaying baseline latencies higher than 10 s were excluded; an automatic 20-s cutoff was used to prevent tissue damage.

Each animal was tested before administration of drugs in order to obtain the baseline; 5 min after i.c.v. treatments, animals were placed on the heated surface and response latency recorded as described above. Data are expressed as mean percent of maximum possible effect (%MPE) ± SEM, according to the following formula (Calcagnetti et al., 1990): %MPE: 100 × (postdrug latency − baseline latency) / (Cutoff time − baseline latency).

4.9. *Hole-board*

The hole-board apparatus (Ugo Basile, 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 automatically recorded 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. Five minutes after i.c.v. treatments, 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 the locomotion (Vinadé et al., 2003).

4.10. *Motor performance*

In order to evaluate non-specific muscle relaxant or neurotoxic effects, we evaluated the effects of GBPs in the rotarod test and on the spontaneous locomotion. The rotarod apparatus (Ugo Basile, Italy) consisted of a rotating (18 rpm) bar (2.5 cm diameter), subdivided by disks into six compartments. As previously described (Leal et al., 2000), mice were initially trained to remain on the rotarod apparatus for 120 s; those not remaining on the bar for at least two out of three consecutive trials were discarded. On the day after training, the latency to fall from the rotarod (one trial with a maximum of 60 s) was determined 5 min after i.c.v. treatments. The method for the spontaneous locomotion was adapted from Creese et al. (1976). Activity cages (45 × 25 × 20 cm, Albarsch Electronic Equipment, Brazil), equipped with three parallel photocells, automatically record the number of crossings. Animals were individually habituated to an activity cage for 10 min before receiving the i.c.v. treatments. The animals returned to the activity cages 5 min after i.c.v. treatments, and the crossings were recorded for 15 min.

4.11. *Cerebrospinal fluid (CSF) sampling*

Mice were treated with i.c.v. administration of vehicle (saline or 10 μM NaOH), guanosine (400 nmol), or GMP (960 nmol). After 5 min, mice were anesthetized with sodium thiopental (60 mg/kg, 10 ml/kg, i.p.) and placed in a stereotaxic apparatus, where the CSF was drawn (10–20 μl per mouse) by direct puncture of the *cisterna magna* with an insulin syringe (27 gauge × 1/2 in length), with the help of a magnifying glass. All samples were centrifuged at 10 000×*g* in an Eppendorf centrifuge during 5 min to obtain cell-free supernatants and stored in separate tubes at −70 °C until analysis were conducted.

4.12. *HPLC procedure*

High-performance liquid chromatography (HPLC) was performed with CSF cell-free supernatants aliquots for determination of purines concentration, according to Domanski et al. (2006). CSF concentrations of the following purines were determined: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine, inosine monophosphate (IMP), inosine, hypoxanthine, xanthine, and uric acid. Analyses were performed with Shimadzu Class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto injector valve with 50 μl loop, and an UV detector. Separations were achieved on a Supelco C18 250 mm × 4.6 mm, 5 μm particle size column. The mobile phase flowed at a rate of 1.2 ml/min and the column temperature was 24 °C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 10 μl were injected into the injection valve loop. Absorbance was read at 254 nm. CSF concentrations of purines are expressed as mean ± SEM in μM.

4.13. *Glutamate uptake*

Mice were treated with an i.c.v. injection of guanosine (400 nmol) or vehicle (10 μM NaOH); after 5 min, animals received an i.pl. injection of capsaicin or vehicle (DMSO 5%). Five minutes after vehicle or capsaicin i.pl. infusion, animals were decapitated, their brains were removed immediately and submerged in Hank's balanced salt solution (HBSS) containing (in mM) 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, and 5.55 glucose, adjusted to pH 7.2. Cortices were dissected into a Petri dish filled with HBSS (room temperature) to obtain the parietal area. Coronal slices (0.4 mm) were obtained using a McIlwain tissue chopper and sections were separated with the help of a magnifying glass. Cortical slices were then transferred to 24-multiwell dishes, containing 500 μl of HBSS solution and pre-incubated for 15 min. Subsequently, cortical slices were washed with 1 ml HBSS, and the pre-incubating medium was immediately replaced by 280 μl HBSS (35°C) (total uptake). Uptake was assessed by addition of 20 μl HBSS containing 0.33 μCi/ml L-[^{3}H]glutamate with 100 μM unlabeled glutamate (final concentration) at 35°C. Incubation was stopped after 7 min by two ice-cold washes with 1 ml HBSS immediately followed by the addition of 0.5 N NaOH, which was kept overnight. Aliquots of lysates were taken for determination of intracellular content of L-[^{3}H]glutamate through scintillation counting. To determine the sodium-dependent glutamate uptake, parallel assays were done under ice using *N*-methyl-D-glucamine instead of sodium chloride in the incubation medium, being subtracted from the uptake at 35 °C with sodium chloride. Protein was measured using the method of

Lowry et al. (1951) using bovine albumin as standard. The experiments were done in triplicate.

4.14. *Statistical analysis*

Data are expressed as mean ± standard error of the mean (SEM), except the ID_{50} values (i.e., the dose of guanosine or GMP necessary to reduce the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} value was determined by linear regression from individual experiments using linear regression GraphPad software (GraphPad software, San Diego, CA, USA). Data were submitted to Kolmogorov–Smirnov, Levene, and Bartlett tests for normality evaluation. Differences among groups were determined by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls test when applicable. $P<0.05$ was considered of statistical significance.

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II.2.c. The NMDA antagonist MK-801 induces hyperalgesia and increases CSF excitatory amino acids in rats: Reversal by guanosine

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The NMDA antagonist MK-801 induces hyperalgesia and increases CSF excitatory amino acids in rats: Reversal by guanosine

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ABSTRACT

Excitatory amino acids (EAAs) and their receptors play a central role in the mechanisms underlying pain transmission. NMDA-receptor antagonists such as MK-801 produce antinociceptive effects against experimental models of chronic pain, but results in acute pain models are conflicting, perhaps due to increased glutamate availability induced by the NMDA-receptor antagonists. Since guanosine and riluzole have recently been shown to stimulate glutamate uptake, the aim of this study was to examine the effects of guanosine or riluzole on changes in nociceptive signaling induced by MK-801 in an acute pain model. Rats received an i.p. injection of vehicle, morphine, guanosine, riluzole or MK-801 or a combined treatment (vehicle, morphine, guanosine or riluzole+MK-801) and were evaluated in the tail flick test, or had a CSF sample drawn after 30 min. Riluzole, guanosine, and MK-801 (0.01 or 0.1 mg/kg) did not affect basal nociceptive responses or CSF EAAs levels. However, MK-801 (0.5 mg/kg) induced hyperalgesia and increased the CSF EAAs levels; both effects were prevented by guanosine, riluzole or morphine. Hyperalgesia was correlated with CSF aspartate and glutamate levels. This study provides additional evidence for the mechanism of action of MK-801, showing that MK-801 induces hyperalgesia with parallel increase in CSF EAAs levels.

1. Introduction

N-methyl-D-aspartate (NMDA) receptors and the excitatory amino acids (EAAs) aspartate and glutamate are implicated in the generation and maintenance of central states of hypersensitivity, whereas glutamate antagonists may prevent hyperalgesia or an enhanced pain state (Bennett, 2000). A number of studies have demonstrated that NMDA antagonists can decrease nociceptive behaviors in animal models of neuropathic pain and potentiate the analgesic effects of opiates (Fisher et al., 2000). However, the effects of NMDA antagonists on acute nociception and the neurobiological mechanisms that mediate their action are still unclear, and of conflicting results have been reported (Al-Amin et al., 2003). Moreover, noncompetitive NMDA-receptor antagonists, such as MK-801, induce several unusual behaviors in rodents, such as hyperlocomotion, stereotyped movements, ataxia, and amnesia (Dall'Igna et al., 2003; Tort et al., 2004). Interestingly, evidence suggests that noncompetitive antagonism of NMDA receptors is also associated with a paradoxical glutamatergic activation of non-NMDA receptors induced by increased glutamate availability, which could underlie the behavioral effects observed (Moghaddam et al., 1997; Tort et al., 2004). However, the role of glutamate and aspartate in the effects of NMDA antagonists has not been studied in pain models to our knowledge.

Extracellular guanine-based purines (GBPs), mainly the nucleoside guanosine, have been shown to exert biological effects not directly related to G-protein 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). In vitro, GBPs inhibit the binding of glutamate and its analogs, prevent cell responses to excitatory amino acids, and present neuroprotective effects in several brain preparations submitted to excitotoxic conditions (Baron et al., 1989; Burgos et al., 1998; Caciagli

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et al., 2000; Frizzo et al., 2001, 2002, 2003; Souza and Ramirez, 1991). In vivo, GBPs prevent seizures induced by glutamatergic agents and present amnesic effects in rodents (Lara et al., 2001; Schmidt et al., 2000, 2005; Soares et al., 2004; Vinadé et al., 2003, 2005). Several studies indicate that these anti-glutamatergic effects seem to be directly related to a guanosine-induced glutamate removal from the synaptic cleft (glutamate uptake) (Frizzo et al., 2001, 2002, 2003; Schmidt et al., 2007; Soares et al., 2004).

Given the pivotal role of EAAs and their receptors in the mechanisms underlying pain transmission, the occurrence of paradoxical behavioral effects of NMDA-receptor noncompetitive antagonists, and the reported antagonism of glutamatergic activity by guanosine, we studied the effects of systemic administration of guanosine and the noncompetitive NMDA-receptor antagonist MK-801 on pain in the tail flick model in rats. Riluzole, a well-known glutamate release inhibitor and glutamate uptake stimulator (Frizzo et al., 2004), and morphine, a well-known opioid receptor agonist, were used as positive controls. Additionally, the effects of guanosine, riluzole, morphine and MK-801 on cerebrospinal fluid (CSF) EAAs levels were determined.

2. Materials and methods

2.1. Animals

Male adult Wistar rats (3–4 months of age, 250–350 g) were used. Animals 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 pellets ad libitum. All behavioral procedures were conducted between 8:00 and 10:00 am. The ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983) and our institutional protocols for experiments with animals, designed to avoid suffering and limit the number of animals sacrificed, were followed throughout. This study was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

2.2. Drugs

Guanosine was purchased from Sigma Chemicals (St Louis, MO, USA). 5-Methyl-10-11-dihydro-5*H*-dibenzo[*a*,*b*]cyclohepta-5-10-imine maleate (MK-801 or dizocilpine) was obtained from RBI-Research Biochemicals (Natick, MA, USA). Riluzole was obtained from Tocris (Ballwin, MO, USA) and morphine from Cristália (São Paulo, Brazil).

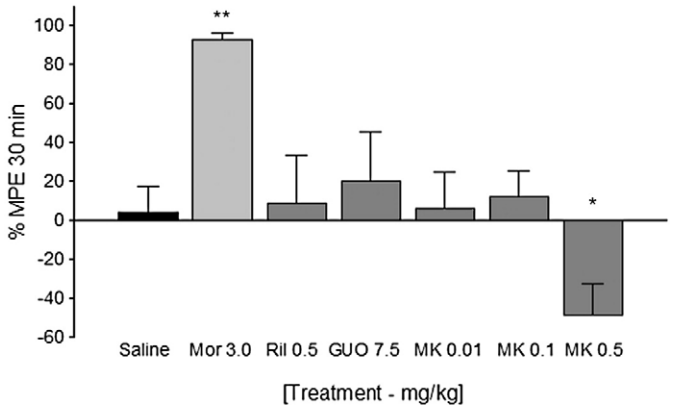

Fig. 1. Effects of i.p. vehicle (saline 0.9%), morphine (Mor — 3 mg/kg), riluzole (Ril – 0.5 mg/kg), guanosine (GUO – 7.5 mg/kg), and MK-801 (MK — 0.01, 0.1 and 0.5 mg/kg) on tail flick latency 30 min after treatments. The columns represent % of Maximum Possible Effect (% MPE) and vertical bars represent SEM. $N=20$ animals per group. * = $P<0.01$ and ** = $P<0.001$ compared to vehicle (control), Kruskal–Wallis followed by Dunn's test.

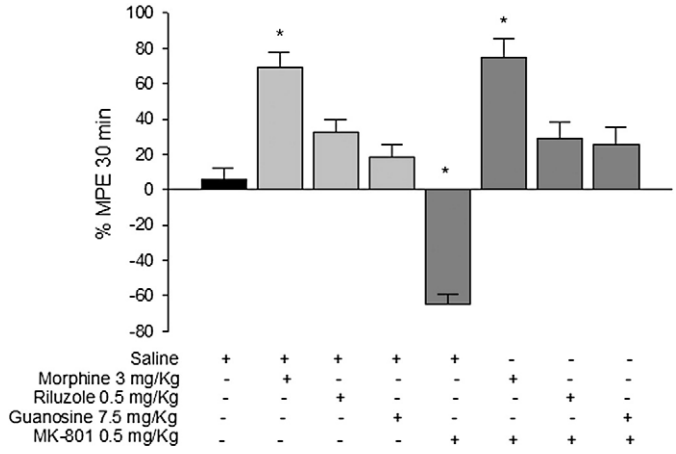

Fig. 2. Effects of i.p. vehicle (saline 0.9%)+vehicle, morphine (3 mg/kg)+vehicle, riluzole (0.5 mg/kg)+vehicle, guanosine (7.5 mg/kg)+vehicle, vehicle+MK-801 (0.5 mg/kg), riluzole+MK-801 or guanosine+MK-801 on tail flick latency 30 min after treatments. The columns represent % of Maximum Possible Effect (% MPE) and vertical bars represent SEM. $N=20$ animals per group. * = $P<0.01$ compared to control (saline), Kruskal–Wallis followed by Dunn's test.

2.3. Tail flick test

Nociception was assessed with a tail flick apparatus (Albrasch Electronic Equipments), as described in detail elsewhere (D'Amour and Smith, 1941). Briefly, a source of heat was positioned above the tail, focused on a point 2.3 cm rostral to the tip of the tail and the latency to withdraw the tail from the noxious luminous stimulus was automatically recorded. The light intensity was adjusted in order to obtain a baseline tail flick latency (TFL) of 3–4 s; a cut-off time of 10 s was employed in order to prevent tissue damage. A rat that did not flick by 10 s was considered as fully responsive to the analgesic. On day one, the animals were habituated with the tail flick apparatus through three separate measures (data not shown). On day two, baseline tail flick latency was measured for each rat prior to the treatments. Immediately after the third TFL measurement, the animals received an i.p. injection of vehicle (saline — NaCl 0.9%), morphine (3 mg/kg), guanosine (0.75, 2.5, or 7.5 mg/kg), MK-801 (0.01, 0.1 or 0.5 mg/kg) or riluzole (0.5 mg/kg). After 30 min, three new tail flick measures were taken at the tail flick apparatus. A separate group of animals received a pretreatment with vehicle, morphine (3 mg/kg), riluzole (0.5 mg/kg), or guanosine (0.75, 2.5 or 7.5 mg/kg) 15 min before MK-801 (0.5 mg/kg) administration. These groups were submitted to the tail flick 30 min after MK-801 administration. MK-801 doses and the 30 min endpoint for tail flick were adapted from elsewhere (Schmidt et al., 2000; Lara et al., 2001; Tort et al., 2004). Data are expressed as mean percent of Maximum Possible Effect (% MPE)±SEM, according to the following formula (Calcagnetti et al., 1990): % MPE: 100×(post drug latency−baseline latency)/(cut-off time−baseline latency).

2.4. CSF sampling

Groups of rats were treated similarly with i.p. administration of vehicle, morphine (3 mg/kg), riluzole (0.5 mg/kg), or guanosine (0.75, 2.5 or 7.5 mg/kg) 15 min before MK-801 i.p. administration; 30 min after MK-801 treatment, rats were anesthetized with sodium thiopental (40 mg/kg, i.p.), placed in a stereotaxic apparatus, and CSF samples (40–60 µL per rat) were drawn by direct puncture of the cisterna magna with an insulin syringe (27 gauge×1/2 in length) (Portela et al., 2002). In order to obtain cell-free supernatants, all samples were centrifuged at 10,000 g

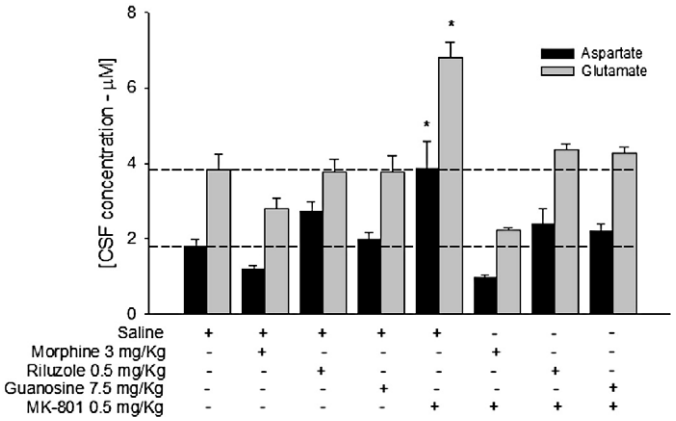

Fig. 3. Effects of i.p. vehicle (saline 0.9%)+vehicle morphine (3 mg/kg)+vehicle, riluzole (0.5 mg/kg)+vehicle, guanosine (7.5 mg/kg)+vehicle, vehicle+MK-801 (0.5 mg/kg), morphine+MK-801, riluzole+MK-801, or guanosine+MK-801 on CSF levels of aspartate and glutamate 30 min after treatments. The columns represent means (µM) and vertical bars represent SEM. Dashed lines represent control values. $N=10$ animals per group. * = $P<0.01$ compared to control (Veh+Veh), ANOVA followed by Tukey–Kramer's test.

in an Eppendorf centrifuge during 5 min and samples stored (−70 °C) until EAAs quantification by HPLC.

2.5. HPLC procedure

High-performance liquid chromatography (HPLC) was performed with CSF cell-free supernatant aliquots to quantify aspartate and glutamate levels (according to Joseph and Marsden, 1986). Briefly, samples were derivatized with *o*-phthalaldehyde and separation was carried out with a reverse phase column (Supelcosil LC-18, 250 mm× 4.6 mm, Supelco) in a Shimadzu Instruments liquid chromatograph (50 µL loop valve injection). The mobile phase flowed at a rate of 1.4 mL/min and column temperature was 24 °C. Buffer composition is A: 0.04 mol/L sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 20% of methanol; B: 0.01 mol/L sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 80% of methanol. The gradient profile was modified according to the content of buffer B in the mobile phase: 0% at 0.00 min, 25% at 13.75 min, 100% at 15.00–20.00 min, 0% at 20.01–25.00 min. Absorbance was read at 360 nm and 455 nm, excitation and emission respectively, in a Shimadzu fluorescence detector. Samples of 50 µL were used and concentration was expressed in µM (as mean±SEM).

2.6. Statistical analysis

Kruskal–Wallis followed by the post-hoc Dunn multiple comparisons test with chi-square distribution was used for behavioral data. One-way ANOVA followed by the post-hoc Tukey–Kramer multiple comparisons test was used for neurochemical data. Correlations are presented as Pearson's coefficient. $P<0.05$ was considered for statistically significant differences.

3. Results

Fig. 1 shows the effects of each drug alone on tail flick latency in rats. As expected, morphine (3.0 mg/kg), which was taken as a positive control, suppressed nociceptive responses in the tail flick test ($P<0.001$). Neither guanosine (0.75, 2.5 or 7.5 mg/kg), MK-801 (0.1 or 0.01 mg/kg) nor riluzole (0.5 mg/kg) treatments altered tail flick latency in rats. However, MK-801 (0.5 mg/kg) induced an increase in nociceptive response ($P<0.01$), compatible with a hyperalgesic profile. As can be observed in Fig. 2, pretreatment with morphine (3 mg/kg), riluzole (0.5 mg/kg) or guanosine (7.5 mg/kg) prevented the MK-801-induced hyperalgesia ($P<0.01$). Note that guanosine and riluzole specifically counteracted MK-801-induced hyperalgesia, as their pain scores were not different from saline controls, in contrast to morphine, which caused analgesia (Fig. 2). Guanosine (0.75 or 2.5 mg/kg) also partially prevented MK-801-induced hyperalgesia (% MPE = 11.5±7.1 and 21.6±7.0, respectively – $P<0.05$ – data not shown).

Fig. 3 shows that MK-801 induces a significant increase of CSF aspartate and glutamate levels, an effect completely prevented by pretreatment with morphine (3 mg/kg), guanosine (7.5 mg/kg) or riluzole (0.5 mg/kg) in doses that per se do not affect CSF aspartate and glutamate levels. Guanosine (0.75 or 2.5 mg/kg) also prevented MK-801-induced increase of CSF aspartate and glutamate levels (guanosine 0.75 mg/kg: 2.5±0.9 µM and 4.1±0.4 µM, respectively — $P<0.05$ and guanosine 2.5 mg/kg: 1.9±0.5 µM and 5.1±0.5 µM, respectively — $P<0.05$). MK-801 (0.01 or 0.1 mg/kg) did not alter CSF aspartate and glutamate levels.

Fig. 4 shows that there was a statistically significant correlation between CSF aspartate and glutamate levels and pain scores in animals that received MK-801 treatment with vehicle, morphine, guanosine or riluzole pretreatment (Pearson's coefficient $r=0.556$;

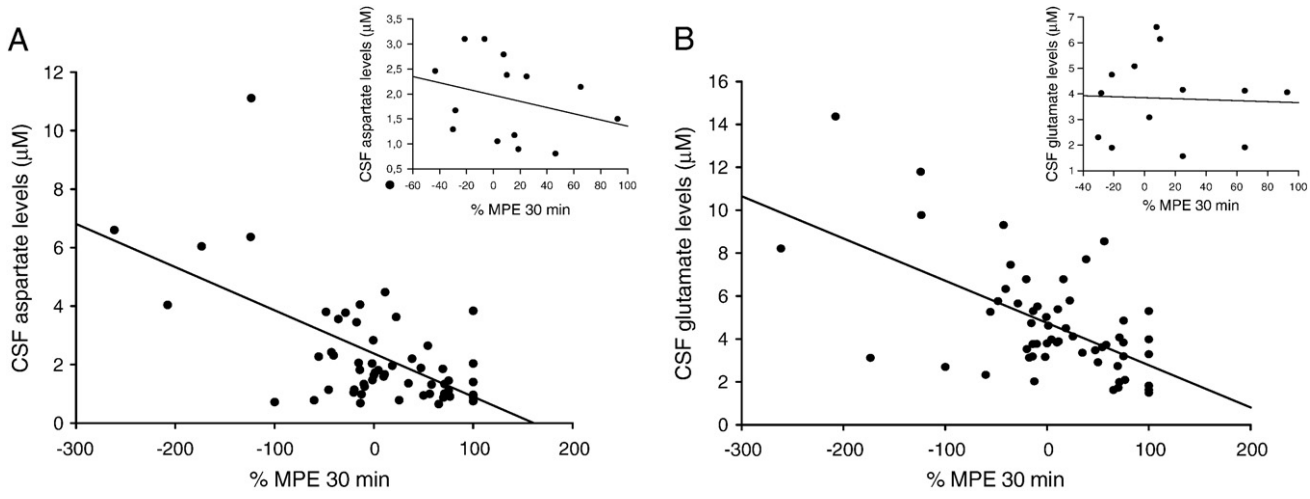

Fig. 4. Correlation between CSF levels of aspartate or glutamate and % of Maximum Possible Effect (% MPE) on the tail flick test. (A) Aspartate CSF levels (Pearson's coefficient $r=0.556$; $P<0.0001$) (insert: control values — Pearson's coefficient $r=0.29$; $P=0.314$); (B) glutamate CSF levels (Pearson's coefficient $r=0.588$; $P<0.0001$) (insert: control values — Pearson's coefficient $r=0.046$; $P=0.88$).

$P<0.0001$ and $r=0.588$; $P<0.0001$, respectively). This correlation was not observed in the control group (Pearson's coefficient $r=0.29$; $P=0.314$ and $r=0.046$; $P=0.88$, respectively).

4. Discussion

The administration of MK-801 (0.5 mg/kg) to rats induced a hyperalgesic state in the tail flick test; this was prevented by pretreatment with morphine, guanosine or riluzole. Guanosine, riluzole or low doses of MK-801 did not affect the tail flick latency. Furthermore, this high dose of MK-801 induced an increase in CSF glutamate and aspartate levels; this was also prevented by pretreatment with morphine, guanosine or riluzole. A significant correlation was found between increased levels of CSF aspartate and glutamate and hyperalgesia induced by MK-801. These results support the notion that a CNS EAAs release may contribute to the atypical behavioral effects commonly induced by moderately high doses of MK-801.

The noncompetitive NMDA antagonist MK-801 has been shown to reduce nociceptive response in neuropathic pain models (Fisher et al., 2000). However, consistent with our data, some previous reports suggest that the NMDA antagonists are ineffective in the tail flick test (Lutfy et al., 1997; Suh et al., 2000; Zhao and Kamei, 1996). Activation of the non-NMDA receptors is necessary for transmission of phasic pain, whereas, activation of NMDA and/or non-NMDA receptors may be involved in mediation of tonic/chronic pain states (Lutfy et al., 1997).

Despite reducing glutamate neurotransmission at NMDA receptors, MK-801 may promote increased activation of non-NMDA receptors consequent to an increased efflux and reduced uptake of EAAs in the CNS (Longuemare et al., 1996). It has been suggested that the EAAs efflux could result from disinhibition of GABAergic or other inhibitory inputs to glutamatergic neurons (Moghaddam et al., 1997; Tort et al., 2004), or from effects on astrocytes not related to the NMDA receptor (Longuemare et al., 1996). Accordingly, Moghaddam et al. have demonstrated that NMDA antagonists provoke an increase in EAAs efflux in prefrontal cortex (PFC) and nucleus accumbens (NAc) (Moghaddam et al., 1997). In this context, non-NMDA-receptor antagonists, or inhibitors of glutamate/aspartate release and/or stimulators of glutamate uptake such as lamotrigine and riluzole have been shown to counteract the behavioral and neurochemical effects of NMDA-receptor antagonists (Anand et al., 2000).

In vitro, high doses of MK-801 inhibit glutamate uptake by both astrocyte and neuronal cultures and induce glutamate efflux from astrocytes. These effects seem to be related to a MK-801-induced membrane depolarization (Longuemare et al., 1996; Moghaddam et al., 1997). Previous reports have demonstrated that pathological conditions not only reduce the electrochemical driving force for glutamate uptake but also stimulate reversal of the transporter such that glutamate is released from the intracellular space to the extracellular space along with its concentration gradient (Longuemare et al., 1996). It is therefore possible that disruption of glutamate transport plays an important role in the increased extracellular EAAs availability and in the neurotoxic effects of MK-801. Likewise, the effects of high doses of MK-801 on EAAs uptake and release by glia and neurons (which could explain the increase in CSF EAAs levels) may contribute to the development of hyperalgesia in the tail flick test in the present study.

The nucleosides guanosine and adenosine interact closely in modulating the glutamatergic system. Given the central role of adenosine in pain transmission and modulation (Sawynok and Liu, 2003), a role for guanosine in pain transmission and nociception could also be considered. In the present study, guanosine did not alter nociception in the tail flick test. The tail flick test is a model designed to investigate phasic pain, mainly peripheral and spinally mediated responses (Lutfy et al., 1997). It remains to be determined whether guanosine has antinociceptive effects in chronic and neuropathic pain models, which actively depend on glutamatergic neurotransmission.

Guanosine and other guanine-based purine effects on the brain have been studied in several in vivo and in vitro models; they exhibit inhibition of glutamate (and analogs) binding (Baron et al., 1989; Souza and Ramirez, 1991), neuroprotection against excitotoxicity (Frizzo et al., 2002; Malcon et al., 1997; Regner et al., 1998), and anticonvulsant properties against glutamatergic agent-induced seizures in rodents (Lara et al., 2001; Schmidt et al., 2000, 2005; Soares et al., 2004; Vinadé et al., 2003, 2005). These anti-glutamatergic effects of guanosine are likely to result from glutamate removal from the synaptic cleft, an outcome of the guanosine-induced stimulation of astrocytic glutamate uptake (Frizzo et al., 2001, 2002, 2003). Since NMDA antagonists such as MK-801 may stimulate the efflux of glutamate and inhibit its uptake, with the overall effect contributing to the hyperalgesia observed in the tail flick test, we suggest that guanosine prevents MK-801-induced hyperalgesia by enhancing glutamate removal from the synaptic cleft, leading to less activation of non-NMDA glutamatergic receptors. Accordingly, we have previously shown that guanosine attenuates MK-801-induced potentiation of kainate toxicity (Lara et al., 2001) and selectively inhibits MK-801-induced hyperlocomotion without any effect on hyperlocomotion produced by amphetamine or caffeine (Tort et al., 2004). Deutsch et al. (2008) have recently shown that guanosine reduces MK-801-induced increase in voltage threshold for electrically-precipitated tonic hindlimb extension in unstressed mice. This modulatory effect was interpreted as resulting from an increased astrocytic glutamate uptake induced by guanosine and subsequent reduced proportion of open NMDA receptor-associated ion channels. It is noteworthy that MK-801 is an “open-channel blocker” and that its pharmacological effects are closely dependent on its access to the open-state channel where it binds to a specific hydrophobic domain (Deutsch et al., 2001). As we recently suggested (Schmidt et al., 2008), guanosine-induced enhancement of glutamate removal from the synaptic cleft, and consequent decrease of paradoxical non-NMDA glutamate receptors activation by MK-801, are alternative interpretations of such results.

Similarly to guanosine, riluzole stimulates glutamate uptake in rat spinal cord synaptosomes (Azbill et al., 2000). This raises the possibility that stimulated glutamate uptake, in addition to an inhibitory action on glutamate release, could contribute to the reversal of MK-801-induced hyperalgesia by riluzole showed here. This hypothesis was supported by the clear increase in CSF EAAs levels observed after high dose of MK-801, which was completely prevented by pretreatment with guanosine or riluzole. An additional interpretation of our results is that the nociceptive input, enhanced by MK-801, specifically caused the increased CSF EAAs, since pretreatment with morphine, an opioid receptor agonist, prevented MK-801-induced behavioral and neurochemical effects. However, there has been growing evidence that opioids also modulate the glutamatergic system, indirectly affecting glutamate receptors, transporters and its extracellular availability (Abarca et al., 2000; Niederberger et al., 2003; Schmidt and Schmidt, 2002; Yamamoto et al., 2003). Therefore, new studies are needed to further clarify the exact correlation between MK-801-induced neurochemical (increased extracellular EAAs availability) and atypical behavioral effects.

In conclusion, this study provides additional evidence for the mechanism of action of MK-801 in the CNS, showing that MK-801 induces hyperalgesia, with parallel increase in CSF EAAs levels in rats. These results suggest that some of the atypical behaviors induced by MK-801, and perhaps other NMDA antagonists, is related to a paradoxical increase of EAAs in the CNS. The prevention of MK-801 effects by guanosine, riluzole or morphine could result, at least partially, from a decrease in extracellular glutamate and aspartate availability. Since guanosine is an endogenous compound and apparently well tolerated and devoid of obvious CNS toxicity, we suggest that the usefulness of guanosine in the management of pain states associated with overstimulation of the glutamatergic system deserves to be further investigated.

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II.2.d. Guanosine and its modulatory effects on the glutamatergic system

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LETTER TO THE EDITOR

Guanosine and its modulatory effects on the glutamatergic system

To the Editor,

We read with great interest the elegant manuscript by Deutsch et al. 2008 showing that guanosine is capable of reducing the ability of MK-801 (a non-competitive NMDA receptor antagonist) to raise the threshold voltage for electrically-precipitated tonic hindlimb extension in unstressed mice. The authors argue that this modulatory effect may be due to guanosine's removal of glutamate from the synaptic cleft by astrocytes, resulting in a reduced proportion of NMDA receptor-associated ion channels in the open configuration. The authors also emphasize that MK-801 is an "open-channel blocker" and its pharmacological effects are closely dependent on its entering the open channel and binding to a specific hydrophobic domain (Deutsch et al., 2001). However, we propose here a supplementary explanation for their results (Deutsch et al., 2008) and similar previous findings (Tort et al., 2004).

Non-competitive NMDA receptor antagonists such as MK-801, phencyclidine (PCP) and ketamine produce several behavioral effects in rodents, such as hyperlocomotion, stereotyped movements, ataxia, and amnesia (Dall'Igna et al., 2003). In humans, the effects of NMDA antagonists closely resemble that observed in schizophrenic patients, making NMDA receptor antagonism one of the best pharmacological models for this disorder (Tort et al., 2004). Although Deutsch and coauthors point out that the primary site of action of MK-801 is the NMDA receptor in an "open-channel configuration", the indirect contribution of other receptors, neurotransmitters and neuromodulators to the expression of its behaviors could not be fully ruled out. This might be especially important when interpreting guanosine effects on MK-801-induced behavioral disturbances.

Recent evidence suggest that NMDA receptor antagonism is also associated with glutamatergic activation of non-NMDA glutamatergic receptors induced by increased glutamate release, which appears to be closely related to the behavioral alterations observed (Moghaddam et al., 1997). Moghaddam and coworkers have further characterized the neurochemical and behavioral effects of non-competitive NMDA antagonists, demonstrating that these compounds induce an increase in the efflux of glutamate in prefrontal cortex and nucleus accumbens (Moghaddam et al., 1997; Takahata and Moghaddam, 2003). Therefore, despite of reducing glutamatergic effects at NMDA receptors, MK-801 may stimulate non-NMDA receptors by increasing the release of glutamate. These effects could be related to MK-801-induced blockade of GABAergic or other inhibitory inputs to glutamatergic neurons, strengthening the glutamatergic neurotransmission (Takahata and Moghaddam, 2003). Moreover, non-NMDA antagonists and inhibitors of glutamate release such as lamotrigine and riluzole have been shown to counteract the behavioral and neurochemical effects of non-competitive NMDA receptor antagonists (Anand et al., 2000). Although speculative, it is thus possible that such an indirect non-NMDA stimulation effect might also be underlying the MK-801 action on the paradigm employed by Deutsch et al.

In the past few years, several *in vitro* and *in vivo* antiglutamatergic effects of guanosine and other guanine-based purines have been extensively demonstrated, as recently reviewed in detail elsewhere (Schmidt et al., 2007). Briefly, we and others have shown that guanosine may be a neuroprotective endogenous compound released under excitotoxic conditions, since it protected brain slices exposed to hypoxia/hypoglycemia (Frizzo et al., 2002), prevented NMDA-induced toxicity in neurons (Caciagli et al., 2000), and induced trophic effects on neural cells (Rathbone et al., 1999). Additionally, we have demonstrated that guanosine is anticonvulsant against glutamatergic agents and amnesic at the inhibitory avoidance task in rodents (Schmidt et al., 2000; 2005; Roesler et al., 2000). Regarding the mechanism of action of guanosine, a direct antagonism of glutamatergic effects is unlikely, since guanosine is a poor displacer of glutamatergic ligands (Souza and Ramirez, 1991). However, as stated by Deutsch and coauthors, guanosine has been shown to promote, mainly in excitotoxic conditions, astrocytic uptake of glutamate, which is known to play a major role in maintaining extracellular glutamate concentration below neurotoxic levels (Schmidt et al., 2007). Guanosine's effects are probably related to its interaction with specific binding sites on brain cell membranes, as previously demonstrated (Traversa et al., 2002; 2003).

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Since NMDA receptor antagonists induce several locomotor effects caused at least partially by a paradoxical increase in glutamate release (Adams and Moghaddam, 2001) and given guanosine effects on the glutamate uptake by astrocytes, we recently investigated the effects of guanosine on hyperlocomotion induced by MK-801 in mice (a pharmacological model of schizophrenia). We showed that guanosine produces a ~60% attenuation of hyperlocomotion induced by MK-801, whereas it does not affect the hyperlocomotion induced by the indirect dopamine agonist amphetamine or by the non-selective adenosine-receptor antagonist caffeine (Tort et al., 2004). Of note, we have recently observed that guanosine is also able to prevent a paradoxical hypernociceptive effect induced by systemic high-dose MK-801 in rats as evidenced by the tail-flick test [unpublished data].

Considering Deutsch's and our results together, we hypothesize that MK-801 induces behavior disturbances due to a paradoxical release of glutamate and further stimulation of non-NMDA glutamate receptors. The attenuation of some behavioral effects of MK-801 by guanosine may be related to an increase of glutamate uptake by astrocytes promoted by guanosine's action at its specific binding site in cell membrane, reducing glutamate levels at the synaptic cleft and leading to less activation of non-NMDA receptors. Further experiments are currently being carried out in our laboratory to investigate this "hyperglutamatergic" hypothesis of NMDA receptor antagonists' action and its reversion by guanosine. These experiments are mainly investigating if neurotransmitter levels are indeed affected by administration of MK-801 and guanosine.

Although the mechanism of action of guanosine and its modulatory effects on MK-801-induced behavioral disturbances are not completely elucidated, these findings point to a potential antipsychotic property of guanosine. This may be especially important in targeting psychotic symptoms that are not generally treated with currently available antipsychotics. Moreover, the neuroprotective and neurotrophic effects of guanosine may also be advantageous for the treatment of schizophrenia and other brain diseases.

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Contributors

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Conflict of interest

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23 November 2007

II.2.e. Characterization of glutamate uptake by spinal cord slices from mice: effects of guanosine

Protocolo experimental em andamento: resultados parciais.

Characterization of glutamate uptake by spinal cord slices from mice: effects of guanosine.

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Abstract:

Glutamate is the major excitatory neurotransmitter found throughout the spinal cord, being involved in many physiologic and pathologic processes, such as pain transmission. However, glutamate can function as a potent neurotoxin and glutamatergic excitotoxicity has been implicated in the pathogenesis of various acute and chronic CNS disorders, including chronic pain. It is of critical importance that the extracellular glutamate concentration be kept at physiological levels, which is achieved through specific glutamate transporters. Guanine-based purines, mainly the nucleoside guanosine, have been shown to modulate pain transmission and glutamate uptake. The aims of this study were to characterize the glutamate uptake by spinal cord slices from mice and investigate the action of guanosine on *in vitro* spinal cord glutamate uptake. Basal glutamate uptake by spinal cord slices was 0.36 ± 0.05 nmol.mg^{-1}.min^{-1} (100 μM) achieving a maximum of 1.63 ± 0.22 nmol.mg^{-1}.min^{-1} at the highest glutamate concentration (1 mM). Values of K_M and V_{max} for glutamate uptake were 100.54 mM and 0.8675 nmol.mg^{-1}.min^{-1}. Basal specific glutamate uptake was significantly stimulated by guanosine up to 73% only at its higher concentration (1 mM). Although this method presents some limitations, we suggest that glutamate uptake by spinal cord slices may be a simple and consistent model to investigate glutamatergic system modulation at the spinal cord. Additionally, we demonstrated that guanosine stimulated glutamate uptake at high concentrations, which may be related to a putative neuroprotective and antinociceptive role of guanosine at the spinal cord.

Section: Sensory and Motor systems

Keywords: Guanosine; Purines; Glutamate uptake; Antinociception; Spinal cord.

Introduction:

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS), being involved in many physiologic and pathologic processes, such as learning and memory [Izquierdo and Medina, 1997], neural development and aging [Meldrum, 2000; Ozawa et al., 1998], environmental adaptation [Segovia et al., 2001], excitatory synaptic transmission and synaptic plasticity [D'Mello and Dickenson 2008]. However, glutamate can function as a potent neurotoxin and glutamatergic excitotoxicity has been implicated in the pathogenesis of various acute and chronic CNS disorders, cell death, stroke, and chronic pain [Lee et al., 1998; Meldrum, 2000; Ozawa et al., 1998; D'Mello and Dickenson 2008; Bleakman 2006].

The spinal cord is an important site at which the various incoming sensory and nociceptive signals undergo convergence and modulation. Glutamate is the major excitatory neurotransmitter found throughout the spinal cord [Millan, 1999]. Thus, as expected, the vast majority of primary afferents synapsing in the dorsal horn of the spinal cord utilize this neurotransmitter [Furst 1999; D'Mello and Dickenson 2008]. It is of critical importance that the extracellular glutamate concentration be kept at physiological levels, as excessive activation of glutamate receptors can lead to excitotoxicity and neuronal death [Danbolt 2001]. The clearance of glutamate from the synaptic cleft, which occurs primarily by neuronal and glial high affinity sodium-dependent transporters, is the major mechanism for modulation of glutamate actions [Danbolt, 2001]. In this context, recent evidence suggests that spinal glutamate transporter might play an important role in normal sensory transmission at the spinal cord, since it is well-documented that glutamate acts as a major excitatory neurotransmitter in primary afferent terminals [Basbaum and Woolf, 1999].

Although the nucleotide ATP and the nucleoside adenosine are usually considered the main effectors of the purinergic system [Burnstock, 2007], extracellular guanine-based purines (GBPs), mainly the nucleoside guanosine, have been shown to exert several biological effects unrelated to the direct G-proteins modulation; these include modulation of glutamatergic activity [Souza and Ramirez, 1991; Regner et al., 1998; Schmidt et al., 2007], trophic effects on neural cells [Ciccarelli, 2001], and behavioral effects [Schmidt et al., 2000, 2005; Lara et al., 2001]. Concerning *in vitro* effects on the glutamatergic system, GBPs inhibit the binding of glutamate and analogs [Baron et al., 1989; Burgos et al., 1998], prevent cell responses to excitatory amino acids [Souza and Ramirez, 1991], present neuroprotective effects in cultured neurons submitted to hypoxia, and increase glutamate uptake in cultured astrocytes and brain cortical slices [Frizzo et al., 2001, 2002, 2003]. *In vivo*, GBPs prevent glutamate-induced seizures, are anxiolytic and amnesic in mice and rats [Roesler et al., 2000; Lara et al., 2001; Schmidt et al., 2000; Vinadé et al., 2003, 2005; Saute et al., 2006], and are neuroprotective against stroke and spinal cord injury [Jiang et al., 2003; 2007; 2008; Chang et al., 2008]; these effects seem to be related to guanosine and may be involved in the physiological neuroprotection against glutamatergic excitotoxicity [Soares et al., 2004; Schmidt et al., 2005, 2007]. Recently, we have demonstrated that intracerebroventricular (i.c.v.) administration of guanosine is antinociceptive against several chemical and thermal pain models in mice [Schmidt et al., 2008]. Additionally, we have shown that spinal administration of guanosine produces significant inhibition of glutamate and non-NMDA agonists-induced biting behavior [personal communication]. Despite having no clearly identified cell surface receptors, guanosine was recently recognized as a putative extracellular signaling molecule [Rathbone *et al.*, 1999], and a high-affinity binding site for guanosine was recently characterized in rat brain membranes

[Traversa *et al.*, 2002, 2003]. Importantly, most of guanosine-induced effects seem to be related, at least partially, to guanosine-induced modulation of the glutamatergic pathways and glutamate uptake [Schmidt et al., 2007; 2008].

Given the pivotal role of glutamatergic system in the mechanisms underlying normal and pathological sensory transmission at the spinal cord, the aim of this study was to characterize the glutamate uptake by spinal cord slices from mice, by using an *in vitro* model, in presence of different glutamate concentrations. Considering that guanosine modulates the glutamatergic system and pain transmission at the spinal cord, we further investigated the action of guanosine on spinal cord glutamate uptake.

Results:

Fig. 1 shows a significant correlation between protein count in the preparation of the spinal cord slices and glutamate uptake (Pearson's r = 0.63, $P = 0.0008$). Saturation studies were also performed in order to characterize spinal cord slices uptake of glutamate. Fig. 2 shows a time course analysis of [^{3}H]-glutamate uptake by spinal cord slices, which was assayed from 2 to 25 min incubations; the transport shows a saturation profile from 10 min incubation (Fig. 2, insert). In order to further characterize glutamate uptake by spinal cord slices, a concentration curve of glutamate (1 to 1000 μM) was performed (Fig.3). Basal glutamate uptake by spinal cord slices was 0.36 ± 0.05 nmol.mg^{-1}.min^{-1} (100 μM) achieving a maximum of 1.63 ± 0.22 nmol.mg^{-1}.min^{-1} at the highest glutamate concentration (1 mM) (Fig. 3). Values of K_M and V_{max} for glutamate uptake were 100.54 μM and 0.8675 nmol.mg^{-1}.min^{-1}.

Considering our previous results showing that guanosine stimulates glutamate uptake by brain slices *in vitro* (Frizzo et al., 2001, 2002, 2003), we investigated whether guanosine could also affect glutamate uptake by spinal cord slices at high (100 μM) glutamate concentrations. Fig. 4 shows that the basal-specific uptake of glutamate in spinal cord slices was not clearly affected by guanosine up to 300 μM. In contrast, when using 1 mM of guanosine, glutamate, the basal specific uptake (0.48 ± 0.04 $nmol.mg^{-1}.min^{-1}$) was significantly stimulated by guanosine up to 73% (0.83 ± 0.15 $nmol.mg^{-1}.min^{-1}$). Sodium-independent uptake of glutamate was not affected by guanosine (data not shown).

In order to evaluate whether preincubation and incubation periods affect cellular integrity, we assessed LDH activity in spinal cord slices from mice. We investigated the injurious effects of guanosine (1 mM) on pre- and incubation periods. Furthermore, we investigated potential deleterious effects of glutamate (100 μM or 1 mM) on the incubation period (7 and 25 min). We demonstrated that there was an important release of LDH during the preincubation period of 120 min (31.4 % from total LDH content). There was no difference between vehicle and guanosine (1 mM) regarding LDH release during the preincubation period (vehicle = 31.7 ± 12.5 % vs. guanosine = 30.6 ± 12.5 %, $P = 0.83$). During the incubation period, no differences between groups were found as well [glutamate 100 μM – 7.7 ± 2.1 % (7 min) and 6.8 ± 3.7 % (25 min) vs. glutamate 1 mM – 7.3 ± 2.0 % (7 min) and 6.1 ± 1.2 % (25 min), $P = 0.49$ and 0.73, respectively]. We additionally investigated the effects of guanosine 1 mM in the incubation period, with or without glutamate 100 μM. A comparison between all groups likewise did not find any difference (guanosine 1 mM – 8.8 ± 2.1 % and guanosine 1 mM + glutamate 100 μM – 7.4 ± 1.7 %, P = 0.6936, one-way ANOVA).

In order to investigate cell viability, we assessed MTT activity in spinal cord slices from mice. Our results indicate that cell viability was maintained throughout the pre-incubation period, since there was no difference in MTT levels between the baseline and preincubation period (2.0 ± 0.4 vs. 2.5 ± 0.5 abs.mg^{-1}, P = 0.15). Considering the incubation period, the cell viability was maintained and no differences between groups were found as well [glutamate 100 μM – 4.7 ± 1.3 abs.mg^{-1} (7 min) and 2.7 ± 0.9 abs.mg^{-1} (25 min) vs. glutamate 1 mM – 3.6 ± 0.4 abs.mg^{-1} (7 min) and 3.7 ± 0.9 abs.mg^{-1} (25 min), P = 0.47 and 0.18, respectively]. We additionally investigated the effects of guanosine (1 mM) in the pre- and incubation periods, with or without glutamate (100 μM). A comparison between all groups likewise did not find any difference (guanosine 1 mM – 3.6 ± 0.4 abs.mg^{-1} and guanosine 1 mM + glutamate 100 μM – 4.0 ± 0.9 abs.mg^{-1}, P = 0.7655, one-way ANOVA).

Discussion:

The present data provide the first evidence that glutamate is taken up into spinal cord slices from mice. A number of observations indicate that glutamate accumulates into spinal cord slices by a carrier mediated transport process: i. accumulation of glutamate presented a consistent time course pattern; ii. glutamate uptake was directly correlated with protein amount and glutamate concentration; iii. glutamate accumulation was saturable, showing an apparent affinity similar to that previously described in glutamate uptake by brain slices [Frizzo et al., 2002; 2005; de Oliveira et al., 2004; Moretto et al., 2005; 2008; Leke et al., 2006; Thomazi et al. 2008]. Interestingly, guanosine, in high concentrations (1 mM), produced an increase in glutamate uptake by spinal cord slices.

Glutamate plays an essential role in pain transmission mechanisms at the spinal cord [Millan, 1999]. Recent evidence suggests that spinal glutamate transporter might play an important role in normal sensory transmission [D'Mello and Dickenson 2008]. Liaw et al. [2005] reported that intrathecal injection of glutamate transporter blockers DL-threo-β-benzyloxyaspartate (TBOA) and dihydrokainate (DHK) produced significant and dose-dependent spontaneous nociceptive behaviors, phenomena similar to the behaviors caused by intrathecal glutamate receptor agonists, when given intrathecally. Thus, spontaneous pain-related behaviors and sensory hypersensitivity evoked by glutamate transporter blockers directly support the involvement of glutamate transporter in normal excitatory synaptic transmission in the spinal cord. *In vivo* microdialysis analysis showed that intrathecal injection of TBOA produced short-term elevation of extracellular glutamate concentration in the spinal cord. These findings indicate that a decrease of spinal glutamate uptake can lead to excessive glutamate accumulation in the spinal cord, which might, in turn, result in over-activation of glutamate receptors, excitotoxicity and production of spontaneous nociceptive behaviors and sensory hypersensitivity [Liaw et al., 2005]. Therefore, glutamate uptake through spinal glutamate transporters is critical for maintaining normal sensory transmission. Previous studies have characterized the glutamate uptake by brain slices from rats and mice [Frizzo et al., 2002; 2005; de Oliveira et al., 2004; Moretto et al., 2005; 2008; Leke et al., 2006; Thomazi et al. 2008], but, to date, no studies have investigated the glutamate uptake capacity of spinal cord slices.

There is compelling evidence suggesting that excitatory amino acids (glutamate and aspartate) are released in the CNS in response to several peripheral noxious stimuli; alteration of glutamatergic neurotransmission within the spinal cord contributes to hyperalgesic and allodynic responses following nerve injury [Willis, 2001]. In particular,

changes in expression and efficacy of glutamate transporters have been reported. A previous study suggests that following chronic constriction injury of the sciatic nerve, glutamate transport proteins show biphasic changes in expression pattern in the superficial dorsal horn [Sung et al., 2003]. An initial up-regulation (1 and 4 days post injury) was followed by a significant down-regulation (7 and 14 days post injury) in glutamate transporter expression in superficial dorsal horn. In the same study, glutamate uptake activity in the dorsal horn was decreased 5 days after sciatic nerve injury. The glutamate transport protein down-regulation is proposed to lead to increased extracellular glutamate levels, which is thought to contribute to central sensitization seen after nerve injury [Willis, 2001]. Recent data suggest that spinal nerve ligation attenuated the glutamate uptake activity extending into the deep dorsal and ventral horn [Binns et al., 2005]. Therefore, plasticity related to spinal nerve injury produces widespread alteration in glutamate transporter function that may contribute to the pathophysiology of chronic pain states such as neuropathic pain. The present study clearly demonstrates data to support the proposition that glutamate uptake by spinal cord slices is a reliable method to evaluate the global glutamate transporter capacity present at the spinal cord from mice.

Recently, we demonstrated that i.c.v. guanosine produces consistent and dose-dependent antinociceptive effects, which may involve, at least in part, the modulation of glutamate uptake at spinal and supraspinal structures [Schmidt et al., 2008]. *In vitro*, guanosine has been shown to prevent ischemic injury [Frizzo et al., 2002] and NMDA-induced excitotoxicity [Ciccarelli et al., 2001]. Additionally, guanosine consistently increases the glutamate uptake by cultured astrocytes and brain slices [Frizzo *et al.*, 2001, 2002, 2003]. *In vivo*, acute or chronic administration of guanosine prevents seizures and toxicity induced by drugs that overstimulate the glutamatergic system [Baron et al., 1989;

Malcon et al., 1997; Schmidt et al., 2000; 2005; Lara et al., 2001; Vinadé et al., 2003], is amnesic and anxiolytic in rodents [Vinadé et al., 2003; 2004; 2005], and is neuroprotective against stroke and spinal cord injury [Jiang et al., 2003; 2007; 2008; Chang et al., 2008]. Although the overall effects of guanosine seem to be related to attenuating glutamatergic overstimulation, its precise mechanism of action remains unclear. Guanosine at high concentrations exerts a small inhibitory action on the binding of glutamate and its analogues, suggesting that inhibition of glutamate binding are unlikely to explain a putative extracellular role of guanosine [Monahan *et al.*, 1988; Souza and Ramirez, 1991]. Therefore, it is tempting to speculate that guanosine could produce its behavioral effects, including antinociception, by modulating glutamate uptake in the CNS.

A previous study from our group has demonstrated that guanosine produces a potent stimulatory effect on glutamate uptake by cortical slices. Surprisingly, the activation of glutamate uptake only occurred when higher concentrations of glutamate (100 μM) were present in the incubation medium, a fact that may reflect a putative role of the nucleoside in maintaining glutamate concentration below excitotoxic levels [Frizzo et al., 2002]. In this study, guanosine, which was tested against glutamate (100 μM), caused a significant increase in glutamate uptake by in spinal cord slices only at its higher concentration (1 mM). A previous study reported a protective effect of moderate-to-high micromolar concentrations of guanosine [>250 μM] in spinal cord cultures submitted to glucose deprivation and mitochondrial inhibition [Jurkowitz *et al.*, 1998]. These effects may represent guanosine receptor-independent mechanisms. Guanosine could act as an alternative source of energy for neural cells due to conversion of ribose-1-phosphate from guanosine to phosphorylated glycolytic intermediates [Jurkowitz et al., 1998; Litsky et al.,

1999]. However, there is data supporting the possible existence of specific receptor-like binding sites for guanosine (*K*d ~ 50 to 100 nM) on membrane preparations from rat brain [Traversa et al., 2002, 2003]. Thus, we also speculate that guanosine, through its specific binding site at spinal cord sites, may promote its effects on glutamate uptake by activating intracellular cAMP-dependent and independent pathway or MAP kinase signalling pathways [Gysbers and Rathbone, 1996; Tomaselli et al., 2005]. However, the exact mechanism of action of extracellular guanosine remains to be clarified.

Among the limitations of our methodology, it can be pointed out that: i. the contribution of neurons and glia for all results was not addressed; ii. glutamate concentrations in slices were high for normal conditions; iii. other mechanisms for the effects of guanosine cannot be ruled out; iv. endogenous levels of guanosine and remaining levels of guanosine after the incubations were not determined; and v. although spinal cord cells seemed to be viable, the prolonged preincubation period was related to significant cell damage and death. These issues limit this methodology and may be addressed in future studies.

In conclusion, this study described for the first time a kinetic and pharmacological characterization of glutamate transport capacity in spinal cord slices from mice. Although this method may present some limitations, we demonstrated that glutamate uptake by spinal cord slices is a simple and consistent model to investigate glutamatergic system modulation at the spinal cord. Additionally, we demonstrated that guanosine stimulated glutamate uptake at high concentrations, which may be related to a putative neuroprotective and antinociceptive role of guanosine at the spinal cord. Therefore, guanosine may be considered an endogenous neuroprotective and antinociceptive compound and may serve as

a basis for the development of new therapeutic strategies. Additional studies should further characterize effects of guanosine at the spinal cord and clarify its mechanism of action.

Experimental Procedure:

Animals: Male adult Swiss albino mice (3-4 months of age, 30-40 g) were kept on a 12 hour light/dark cycle (light on at 7:00 am) at temperature of 22 ± 1ºC, housed in plastic cages (five per cage) with tap water and commercial food *ad libitum.* Our institutional protocols for experiments with animals, designed to avoid suffering and limit the number of animals sacrificed, were followed throughout. This study was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The number of animals was the minimum necessary to demonstrate the consistent effects of the drug treatments.

Chemicals: Guanosine, L-glutamic acid hydrochloride (glutamate), and N-methyl-D-glucamine were purchased from Sigma Chemicals (St. Louis, MO, USA). L-(2,3-^{3}H)-glutamate (45 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ, USA). Guanosine was dissolved in 0.1 mN NaOH and buffered to pH 7.4. The amount of NaOH caused no detectable effect. All other solutions were dissolved in saline (NaCl 0.9%) and buffered with 0.1 N NaOH or 0.1 N HCl to pH 7.4 when necessary.

Spinal cord preparation and glutamate uptake: Animals were firstly acclimatized to the laboratory for at least 60 min before testing. Mice were decapitated, their spinal cords were removed immediately and submerged in a ice-cold Hank's balanced salt solution (HBSS) containing [in mM]: 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, adjusted to pH 7.2.

Spinal cords were dissected into a Petri dish filled with ice-cold HBSS. Transversal slices (0.4 mm) were obtained using a McIlwain tissue chopper and sections were separated with the help of a magnifying glass. Spinal cord slices were then transferred to 24-multiwell dishes, containing 500 μL of HBSS solution and pre-incubated for 120 minutes at 35°C. Subsequently, spinal cord slices were washed with 1 ml HBSS, and the total glutamate uptake was assessed by addition of 0.33 μCi/ml L-[^{3}H]glutamate with unlabeled glutamate (concentrations: 1, 3, 10, 30, 100, 300 or 1000 μM) in HBSS solution at 35°C. Incubation was stopped after 2, 5, 7, 10, 15 or 25 min by two ice-cold washes with 1 ml HBSS immediately followed by the addition of 0.5 N NaOH, which was kept overnight. Lysates were taken for determination of intracellular content of L-[^{3}H]glutamate through scintillation counting. To determine the sodium-independent glutamate uptake, parallel assays were done under ice using *N*-methyl-D-glucamine instead of sodium chloride in the incubation medium. Sodium-dependent glutamate uptake was obtained by subtracting the sodium-independent uptake from the total in order to obtain the specific uptake. Protein was measured using the method of Peterson [1977] using bovine albumin as standard. The experiments were done in triplicate.

Assessment of Cell Death – metabolic parameters: Slice injury was quantified by the measurement of lactate dehydrogenase (LDH) released from damaged cells into the extracellular fluid [Koh and Choi, 1987]. LDH efflux occurs from either necrotic or apoptotic cells, and is proportional to the number of damaged cells [Koh and Choi, 1987]. LDH activity was determined using a colorimetric LDH assay kit (Doles, Brazil); the activity of this enzyme was assessed in the bathing fluid. Following the conversion of exogenously added lactate to pyruvate, 1,10-phenantroline is converted to a colored

complex, after a chain of reactions resulting from the NADH formed by the enzymatic reaction, which is measured using a spectrophotometric method (490 nm). Slice viability assay was also performed by the colorimetric 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method [Hansen et al., 1989]. Slices were incubated with 0.5 $mg.ml^{-1}$ of MTT, followed by incubation at 37 ^{o}C for 45 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide (DMSO) and measured at 560 and 630 nm. Protein content was measured in aliquots of the dissociated slices following the method described by Peterson [1977]. Results are expressed as a percentage of the control.

Guanosine treatment: The effect of guanosine [0.1, 1, 10, 100, 300 or 1000 μM] was assessed in normal conditions for glutamate uptake and cell damage assays. Guanosine was maintained in the medium during 120 min [preincubation period]. Before glutamate uptake assays, the medium was removed and replaced with medium containing the same previous treatment. Control group received 0.1 mN NaOH instead of guanosine.

Statistical analysis: Data are expressed as mean ± standard error of the mean (SEM). Data were submitted to Kolmogorov-Smirnov test for normality evaluation. Statistical analysis among groups was performed using one-way analysis of variance (ANOVA) followed by the *post-hoc* Student-Newman-Keuls test when necessary. Correlations are presented as linear Pearson's coefficient. Kinetics analysis of glutamate transport was determined by using GraphPad software (GraphPad software, San Diego, CA, USA). All results with $P < 0.05$ were considered statistically significant.

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Legends:

Figure 1: Correlation between protein count and glutamate uptake by spinal cord slices. (Pearson's coefficient r = 0.63, $P = 0.0008$). N = 24 slices. Data for glutamate uptake are expressed in $nmol.min^{-1}$.

Figure 2: Time course analysis of [^{3}H]-glutamate uptake into spinal cord slices from mice. Total glutamate uptake was assessed by addition of 0.33 μCi/ml L-[^{3}H]glutamate with unlabeled glutamate (concentrations: 100 μM) in HBSS solution at 35°C. Incubation was stopped after 2, 5, 7, 10, 15 or 25 min. Data are mean ± standard deviation and expressed in $nmol.mg^{-1}$ of protein or $nmol.mg^{-1}.min^{-1}$ (insert). N = 24 slices per group. * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$ as compared to baseline, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 3: Concentration dependence of glutamate into spinal cord slices from mice. Total glutamate uptake was assessed by addition of 0.33 μCi/ml L-[^{3}H]glutamate with unlabeled glutamate (concentrations: 1, 3, 10, 30, 100, 300 or 1000 μM) in HBSS solution at 35°C. Incubation was stopped after 7 min. Data are mean ($nmol.mg^{-1}.min^{-1}$) ± standard deviation. N = 18 slices per group. ** = $P < 0.01$ and *** = $P < 0.001$ as compared to baseline, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 4: Guanosine effects on basal-specific uptake of glutamate by spinal cord slices. The columns represent mean ($nmol.mg^{-1}.min^{-1}$) and vertical bars represent standard deviation. N

= 16 slices per group. * = $P < 0.05$ as compared to control (vehicle), one-way ANOVA followed by Student-Newman-Keuls test.

Figure 1:

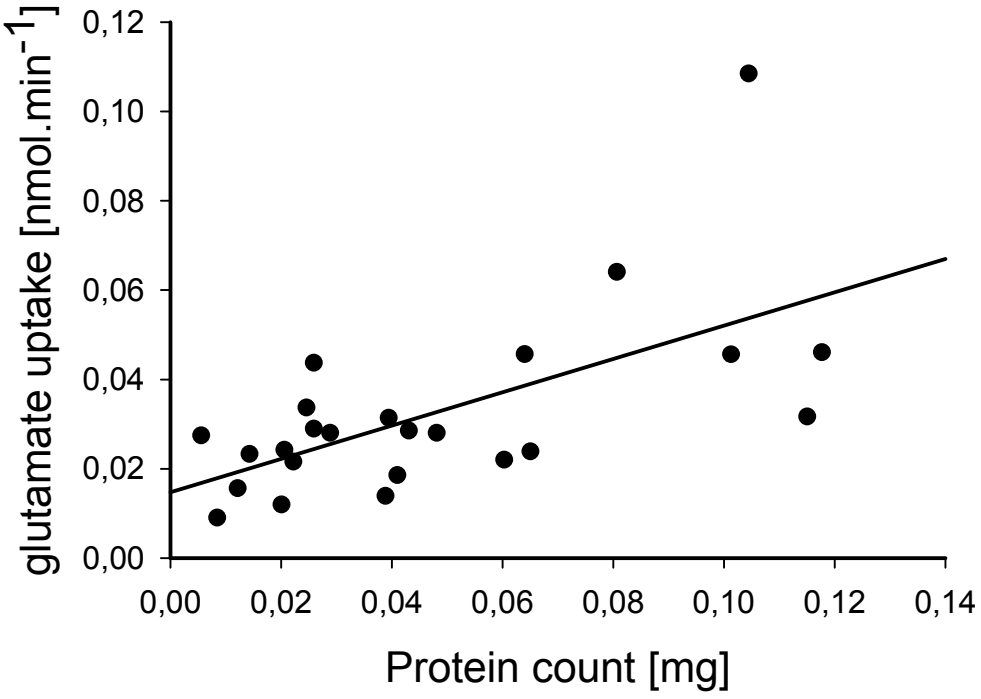

glutamate uptake [nmol.min-1]
0,12
0,10
0,08
0,06
0,04
0,02
0,00
0,00
0,02
0,04
0,06
0,08
0,10
0,12
0,14
Protein count [mg]

Figure 2:

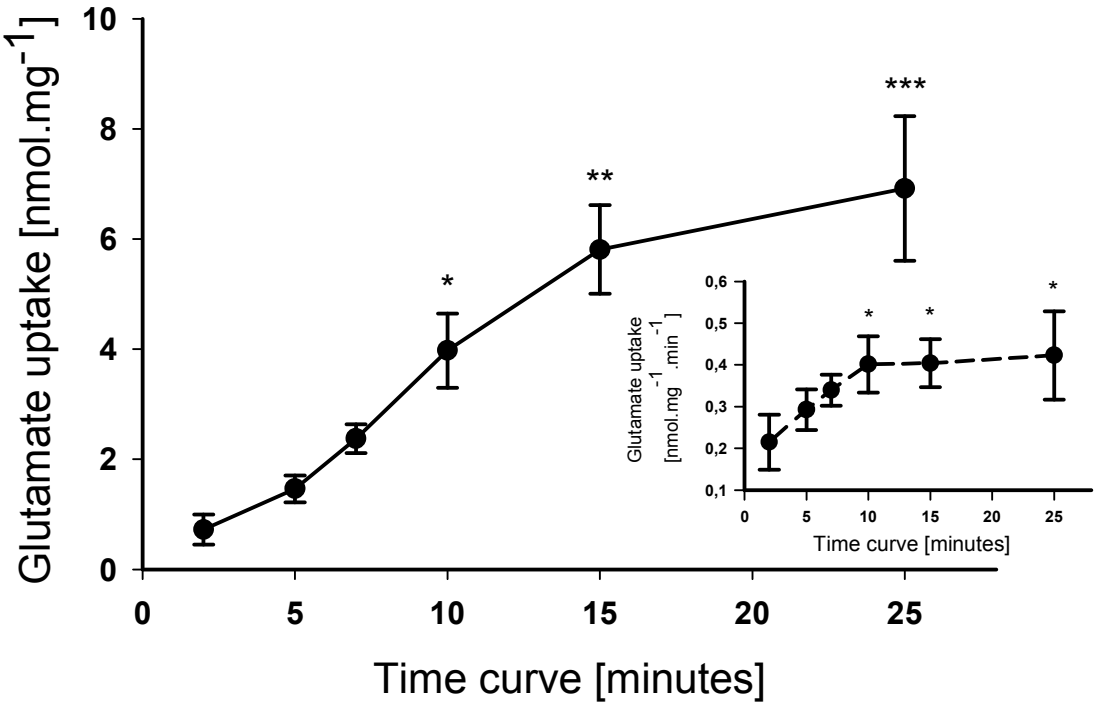

Glutamate uptake [nmol.mg-1]
Time curve [minutes]
*
**
***
Glutamate uptake [nmol.mg-1.min-1]
Time curve [minutes]

Figure 3:

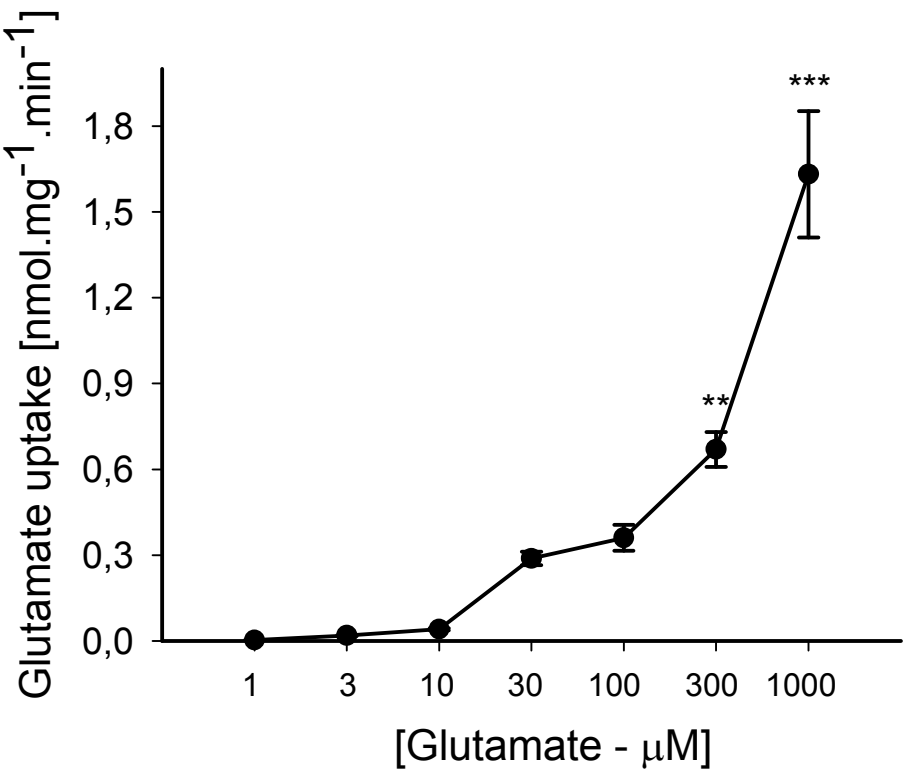
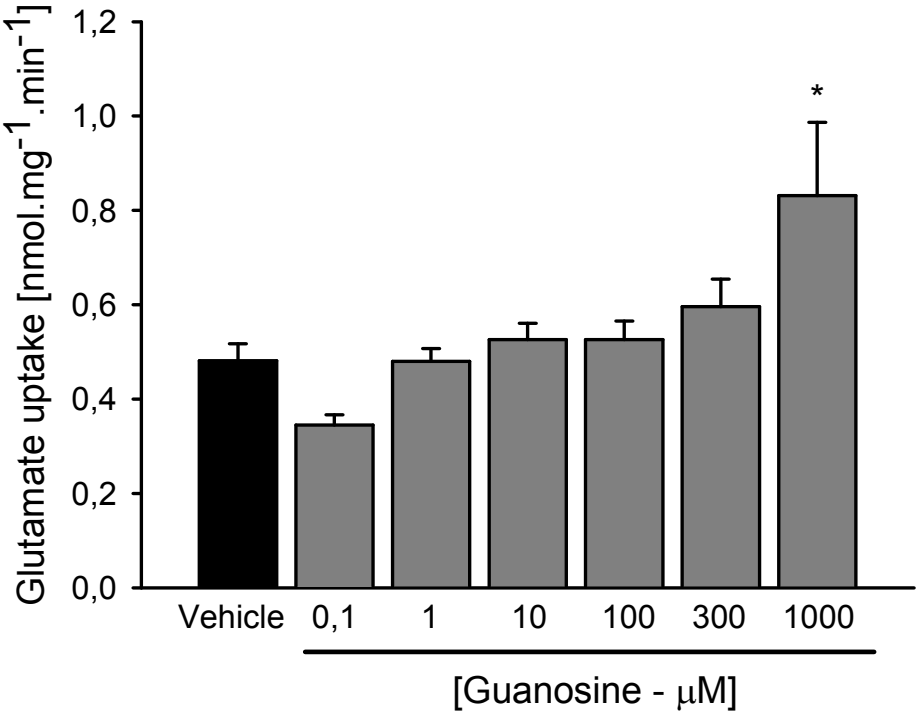

Glutamate uptake [nmol.mg-1.min-1]
1,8
1,5
1,2
0,9
0,6
0,3
0,0
***
**
1
3
10
30
100
300
1000
[Glutamate - μM]

Figure 4:


Glutamate uptake [nmol.mg-1.min-1]
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
Vehicle
0,1
1
10
100
300
1000
[Guanosine - μM]

II.2.f. Spinal mechanisms of antinociceptive action caused by guanosine in mice

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Spinal mechanisms of antinociceptive action caused by guanosine in mice.

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Abstract:

It is well known that adenine-based purines exert multiple effects on pain transmission. Recently, we have demonstrated that intracerebroventricular (i.c.v.) administered guanine-based purines are antinociceptive against chemical and thermal pain models in mice. The present study was designed to further investigate the antinociceptive effects of guanosine in mice. Animals received an intrathecal (i.t.) injection of vehicle (0.1 mN NaOH) or guanosine (10 to 400 nmol). Measurements of cerebrospinal fluid (CSF) purine levels and spinal cord glutamate uptake were performed. Guanosine produced dose-dependent antinociceptive effects against tail-flick, hot-plate, intraplantar (i.pl.) capsaicin, and i.pl. glutamate tests. Additionally, i.t. guanosine produced significant inhibition of the biting behavior induced by i.t. injection of glutamate (175 nmol/site), AMPA (135 pmol/site), kainate (110 pmol/site), trans-ACPD (50 nmol/site), and substance P (135 ng/site), with mean ID_{50} values of 140 (103–190), 136 (100–185), 162 (133–196), 266 (153–461) and 28 (3–292) nmol, respectively. However, guanosine failed to affect the nociception induced by NMDA (450 pmol/site) and capsaicin (30 ng/site). Intrathecal administration of guanosine (200 nmol) induced an approximately 120-fold increase on CSF guanosine levels. Guanosine prevented the increase on spinal cord glutamate uptake induced by i.pl. capsaicin. This study provides new evidence on the mechanism of action of guanosine presenting antinociceptive effects at spinal sites. This effect seems to be at least partially associated with modulation of glutamatergic pathways by guanosine.

Keywords: Guanosine; Pain; Nociception; Spinal cord; Purines; Glutamate.

1. Introduction:

It is well known that the extracellular adenine-based purines (ABPs) exert multiple effects on pain transmission (Sawynok and Liu, 2003; Donnelly-Roberts et al., 2008). There is abundant evidence that extracellular ATP and other nucleotides have an important role in pain signaling at both the periphery and in the Central Nervous System (CNS) (Inoue et al., 2005). ATP can stimulate sensory nerve endings causing pain and a marked increase in the discharge from sensory neurons, and is involved in the initiation of different types of nociception and pain (Burnstock, 2007). Adenosine presents antinociceptive effects by acting at spinal, supraspinal and peripheral sites (Sawynok, 1998), plays a pivotal role in inflammatory and neuropathic pain (McGaraughty and Jarvis, 2005), and mediates aspects of opioid-induced analgesia (Sawynok, 1998; Eisenach et al., 2004). The antinociceptive effects of adenosine are particularly related to the activation of adenosine A_1 receptors in the spinal cord, where inhibition of intrinsic neurons and sensory nerve terminals simultaneously with a diminished release of substance P and glutamate contributes to its actions (Sawynok and Liu, 2003).

Although the nucleotide ATP and the nucleoside adenosine are usually considered the main effectors of the purinergic system (Burnstock, 2007), extracellular guanine-based purines exert biological effects unrelated to the direct G-proteins modulation; these include trophic effects on neural cells (Ciccarelli, 2001), and the modulation of glutamatergic activity (Schmidt et al., 2007). Concerning *in vitro* effects on the glutamatergic system, guanine-based purines inhibit the binding of glutamate and analogs (Baron et al., 1989; Burgos et al., 1998), prevent cell responses to excitatory amino acids (Souza and Ramirez, 1991), present neuroprotective effects in cultured neurons submitted

to hypoxia and increase glutamate uptake in cultured astrocytes (Frizzo et al., 2001, 2002, 2003). *In vivo*, guanine-based purines, mainly guanosine, prevent glutamate-induced seizures, are anxiolytic and amnesic in rodents (Roesler et al., 2000; Lara et al., 2001; Schmidt et al., 2000, 2005; Soares et al., 2004; Vinadé et al., 2003, 2004, 2005; Saute et al., 2006). Recently, we have demonstrated that i.c.v. administered guanosine or GMP are antinociceptive against several chemical and thermal pain models in mice. Most of these effects are probably related to guanosine-induced modulation of the glutamatergic system.

This study was designed to investigate the antinociceptive effects of intrathecal (i.t.) administration of guanosine in mice. By means of pharmacological and neurochemical approaches, we examined the mechanisms underlying guanosine-induced spinal antinociception in mice.

2. Methods:

2.1. Animals: Male adult Swiss albino mice (3-4 months of age, 30-40 g) were kept on a 12 hour light/dark cycle (light on at 7:00 am) at temperature of 22 ± 1ºC, housed in plastic cages (five per cage) with tap water and commercial food *ad libitum*. In all nociceptive behavioral experiments, the animals were acclimatized to the laboratory for at least 60 min before testing and were used only once throughout the experiments. The ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983) and our institutional protocols for experiments with animals, designed to avoid suffering and limit the number of animals sacrificed, were followed throughout. This study was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The number of animals and

intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

2.2. Drugs: Guanosine, L-glutamic acid hydrochloride (glutamate), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid (kainate), N-methyl-D-aspartic acid (NMDA), substance P, (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (trans-ACPD), and capsaicin were purchased from Sigma Chemicals (St Louis, MO, USA). The anesthetic sodium thiopental and morphine sulphate, which was taken as a positive control for some experiments, were obtained from Cristália (SP, Brazil). Guanosine was dissolved in 0.1 mN NaOH. The amount of NaOH caused no detectable effect. Capsaicin was diluted in DMSO (dimethyl sulfoxide, 5%). All other solutions were dissolved in saline (NaCl 0.9%) and buffered with 0.1 N NaOH or 0.1 N HCl to pH 7.4 when necessary.

2.3. Drugs administration and intrathecal injection: Experiments were performed according to Schmidt et al. (2008): 20 minutes before the experiment, animals were placed individually in acrylic boxes, which served as observation chambers. After this adaptation period, i.t. injections were given to animals using the method described by Hylden and Wilcox (1980). Briefly, the animals were restrained manually and a 30-gauge needle, attached to a 10 μl microsyringe, was inserted through the skin and between the vertebrae into the subdural space of the L_5–L_6 spinal segments. Injections were given over a period of 5 s. Animals were treated with an i.t. injection (8 μl) of vehicle (0.1 mN NaOH) or guanosine 5 min before behavioral evaluation.

2.4. Capsaicin-induced nociception: The method used for capsaicin-induced licking was similar to that described by Sakurada et al. (1993). Five minutes after i.t.

treatments, 20 μL of capsaicin (1.6 μg/paw) was injected intraplantarly (i.pl.) under the plantar skin of the right hindpaw (Hamilton microsyringe with a 26-gauge needle). Animals were observed individually for 5 minutes after capsaicin administration for the time spent licking the injected paw, which was considered as indicative of nociception.

2.5. Glutamate-induced nociception: The procedure used was similar to Beirith et al., (2002). Five minutes after the i.t. treatments, 20 μl of glutamate solution (10 μmol/paw prepared in saline) was injected i.pl. under the plantar skin of the right hindpaw. The mice were observed individually for 15 min following glutamate injection and the amount of time spent licking the injected paw was considered as indicative of nociception.

2.6. Tail-flick: Nociception was assessed with a tail-flick apparatus (Albrasch Electronic Equipments, Brazil), as described in detail elsewhere (D'Amour and Smith, 1941), with minor modification. A source of light was positioned below the tail and the time that the mouse took to withdraw its tail from the noxious stimulus was recorded. The trial was automatically terminated when the mouse tail deflected activating a photocell that turns off the light. A cut-off time of 10 s was employed in order to prevent tissue damage (a mouse that did not flick by 10 s was considered as fully analgesic). On day one, the animals were habituated with the tail-flick apparatus through three separate measures (data not shown). On day two, baseline tail-flick latency was measured for each mouse prior to the treatments. Animals displaying at least two tail-flick latencies of 10 s on the baseline were excluded from the study. Immediately after the third tail-flick latency measurement, animals received i.t. treatments and 5 minutes thereafter were submitted to the tail-flick apparatus for tail-flick latency measurement. Data are

expressed as mean percent of maximum possible effect (%MPE) ± S.E.M., according to the following formula (Calcagnetti et al., 1990): %MPE: 100 x (post drug latency – baseline latency) / (Cut-off time – baseline latency).

2.7. Hot-plate: The hot-plate test was used to measure the response latencies according to the method described by Eddy and Leimback (1953), with minor modification. The hot-plate apparatus (Ugo Basile, model-DS 37, Italy) was maintained at 55 ± 0.5 °C. Animals were placed into a glass cylinder of 24 cm diameter on the heated surface, and the time between placement of the animal on the hot-plate and the occurrence of licking hindpaws or jumping off the surface was recorded as response latency. On day one, the animals were first habituated with the apparatus. On day two, mice were tested and animals displaying baseline latencies of more than 15 s were excluded from the study. An automatic 20 s cut-off was used to prevent tissue damage. Each animal was tested before administration of drugs in order to obtain the baseline. Five minutes after i.t. treatments, animals were placed on the heated surface and response latency recorded as described above. Data are expressed as mean percent of maximum possible effect (%MPE) ± S.E.M., according to the following formula (Calcagnetti et al., 1990): %MPE: 100 x (post drug latency – baseline latency) / (Cut-off time – baseline latency).

2.8. Spinal algogen-induced nociception-related behavior in mice: To test the hypothesis that excitatory amino acids, substance P or capsaicin might be involved in the antinociception caused by guanosine, we examined the effect of guanosine on the biting response induced by these algogens. For this purpose, mice received guanosine by i.t. route (8 μl, dose range: 50 to 200 nmol) or vehicle (0.1 mN NaOH) 5 min before i.t.

injection of 5 μl of the excitatory amino acids, substance P or capsaicin. The nociceptive response was elicited by glutamate (an excitatory amino acid, 175 nmol/site, i.t.) (Scheidt et al., 2002), NMDA (a selective agonist of NMDA subtype of glutamatergic ionotropic receptors, 450 pmol/site, i.t.) (Urca and Raigorodsky, 1988), AMPA (a selective agonist of AMPA-subtype of glutamatergic ionotropic receptors, 135 pmol/site, i.t.) (Brambilla et al., 1996), kainate (a selective agonist of kainate subtype of glutamatergic ionotropic receptors, 110 pmol/site, i.t.), trans-ACPD (a metabotropic glutamate receptor agonist, 50 nmol/site, i.t.) (Boxall et al., 1998), substance P (NK_1 receptor-selective agonist, 135 ng/site, i.t.) (Sakurada et al., 1990), capsaicin ($TRPV_1$ receptor agonist, 30 ng/site, i.t.) (Sakurada et al., 1996). A group of mice received only vehicle (saline) by i.t. route and no significant biting behavior was observed (data not shown). Immediately after i.t injection of each nociceptive agent, mice were placed individually in observation chambers, and the amount of time (s) the animal spent biting itself was evaluated: glutamate (3 min); NMDA (5 min); AMPA (1 min); kainate (4 min); trans-ACPD (15 min); substance P and capsaicin (6 min). A bite was defined as a single head movement directed at the flanks or hind limbs, resulting in contact of the animal's snout with the target organ.

2.9. Hole-board test: The hole-board apparatus (Ugo Basile, Italy) consisted of gray Perspex panels (40 x 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 x 10 cm with a water-resistant marker. Five minutes after i.t. treatments, 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 the locomotion (Vinadé et al., 2003).

2.10. Measurement of motor performance: In order to evaluate non-specific muscle relaxant or neurotoxic effects, we evaluated the effects of i.t. guanosine in the rotarod test and on the spontaneous locomotor activity test. The rotarod apparatus (Ugo Basile, Italy) consisted of a rotating (18 rpm) bar (2.5 cm diameter), subdivided by disks into six compartments. As previously described (Vinadé et al., 2003), mice were initially trained to remain on the rotarod apparatus for 120 s. Those not remaining on the bar for at least two out of three consecutive trials were discarded. After 24 h, the latency to fall from the rotarod (one trial with a maximum of 60 s) was determined 5 minutes after i.t. treatments. The method for the spontaneous locomotor activity was adapted from Creese et al. (1976). Activity cages (45 x 25 x 20 cm, Albarsch Electronic Equipment, Brazil), equipped with three parallel photocells, automatically record the number of crossings. Animals were individually habituated to an activity cage for 10 min before receiving the i.t. treatments. The animals returned to the activity cages 5 min after i.t. treatments, and the crossings were recorded for 15 min.

2.11. Cerebrospinal fluid (CSF) sampling: Mice were treated with i.t. injection of vehicle or guanosine (200 nmol). After 5 min, mice were anesthetized with sodium thiopental (60 mg/kg, 10 ml/kg, i.p.) and placed in a stereotaxic apparatus, where the CSF was drawn (10 - 20 μl per mouse) by direct puncture of the *cisterna magna* with an insulin syringe (27 gauge x 1/2 in length), with the help of a magnifying glass. All

samples were centrifuged at 10,000g in an Eppendorf centrifuge during 5 min to obtain cell-free supernatants and stored in separate tubes in -70^{o}C.

2.12. HPLC procedure: High-performance liquid chromatography (HPLC) was performed with CSF cell-free supernatants aliquots for determination of purines concentration, according to Domanski et al (2006). CSF concentrations of the following purines were determined: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine, inosine monophosphate (IMP), inosine, hypoxanthine, xanthine, and uric acid. Analyses were performed with Shimadzu Class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto injector valve with 50 μL loop, and an UV detector. Separations were achieved on a Supelco C18 250 mm x 4.6 mm, 5 μm particle size column. The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24 ^{o}C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 10 μL were injected into the injection valve loop. Absorbance was read at 254 nm. CSF concentrations of purines are expressed as mean ± S.E.M. in μM.

2.13. Glutamate uptake by spinal cord slices: Mice were treated with an i.t. injection of guanosine (200 nmol) or vehicle (0.1 mN NaOH); after 5 min, animals received an i.pl. injection of capsaicin or vehicle (DMSO 5%). Five minutes after vehicle

or capsaicin i.pl. injection, animals were decapitated, their spinal cords were removed immediately and submerged in a ice-cold Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, adjusted to pH 7.2. Spinal cords were dissected into a Petri dish filled with ice-cold HBSS. Transversal slices (0.4 mm) were obtained using a McIlwain tissue chopper and sections were separated with the help of a magnifying glass. Spinal cord slices were then transferred to 24-multiwell dishes, containing 500 μl of HBSS solution and pre-incubated for 120 minutes at 35°C. Subsequently, spinal cord slices were washed with 1 ml HBSS, and the total glutamate uptake was assessed by addition of 0.33 μCi/ml L-(^{3}H)glutamate with 100 μM unlabeled glutamate in HBSS solution at 35°C. Incubation was stopped after 7 min by two ice-cold washes with 1 ml HBSS immediately followed by the addition of 0.5 N NaOH, which was kept overnight. Lysates were taken for determination of intracellular content of L-(^{3}H)glutamate through scintillation counting. To determine the sodium-independent glutamate uptake, parallel assays were done under ice using *N*-methyl-D-glucamine instead of sodium chloride in the incubation medium. Sodium-dependent glutamate uptake was obtained by subtracting the sodium-independent uptake from the total in order to obtain the specific uptake. Protein was measured using the method of Peterson et al. (1977) using bovine albumin as standard. The experiments were done in triplicate.

2.14. Statistical analysis: Data are expressed as mean ± standard error of the mean (S.E.M.), except the ID_{50} values (i.e., the dose of guanosine necessary to reduce the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} value

was determined by linear regression from individual experiments using linear regression GraphPad software (GraphPad software, San Diego, CA, USA). Data were submitted to Kolmogorov-Smirnov test for normality evaluation. Statistical analysis among groups was performed using one-way analysis of variance (ANOVA) plus the *post-hoc* Student-Newman-Keuls test when necessary. All results with $P < 0.05$ were considered statistically significant.

3. Results:

The results presented in Figure 1 show that i.t. administration of guanosine (10 – 400 nmol) produces antinociception against i.pl. capsaicin (1A) and i.pl. glutamate (1B) induced pain, as well as in the tail-flick (1C) and hot-plate (1D) tests. Neither i.t. saline nor 0.1 mN NaOH affected nociception as compared to control (sham) animals (data not shown). Mean ID_{50} values (95% confidence limits) for i.t. guanosine in the capsaicin and glutamate tests were 241 (158 – 366) and 165 (109 – 251) nmol, respectively; maximal inhibitions of 68±10% and 69±12%, respectively.

Figure 2 shows that i.t. guanosine (50 – 200 nmol), significantly inhibited the nociceptive response induced by i.t. injection of glutamate (2A), AMPA (2C), kainate (2D), trans-ACPD (2E), and substance P (2F) when compared to the control group. The calculated mean ID_{50} values for the antinociceptive effect of guanosine against glutamate, AMPA, kainate, trans-ACPD, and substance P were 140 (103–190), 136 (100–185), 162 (133–196), 266 (153–461) and 28 (3–292) nmol and maximal inhibitions of 69±10, 74±8, 68±7, 47±12, and 88±5%, respectively. In contrast, guanosine had no significant effect against NMDA- (2B) and capsaicin- (2G) mediated biting response in mice.

In the hole-board test, i.t. guanosine (50 - 200 nmol) did not affect latency to first head-dip, and the number of head-dips, crossings, rearings, groomings and defecations as compared to control (Table 1). Intrathecal guanosine did not cause motor deficits or ataxia, as evaluated by the performance in the rotarod test and did not affect spontaneous locomotor activity as measured by activity cages (Table 1).

As evidenced in the Figure 3, i.t. administration of guanosine (200 nmol) produced a significant increase in guanosine, inosine, xanthine and uric acid CSF levels. Administration of i.t. guanosine did not affect hypoxanthine (Figure 3). Moreover, the adenosine, ATP, ADP, AMP, GTP, GDP, and IMP CSF levels were not affected by guanosine administration (data not shown).

Figure 4 shows the effects of i.t. guanosine or vehicle (0.1 mN NaOH) followed by i.pl. capsaicin or vehicle (5% DMSO) on glutamate uptake by mice spinal cord slices. Capsaicin produced a significant increase in spinal cord glutamate uptake, an effect prevented by pretreatment with guanosine ($P < 0.05$).

4. Discussion:

The results of the present study extend previous data from our group (Schmidt et al., 2008) and clearly demonstrate that the glutamatergic system, more specifically via interaction with non-NMDA receptors, plays a critical role in the antinociceptive effect caused by guanosine. Intrathecal injection of guanosine caused significant inhibition of i.t. glutamate and non-NMDA agonists-induced biting behavior, but did not inhibit the biting response caused by NMDA. Additionally, guanosine also inhibited biting response induced by i.t. substance P, but not against capsaicin.

ABPs have been considered important targets for the development of new drugs for treating pain, since the nucleoside adenosine and its analogs induce antinociceptive effects following both systemic and central administration (Sawynok, 1998; Sawynok and Liu, 2003). Since nucleosides guanosine and adenosine closely interact in modulating several functions of the CNS (Dobolyi et al., 2000), guanosine might well play a significant role on pain transmission and nociception. More recently, we demonstrated that i.c.v. guanosine is antinociceptive in several pain tests (Schmidt et al., 2008). Interestingly, we also demonstrated that GMP-induced antinociceptive effects were prevented by the 5'-nucleotidase inhibitor AOPCP, suggesting that GMP antinociception results from its conversion to guanosine (Schmidt et al., 2008). This effect was similarly demonstrated in the investigation of GMP anticonvulsant effects (Soares et al., 2004) and GMP-induced increase in glutamate uptake (Frizzo et al., 2003). Therefore, in the present study, only guanosine was used to investigate spinal antinociceptive effects of guanine-based purines.

Previous studies have suggested involvement of the adenosinergic system in effects of guanosine, since guanosine stimulates the release of adenosine in cultured astrocyte, and both are released under excitotoxic conditions (Ciccarelli et al., 1999, 2001). Besides, guanosine induces trophic effects that are partially inhibited by adenosine deaminase and adenosine receptor antagonists (Ciccarelli et al., 2000). In contrast, some studies exclude the involvement of adenosine and its receptors to play a role in guanosine effects: guanosine-induced enhancement of neurite outgrowth in PC12 cells was not affected by adenosine receptor antagonists (Gysbers et al., 1996), nor were the effect of guanosine on glutamate uptake (Frizzo et al., 2001), seizures induced by glutamatergic

agents (Lara et al., 2001), or impairment in the inhibitory avoidance task (Roesler et al., 2000; Vinadé et al., 2004). Additionally, i.c.v. guanosine-induced antinociception was not prevented by adenosine-receptor antagonists, and i.c.v. guanosine or GMP (which would latter produce guanosine) failed to increase ABPs levels in the CSF (Schmidt et al., 2008). In the present study, i.t. injection of guanosine also failed to increase CSF ABPs levels. Considering that high affinity binding sites for guanosine have been reported in the rat brain (Traversa et al., 2002, 2003), this study reinforces the proposal that guanosine could act independently from adenosinergic system in inhibiting nociception. Notably, i.t. administration of guanosine caused a significant increase in CSF levels of oxypurines, mainly xanthine, which probably indicate an *in vivo* degradation. The significant production of oxypurines (xanthine and uric acid) and inosine can not be excluded to play a role in the antinociceptive effects of guanosine. Since extracellular guanine also exerts several biological effects (Rathbone et al., 2008), the antinociceptive effects of guanosine may likely be regulated by its conversion to guanine by a membrane located purine nucleoside phosphorylase (ecto-PNP). A previous study has demonstrated that after a systemic administration of guanosine, the amount of both guanosine and guanine at the spinal cord increased, reaching a maximum effect by 30 min (Jiang et al., 2008). However, our study was not accurate to measure CSF guanine levels.

With regard the anatomical area in which guanosine induces antinociception, it is difficult to evaluate if the effect here observed is related to spinal and/or supraspinal mechanisms, since it is shown that i.t. guanosine leads to marked increases in CSF guanosine levels. Because CSF was obtained from *cisterna magna* 5 min after the i.t.

administration of guanosine, a wide dispersion of guanosine throughout the CNS (brain and spinal cord) could have occurred. Given that there are studies showing that guanosine produces neuroprotective effects at the spinal cord level (Jurkowitz et al., 1998; Litsky et al., 1999; Jiang et al., 2003, 2007, 2008), it is tempting to speculate that guanosine may act at spinal cord in addition to the brain.

Glutamate and its receptors play a crucial role in pain transmission mechanisms and modulation of glutamate receptors may have therapeutic potential for several categories of pain (Millan, 1999). Therefore, the search for new agents which impact upon glutamatergic mechanisms is important in the understanding of pain, and the search for new analgesics. Guanosine has been shown to prevent ischemic injury (Frizzo et al., 2002) and NMDA-induced excitotoxicity (Ciccarelli et al., 2001). Guanosine prevents seizures and toxicity induced by drugs that overstimulate the glutamatergic system (Baron et al., 1989; Schmidt et al., 2000; 2005; Lara et al., 2001; Vinadé et al., 2003; Losch et al., 2004), and is neuroprotective against stroke and spinal cord injury (Jiang et al., 2003; 2007; 2008; Chang et al., 2008). Although the overall effects of guanosine seem related to attenuating glutamatergic overstimulation, its precise mechanism of action remains unclear. Guanosine is a poor displacer of glutamate ligands (Souza and Ramirez, 1991), so direct antagonism on glutamatergic receptors seems unlikely. Surprisingly, in this study, guanosine produced a significant inhibition of the biting behavior induced by i.t. injection of glutamate or non-NMDA agonists (AMPA, kainate and trans-ACPD), but not against NMDA. Additionally, glutamate, substance P, capsaicin and their receptors closely interact in central and peripheral mechanisms of pain transmission (Siebel et al., 2004). In fact, substance P co-exists with glutamate in sensory

fibers (Biasi et al., 1988) and capsaicin elicits a significant release of glutamate and substance P from spinal cord (Skilling et al., 1993). In this study, guanosine partially inhibited the biting response induced by i.t. injection of substance P, but not of capsaicin, indicating that NK_1 rather than vanilloid ($TRPV_1$) receptors may play a role in guanosine-induced antinociception. Considering these findings, we hypothesize that antinociceptive effects caused by guanosine involve a possible interaction with glutamate (mainly non-NMDA receptors) and substance P receptors and/or with their signal transduction mechanisms.

There is compelling evidence suggesting that excitatory amino acids (glutamate and aspartate) are released in the CNS in response to several peripheral noxious stimuli, including i.pl. administration of capsaicin (Ueda et al, 1993). Since the i.pl. capsaicin test involves peripheral and central mechanisms, induces glutamate and aspartate release from dorsal spinal cord *in vivo* (Sorkin and McAdoo 1993) and the nociceptive response may be prevented by glutamate-receptor antagonists (Sakurada et al., 1998), it is not surprising that an i.pl. injection of capsaicin modulated glutamate uptake at the spinal cord. Therefore, the capsaicin pain test seems to be a reliable model to investigate the mechanisms involved in guanosine-induced antinociceptive effects. Recently, we demonstrated that an i.pl. administration of capsaicin caused a decrease in cortical glutamate uptake, an effect prevented by i.c.v. guanosine (Schmidt et al., 2008). Since guanosine has been shown to stimulate glutamate uptake *in vitro* (Frizzo et al., 2001, 2002, 2003), it was speculated that the *in vivo* antinociceptive effect of i.c.v. guanosine against capsaicin could result from its effect on glutamate removal from the synaptic cleft, leading to less activation of glutamatergic receptors. However, in the present study,

we showed that an i.pl. capsaicin produced an increase in the spinal cord glutamate uptake, an effect prevented by i.t. guanosine as well. Notably, either i.c.v. or i.t. guanosine did not alter basal glutamate uptake at both sites (brain and spinal cord). It is not possible to determine whether the changes in the spinal cord glutamate uptake were responsible for nociceptive behavior or it caused the changes. However, considering our results, we suggest that the changes in the spinal cord glutamate uptake induced by capsaicin and guanosine were probably produced by nociceptive stimulus modulation rather to an underlying mechanism of action. Considering that there is growing evidence suggesting that alteration of glutamatergic neurotransmission within the spinal cord contributes to hyperalgesic responses (Woolf and Thompson, 1999) and spinal glutamate uptake is critical for normal sensory transmission (Sung et al., 2003; Liaw et al., 2005), it is tempting to speculate that the increase in the global glutamate uptake at spinal cord slices may represent a protective response of spinal cord cells to the increased extracellular availability of glutamate caused by i.pl. capsaicin injection. Consequently, we may speculate that guanosine, perhaps by modulating glutamatergic receptors or inhibiting glutamate release, could reduce glutamate levels at the synaptic cleft, decreasing its uptake even after capsaicin administration.

Guanosine may exert antinociceptive effects through one or more mechanisms, which might include effects in tissues outside the CNS which could influence nociception, production and release of trophic factors, and/or reduction of glutamate toxicity (by modulating glutamate uptake or glutamatergic receptors). Since guanosine was directly administrated to the CNS, a peripheral mechanism is unlikely. In addition to the modulation of glutamatergic system, we may speculate that guanosine could influence

pain transmission by releasing trophic factors. Purines, including guanosine, stimulate the synthesis and release of several protein trophic factors by astrocytes, including bFGF (basic fibroblast growth factor), nerve growth factor (NGF), neurotrophin-3, ciliary neurotrophic factor, S-100β protein and others (Rathbone et al., 1999). Although endogenous release of neurotrophic factors is triggered by nociceptive stimuli and may be involved in the induction changes in spinal transmission in various pain states (Jongen et al., 2005), it has been demonstrated that i.t. administration of some neurotrophic factors may be efficient in the treatment of neuropathic pain (Pezet et al., 2008). However, an *in vivo* correlation between guanosine, neurotrophic factors and pain transmission remains to be further investigated.

As commented above, there is data supporting the possible existence of specific receptor-like binding sites for guanosine on membrane preparations from rat brain (Traversa et al., 2002, 2003). The addition of GTP produces a small concentration-dependent decrease in guanosine binding, which could suggest that this site is linked to a G protein. We speculate that guanosine, through its specific binding site, may promote its extracellular effect by activating intracellular cAMP-dependent and independent pathway (PKC-ERK 1/2 pathway), MAP kinase and/or PI3-kinase signalling pathways (Gysbers and Rathbone, 1996; Tomaselli et al., 2005). Guanosine also could act as an alternative source of energy for neural cells after further metabolism, as previously demonstrated in spinal cord cultures (Jurkowitz et al., 1998; Litsky et al., 1999). Intracellular mechanisms underlying guanosine effects remain to be investigated in future studies.

Regarding side effects, a significant point to be considered is that ABPs, even by an intrathecal route, may cause important side effects such as impaired motor function

and sedation (Sawynok and Liu, 2003). It is possible that such alterations could have influenced guanosine-induced effects as well. However, our results showed that i.t. guanosine did not induce obvious behavioral disturbances (hole-board), altered coordination (rotarod) or locomotion (activity cages), consistent with results previously demonstrated (Lara et al., 2001; Vinadé et al., 2003; Schmidt et al., 2008).

In summary, this is the first study showing an antinociceptive action by spinal administration of guanosine. It provides new evidence on the role of extracellular guanosine in the CNS. Because guanosine is an endogenous compound apparently well tolerated, it could eventually be developed as a drug useful for pain treatment. These results do not exclude the involvement of other neurochemical parameters rather than glutamatergic system in the guanosine effects. We are continuing to investigate the mechanisms by which guanosine produces antinociception.

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Legends:

Table 1: Guanosine (50 – 200 nmol) or vehicle (0.1 mN NaOH) were i.t. administered 5 min prior to the behavior measurements: latency to the first head-dip; head-dips; squares crossed; rearings; groomings; fecal boli; latency to fall (rotarod); number of crossings (spontaneous locomotor activity test). Data are mean ± S.E.M. N = 8 animals per group; one-way ANOVA.

Figure 1: Effects of i.t. (8 μl) vehicle (0.1 mN NaOH) or guanosine (10 to 400 nmol) against i.pl. capsaicin (A), i.pl. glutamate (B), tail-flick (C) and hot-plate (D) tests in mice. (A and B) Columns represent mean time spent licking the injected hindpaw and vertical bars represent S.E.M. (C and D) Columns represent mean percent of maximum possible effect (%MPE) and vertical bars represent S.E.M. N = 10 – 12 animals per group. * = $P < 0.05$ and ** = $P < 0.01$ compared to vehicle (control), one-way ANOVA followed by Student-Newman-Keuls test.

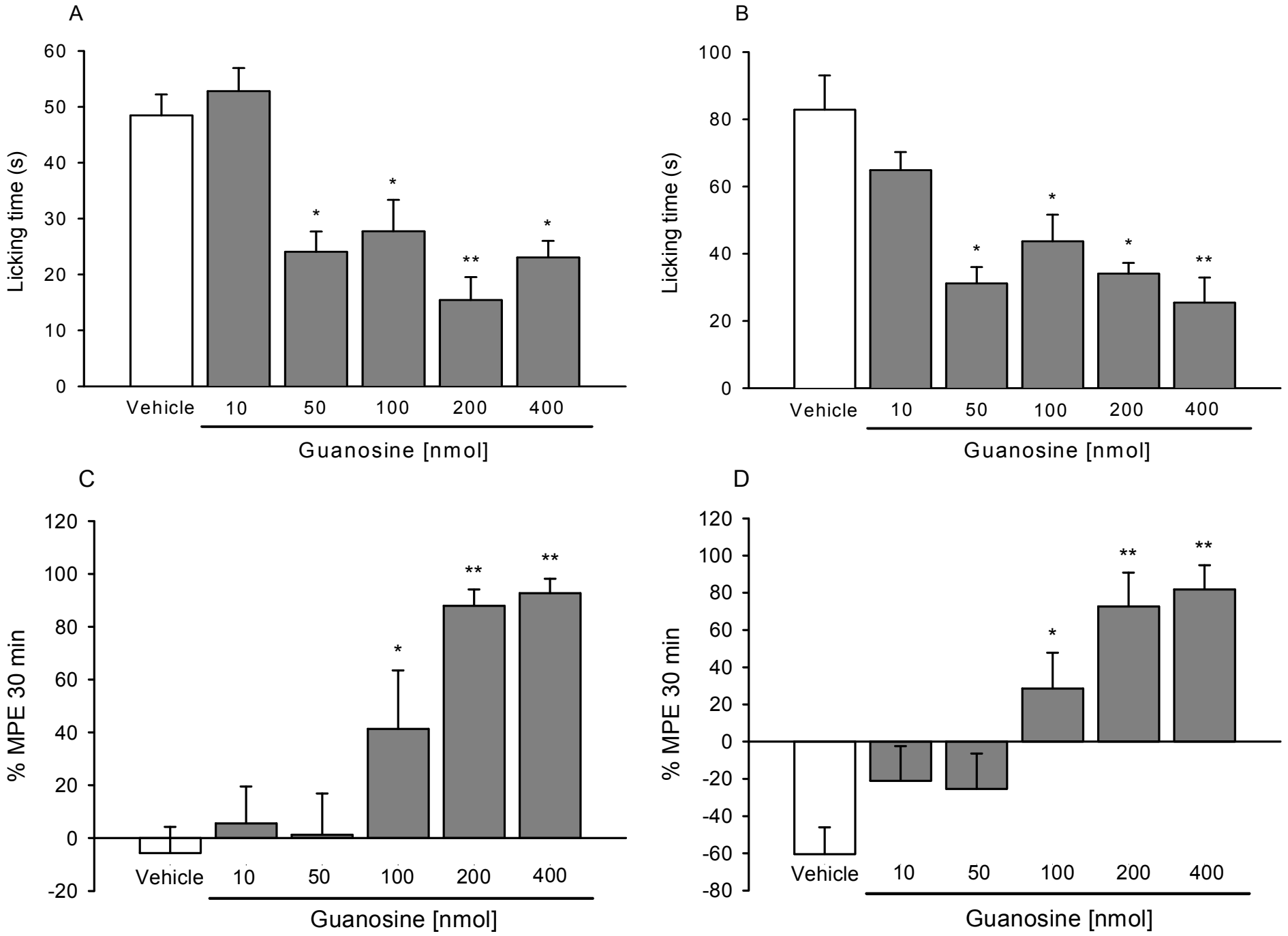
Figure 2: Effects of i.t. guanosine (50 – 200 nmol), against glutamate (175 nmol/site, i.t., A)-, NMDA (450 pmol/site, i.t., B)-, AMPA (135 pmol/site, i.t., C)-, kainate (110 pmol/site, i.t., D)-, trans-ACPD (50 nmol/site, i.t., E)-, substance P (135 ng/site, i.t., F)-, or capsaicin (30 ng/site, i.t., G)-induced biting in mice. Columns represent mean and vertical bars represent S.E.M. N = 8 animals per group.* = $P < 0.05$ and ** = $P < 0.01$ compared to the control group values (closed columns), one-way ANOVA followed by Student-Newman-Keuls test.

Figure 3: Effects of i.t. guanosine (GUO - 200 nmol) or vehicle (0.1 mN NaOH) on CSF concentration of purines. The columns represent mean (μM) and vertical bars represent S.E.M. Guanosine or vehicle was i.t. administered 5 min prior to the CSF sampling. UA = uric acid; XA = xanthine; HX = hypoxanthine; GUO = guanosine; INO = inosine. N = 8 animals per group. * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$ compared to vehicle (control), one-way ANOVA followed by Student-Newman-Keuls test.

Figure 4: Effects of i.t. guanosine and i.pl. capsaicin on glutamate uptake by spinal cord slices from mice. Animals were treated with an i.t. injection of guanosine (200 nmoles) or vehicle (0.1 mN NaOH); after 5 min, mice received an i.pl. injection of capsaicin or vehicle (5% DMSO). After behavioral evaluation, mice were sacrificed and the spinal cord slices processed for glutamate uptake assay. Data are mean ± S.E.M. N = 10 animals per group. * = $P < 0.05$ compared to other groups, one-way ANOVA followed by Student-Newman-Keuls test.

Figure

Figure 1



Figure

Figure 2

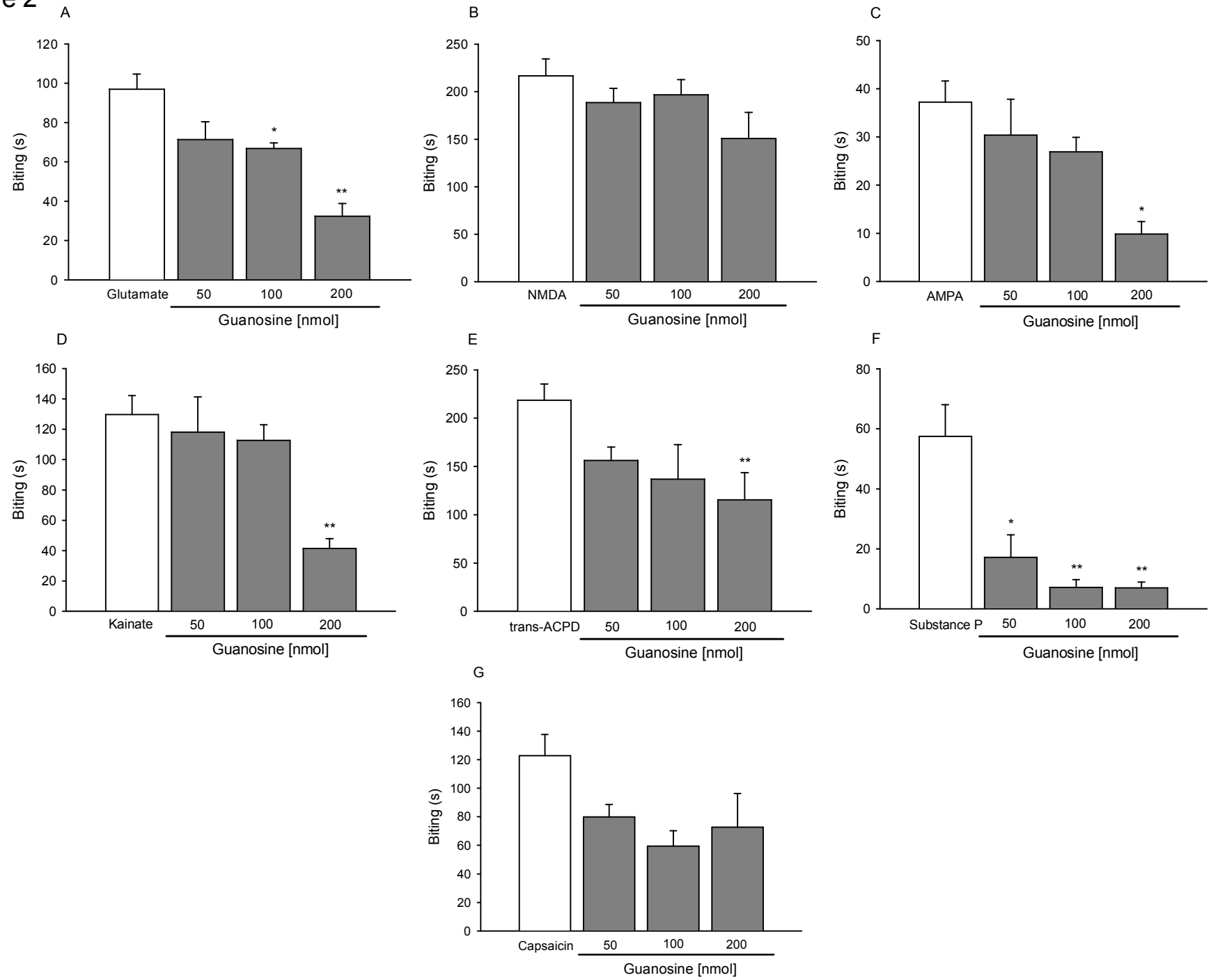
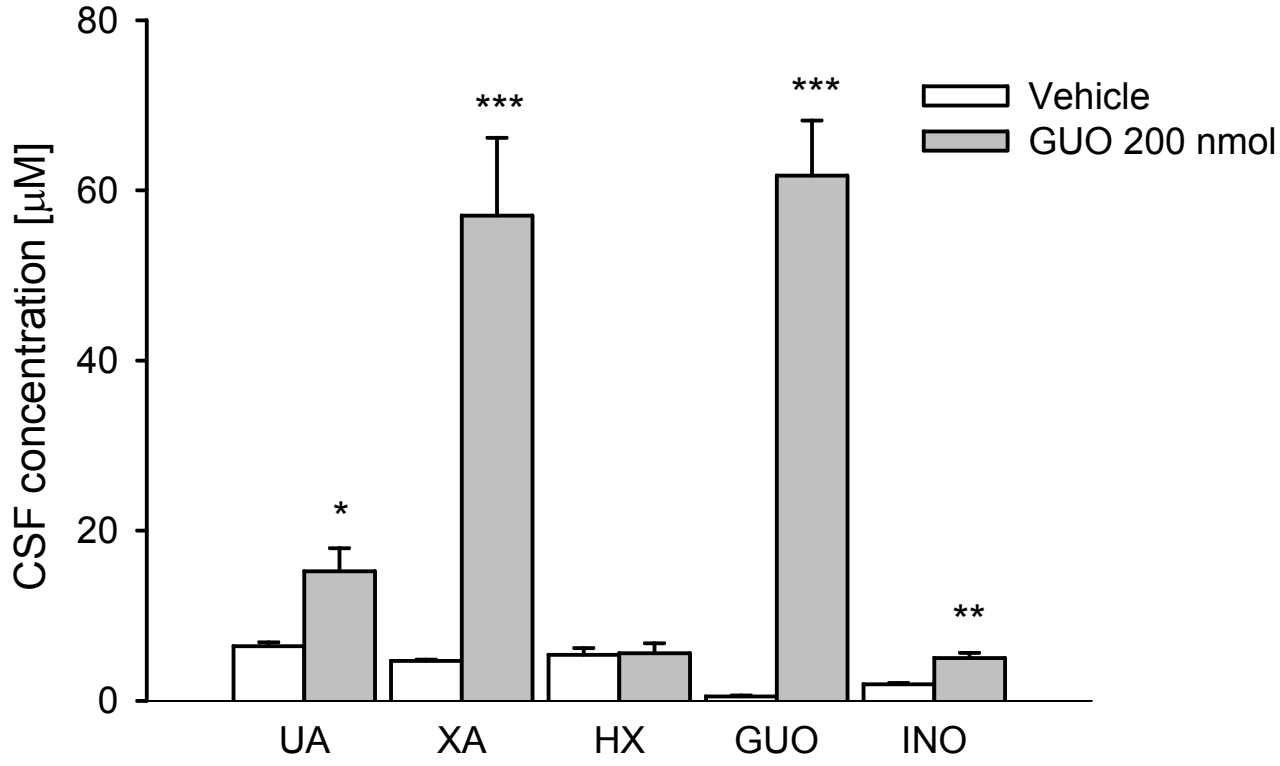


Figure 3


80
60
40
20
0
CSF concentration [µM]
*
***
***
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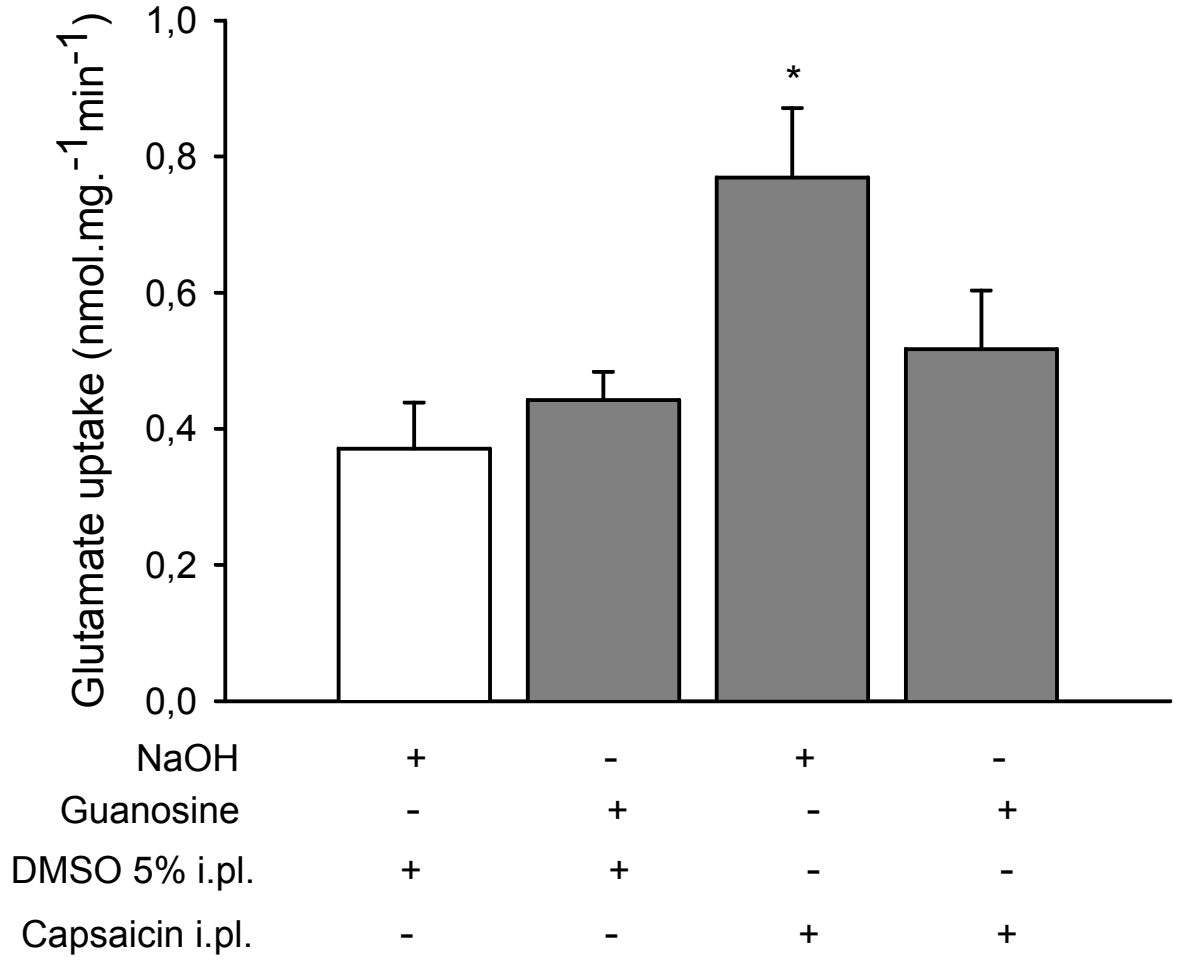
Vehicle
GUO 200 nmol
UA
XA
HX
GUO
INO

Figure

Figure 4



Table

Table 1 - Effects of i.t. vehicle (0.1 mN NaOH) or guanosine (50 – 200 nmol) on the mice hole-board, rotarod and spontaneous locomotor activity tests:

Treatment	NaOH	Guanosine [nmol]		
		50	100	200
Latency to head-dip (s)	4.4 (1.2)	5.0 (1.3)	5.4 (1.6)	6.2 (1.7)
Head-dips (n)	84.8 (5.1)	84.7 (8.4)	70.2 (4.7)	73.8 (4.8)
Squares crossed (n)	45.9 (5.3)	54.6 (8.2)	57.4 (10.7)	50.6 (7.0)
Rearings (n)	1.5 (0.8)	1.5 (0.9)	1.7 (0.6)	1.5 (0.5)
Groomings (n)	1.7 (0.5)	1.2 (0.5)	1.4 (0.6)	1.2 (0.3)
Fecal boli (n)	1.0 (0.4)	1.3 (0.6)	1.5 (0.6)	1.0 (0.5)
Latency to fall (s)	55.6 (3.6)	60.0 (0)	57.5 (2.0)	58.3 (1.1)
Crossings (n)	136.3 (24.7)	152.2 (32.8)	158.2 (25.7)	122.4 (18.3)

II.2.g. Mechanisms involved in the antinociception induced by systemic administration of guanosine in mice

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Mechanisms involved in the antinociception induced by systemic administration of guanosine in mice.

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Running title: Guanosine-induced antinociception in mice.

Summary:

Background and purpose: It is well known that adenine-based purines exert multiple effects on pain transmission. However, less attention has been given to the potential effects of guanine-based purines on pain transmission. The aim of this study was to investigate the effects of intraperitoneal (i.p.) or oral (p.o.) administration of guanosine on mice pain models. Additionally, investigation on the mechanism of action of guanosine, its potential toxicity and measurement of CSF purine levels were also performed.

Experimental approach: Mice received an i.p. or p.o. administration of vehicle (0.1 mN NaOH) or guanosine (up to 240 $mg.kg^{-1}$) and submitted to several pain models.

Key results: Guanosine produced dose-dependent antinociceptive effects against hot-plate, glutamate, capsaicin, formalin and acetic acid models, but it was ineffective in the tail-flick test. Additionally, i.p. guanosine produced significant inhibition of the biting behavior induced by i.t. injection of glutamate, AMPA, kainate and trans-ACPD, but not against NMDA, substance P or capsaicin. Systemic administration of guanosine (120 $mg.kg^{-1}$) induced an approximately 7-fold increase on CSF guanosine levels. Guanosine prevented the increase on spinal cord glutamate uptake induced by intraplantar capsaicin.

Conclusions and implications: This study provides new evidence on the mechanism of action of guanosine presenting antinociceptive effects after systemic administration. This effect seems to be partially associated with the modulation of non-NMDA receptors at spinal cord sites.

Keywords: Guanosine; Purines; Pain; Glutamate; Antinociception.

Abbreviations: A_1, adenosine receptor type 1; ABPs, adenine-based purines; AOPCP, α,β-methyleneadenosine 5'-diphosphate; DMSO, dimethyl sulfoxide; GBPs, guanine-based purines; HBSS, Hank's balanced salt solution; HPLC, high-performance liquid chromatography; L-NOARG, *N*-nitro-L-arginine; MPE, maximum possible effect; NO, nitric oxide; TFL, tail-flick latency; trans-ACPD, (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid.

Introduction:

It is well known that extracellular adenine-based purines (ABPs), namely the nucleotide ATP and the nucleoside adenosine, have an important role in pain signaling at both the periphery and in the central nervous system (CNS) [Sawynok, 1998; Sawynok and Liu, 2003; Inoue et al., 2005]. ATP can stimulate sensory nerve endings causing pain and a marked increase in the discharge from sensory neurons and is involved in the initiation of different types of nociception and pain [Burnstock, 2007]. Adenosine presents antinociceptive effects by acting at spinal, supraspinal and peripheral sites [Sawynok, 1998], plays a pivotal role in inflammatory and neuropathic pain [McGaraughty and Jarvis, 2005], and mediates aspects of opioid-induced analgesia [Sawynok, 1998; Eisenach et al., 2004]. Antinociceptive effects of adenosine are particularly related to the activation of adenosine A_1 receptors in the spinal cord, where inhibition of intrinsic neurons and sensory nerve terminals simultaneously with a diminished release of substance P and glutamate contributes to its actions [Sawynok and Liu, 2003].

Although ATP and adenosine are usually considered the main effectors of the purinergic system [Burnstock, 2007], extracellular guanine-based purines (GBPs) exert biological effects unrelated to the direct G-proteins modulation; these include modulation of glutamatergic activity [Souza and Ramirez, 1991; Schmidt et al., 2007], trophic effects on neural cells [Ciccarelli et al., 2001], and behavioral effects [Schmidt et al., 2000; Lara et al., 2001; Schmidt et al., 2005]. Concerning *in vitro* effects on the glutamatergic system, GBPs inhibit the binding of glutamate and analogs [Baron et al., 1989; Burgos et al., 1998], prevent cell responses to excitatory amino acids [Souza and Ramirez, 1991],

present neuroprotective effects in cultured neurons submitted to hypoxia and increase glutamate uptake in cultured astrocytes [Frizzo et al., 2001, 2002, 2003]. *In vivo*, GBPs prevent glutamate-induced seizures and toxicity [Malcon et al., 1997; Regner et al., 1998; Lara et al., 2001; Schmidt et al., 2000, 2005; Saute et al., 2006], and are anxiolytic/amnesic in rodents [Roesler et al., 2000; Vinadé et al., 2003, 2004, 2005]; these effects seem to be related to conversion to guanosine [Soares et al., 2004; Schmidt et al., 2005]. Recently, we have demonstrated that intracerebroventricular (i.c.v.) injection of guanosine or GMP was antinociceptive against several chemical and thermal pain models in mice [Schmidt et al., 2008]. Additionally, we have shown that spinal administration of guanosine produces significant inhibition of glutamate, non-NMDA agonists and substance P-induced biting behavior (personal communication). Importantly, most of these effects seem to be related, at least partially, to guanosine-induced modulation of the glutamatergic pathways.

This study was designed to investigate the antinociceptive effects of intraperitoneal (i.p.) or oral (p.o.) administration of guanosine in mice. Attempts have been made to further investigate some of the possible mechanisms that underlie the antinociceptive action of guanosine and acute toxicity induced by its systemic administration.

Methods:

Animals: Male adult Swiss albino mice (3-4 months of age, 30-40 g) were kept on a 12 hour light/dark cycle (light on at 7:00 am) at temperature of 22 ± 1ºC, housed in plastic cages (five per cage) with tap water and commercial food *ad libitum.* In all

nociceptive behavioral experiments, the animals were acclimatized to the laboratory for at least 1 h before testing. The ethical guidelines for investigations of experimental pain in conscious animals [Zimmermann, 1983] and our institutional protocols for experiments with animals, designed to avoid suffering and limit the number of animals sacrificed, were followed throughout. This study was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Drugs: Guanosine, L-glutamic acid hydrochloride (glutamate), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid (kainate), N-methyl-D-aspartate (NMDA), Substance P, and (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (trans-ACPD), capsaicin and dexamethasone were purchased from Sigma Chemicals (St. Louis, MO, USA). The anesthetic sodium thiopental and morphine sulphate were obtained from Cristália (SP, Brazil). Guanosine was dissolved in 0.1 mN NaOH and buffered to pH 7.4. The amount of NaOH caused no detectable effect. Capsaicin was diluted in DMSO (dimethyl sulfoxide, 5%). All other solutions were dissolved in saline (NaCl 0.9%) and buffered with 0.1 N NaOH or 0.1 N HCl to pH 7.4 when necessary. Drug and molecular target nomenclature used in this manuscript conforms with the Guide to Receptors and Channels [Alexander et al., 2008].

Drugs administration: Experiments were performed according to Schmidt et al. [2000]: 20 min before the experiment, animals were placed individually in acrylic boxes, which served as observation chambers. After this adaptation period, treatments were performed. Animals were treated with an i.p. or p.o. injection (10 $ml.kg^{-1}$) of vehicle (0.1

mN NaOH) or guanosine (up to 240 mg.kg^{-1}) and submitted to pain tests 30 or 45 min thereafter, respectively. Morphine (6 mg.kg^{-1}) and/or dexamethasone (30 mg.kg^{-1}) were used in some experiments as positive controls. The doses and time of drug administration were selected on the basis of literature data and also based in previous results from our laboratory [Schmidt et al., 2000; Lara et al., 2001; Vinadé et al., 2003, 2004, 2005]. In order to minimize number of animals used, some mechanistic studies of guanosine effects and toxicity tests were performed only using i.p. treatment.

Capsaicin-induced nociception: The method used for capsaicin-induced licking was similar to that described by Sakurada et al. [1993]. Thirty or 45 min after i.p. or p.o. treatments respectively (vehicle, morphine or guanosine 7.5 to 240 mg.kg^{-1}), 20 µl of capsaicin (1.6 µg/paw) were injected intraplantarly (i.pl.) under the plantar surface of the right hindpaw (Hamilton microsyringe with a 26-gauge needle). Animals were observed individually for 5 min after capsaicin administration for the time spent licking the injected paw, which was considered as indicative of nociception. Considering that the capsaicin test involves peripheral and central mechanisms of nociception, pain induced by capsaicin may be prevented by glutamate receptor antagonists [Sakurada et al. 1998] and capsaicin induces glutamate and aspartate release from dorsal spinal cord [Sorkin and McAdoo 1993, Jeftinija et al. 1991; Ueda et al. 1993], the capsaicin pain model was chosen for a wide dose-response curve of guanosine.

Tail-flick test: Nociception was assessed with a tail-flick apparatus (Albrasch Electronic Equipments, Brazil), as described in detail elsewhere [D'Amour and Smith, 1941]. A source of light was positioned below the tail and the time that the mouse took to withdraw its tail from the noxious stimulus was recorded. The trial was automatically

terminated when the mouse tail deflected activating a photocell that turns off the light. A cut-off time of 10 s was employed in order to prevent tissue damage (a mouse that did not flick by 10 s was considered as fully analgesic). On day one, the animals were habituated with the tail-flick apparatus through three separate measures (data not shown). On day two, baseline tail-flick latency (TFL) was measured for each mouse prior to the treatments. Animals displaying at least two TFL of 10 s on the baseline were excluded from the study. Immediately after the third TFL measurement, animals received i.p. or p.o. treatments (vehicle, morphine or guanosine 30, 60 or 120 $mg.kg^{-1}$) and, after 30 or 45 min respectively, were submitted to the tail-flick apparatus for TFL measurement. Data are expressed as mean percent of maximum possible effect (%MPE) ± SEM, according to the following formula [Calcagnetti et al., 1990]: %MPE: 100 x (postdrug latency – baseline latency) / (Cutoff time – baseline latency).

Hot-plate test: Response latencies were measured according to the method described by Eddy and Leimback [1953], with minor modification. The hot-plate apparatus (Ugo Basile, model-DS 37, Italy) was maintained at 55 ± 0.5 °C. Animals were placed into a glass cylinder of 24 cm diameter on the heated surface, and the time between placement of the animal on the hot-plate and the occurrence of licking hindpaws or jumping off the surface was recorded as response latency. On day one, the animals were first habituated with the apparatus. On day two, mice were tested and animals displaying baseline latencies of more than 15 s were excluded from the study. An automatic 20 s cut-off was used to prevent tissue damage. Each animal was tested before administration of drugs in order to obtain the baseline. Thirty or 45 min after i.p. or p.o. treatments respectively (vehicle, morphine or guanosine 30, 60 or 120 $mg.kg^{-1}$), animals

were placed on the heated surface and response latency recorded as described above. Data are expressed as mean percent of maximum possible effect (%MPE) ± SEM, according to the following formula [Calcagnetti et al., 1990]: %MPE: 100 x (postdrug latency – baseline latency) / (Cutoff time – baseline latency).

Formalin-induced nociception: The formalin test was carried out as described by Hunskaar and Hole [1987]. Animals received 20 µl of 2.5% formalin solution (0.92% of formaldehyde), injected i.pl. under the plantar surface of the right hindpaw. Animals were pretreated with i.p. or p.o. administration of vehicle, morphine, dexamethasone or guanosine 30, 60 or 120 $mg.kg^{-1}$, 30 or 45 min before formalin injection, respectively. After i.pl. injection of formalin, the animals were observed from 0–5 min (neurogenic phase) and 15–30 min (inflammatory phase) and the time spent licking the injected paw was timed with a chronometer and considered as indicative of nociception.

Acetic acid-induced abdominal constriction: The abdominal constriction was induced according to procedures described previously by Corrêa et al. [1996] and resulted in contraction of the abdominal muscle together with a stretching of the hind limbs in response to an i.p. injection of acetic acid (1.6%) at time of the test. Mice were pretreated with i.p. or p.o. vehicle, morphine or guanosine 30, 60 or 120 $mg.kg^{-1}$, 30 or 45 min before the irritant injection. After the challenge, the abdominal constrictions were counted cumulatively over a period of 20 min. Antinociceptive activity was expressed as the reduction in the number of abdominal constrictions, i.e. the difference between control animals (mice pretreated with vehicle) and animals pretreated with guanosine.

Glutamate-induced nociception and paw oedema: The procedure used was similar to Beirith et al. [2002]. Thirty or 45 min after i.p. or p.o. treatments respectively (vehicle,

morphine or guanosine 30, 60 or 120 mg.kg^{-1}), 20 µl of glutamate solution (10 µmol/paw prepared in saline) was injected i.pl. under the plantar surface of the right hindpaw. The mice were observed individually for 15 min following glutamate injection and the amount of time spent licking the injected paw was considered as indicative of nociception. In order to verify whether the antinociceptive activity produced by i.p. guanosine in glutamate-induced nociception was associated with development of oedema formation, we measured the paw oedema by comparing the difference between the weight of the glutamate-treated paw and the weight of the contralateral paw (nontreated paw). For this purpose, animals were sacrificed 15 min after glutamate injection by cervical dislocation, and both paws were cut at the ankle joint and weighed on an analytical balance. To address some mechanisms involved in local effect caused by guanosine on glutamate-induced nociception and paw oedema, distinct groups of animals were treated with i.pl. guanosine (200 nmol) or vehicle, both locally co-administered with glutamate (10 µmol/paw).

Analysis of the mechanisms involved in guanosine action on the glutamate test: To explore the possible involvement of nitric oxide-L-arginine-cGMP pathway in the antinociceptive action caused by guanosine, mice were pretreated with L-arginine (600 mg.kg^{-1}, i.p., a nitric oxide precursor) or D-arginine (600 mg.kg^{-1}, i.p., an inactive isomer of L-arginine), and after 20 min, they received guanosine (60 mg.kg^{-1}, i.p.), *N*-nitro-L-arginine (L-NOARG; 75 mg.kg^{-1}, i.p., an inhibitor of nitric oxide synthesis), or vehicle (0.1 mN NaOH, i.p.). The nociceptive responses to i.pl. glutamate were recorded 30 min after the administration of guanosine, L-NOARG, or vehicle. A separate group of animals were pretreated with L-NOARG (30 mg.kg^{-1}, i.p.), methylene blue (a non-specific

inhibitor of NO/guanylyl cyclase; 1 $mg.kg^{-1}$, i.p.), or vehicle (saline, i.p.) and after 15 min received guanosine (60 $mg.kg^{-1}$, i.p.) or vehicle (0.1 mN NaOH, i.p.) 30 min before i.pl. glutamate injection [Abacioglu et al., 2001; Duarte and Ferreira, 2000].

Spinal algogen-induced nociception-related behavior in mice: To test the hypothesis that spinal excitatory amino acids, substance P, or capsaicin might be involved in the antinociceptive effect caused by guanosine, we assessed the effect of guanosine (30, 60 or 120 $mg.kg^{-1}$ or vehicle, i.p.) 30 min in advance on the biting response induced by i.t. injection of 5 μl of these algogens. The nociceptive response was elicited by glutamate (an excitatory amino acid, 175 nmol/site, i.t.), NMDA (a selective agonist of NMDA-subtype of glutamatergic ionotropic receptors, 450 pmol/site, i.t.) [Urca and Raigorodsky, 1988], AMPA (a selective agonist of AMPA-subtype of glutamatergic ionotropic receptors, 135 pmol/site, i.t.) [Brambilla et al., 1996], kainate (a selective agonist of kainate-subtype of glutamatergic ionotropic receptors, 110 pmol/site, i.t.), trans-ACPD (a metabotropic glutamate agonist, 50 nmol/site, i.t.) [Boxall et al., 1998], substance P (NK_1 receptor-selective agonist, 135 ng/site) [Sakurada et al., 1990], capsaicin ($TRPV_1$ receptor agonist, 30 ng/site) [Sakurada et al., 1996]. A group of mice received only vehicle (saline) by i.t. route and no significant biting behavior was observed (data not shown). Immediately after i.t injection of each agonist, mice were placed individually in observation chambers, and the amount of time (s) the animal spent biting itself was evaluated: glutamate (3 min); AMPA (1 min); kainate (4 min); NMDA (5 min); substance P and capsaicin (6 min); and trans-ACPD (15 min). A bite was defined as a single head movement directed at the flanks or hind limbs, resulting in contact of the animal's snout with the target organ.

Hole-board test: The hole-board apparatus (Ugo Basile, Italy) consisted of gray Perspex panels (40 x 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 x 10 cm with a water-resistant marker. Thirty min after i.p. treatments (vehicle or guanosine 30, 60 or 120 $mg.kg^{-1}$), 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 the locomotion [Vinadé et al., 2003].

Measurement of motor performance: In order to evaluate non-specific muscle relaxant or neurotoxic effects, we evaluated the effects of guanosine (30, 60 or 120 $mg.kg^{-1}$ or vehicle, i.p.) in the rotarod test and on the spontaneous locomotor activity. The rotarod apparatus (Ugo Basile, Italy) consisted of a rotating (18 rpm) bar (2.5 cm diameter), subdivided by disks into six compartments. As previously described [Vinadé et al., 2003], mice were initially trained to remain on the rotarod apparatus for 120 s. Those not remaining on the bar for at least two out of three consecutive trials were discarded. On the day after training, the latency to fall from the rotarod (one trial with a maximum of 60 s) was determined 30 min after i.p. treatments. The method for the spontaneous locomotor activity was adapted from Creese et al. [1976]. Activity cages (45 x 25 x 20 cm, Albarsch Electronic Equipment, Brazil), equipped with three parallel photocells, automatically record the number of crossings. Animals were individually

habituated to an activity cage for 10 min before treatments. The animals returned to the activity cages 30 min after i.p. treatments, and the crossings were recorded for 15 min.

Potentiation of barbiturate sleeping time in mice: In order to investigate sedative properties of guanosine, mice pretreated with an i.p. administration of guanosine (30, 60 or 120 $mg.kg^{-1}$) or vehicle 30 min before an i.p. injection of sodium pentobarbital (30 $mg.kg^{-1}$). After the barbiturate injection, the sleeping time (time elapsed between loss and recuperation of righting reflex) was recorded. Criterion for recuperation of righting reflex is that animals have to regain their normal posture for three consecutive times when challenged to remain on their backs [Costa-Campos et al., 1998].

Rectal temperature: This was measured by using a flexible probe before and 30 min after an i.p. injection of guanosine (30, 60 or 120 $mg.kg^{-1}$) or vehicle.

General toxicity and lethal-dose: To investigate the potential toxicity of guanosine, mice received a single i.p. administration of guanosine (7.5 to 960 $mg.kg^{-1}$) or vehicle. After the compound administration, animals were observed up to 72 h to determine the lethal potential of guanosine. The body weight gain of animals was also recorded every 24 hours as a sign of general toxicity. After 72 h of exposure, mice were slightly anesthetized for blood collection by heart puncture. Serum was obtained by centrifugation at 5,000 g for 10 min (hemolyzed serum was discarded) and used for biochemical assays. The following biochemical parameters were measured using commercial kits: serum enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used as the biochemical markers for the early acute hepatic damage; renal function was analyzed by determining serum urea and creatinine levels.

Cerebrospinal fluid (CSF) sampling: Another group of mice was similarly treated with i.p. or p.o. administration of vehicle or guanosine (60 $mg.kg^{-1}$). After 30 or 45 min for i.p. or p.o. treatments respectively, mice were anesthetized with sodium thiopental (60 $mg.kg^{-1}$, 10 $ml.kg^{-1}$, i.p.) and placed in a stereotaxic apparatus, where the CSF was drawn (10 - 20 µl per mouse) by direct puncture of the *cisterna magna* with an insulin syringe (27 gauge x 1/2 in length), with the help of a magnifying glass. All samples were centrifuged at 10,000 g in an Eppendorf centrifuge during 5 min to obtain cell-free supernatants and stored in separate tubes in -70^{o}C.

HPLC procedure: High-performance liquid chromatography (HPLC) was performed with aliquots obtained from the CSF cell-free supernatants in order to measure the concentration of purines. The measurement was done according to Domanski et al. [2006]. It was measured the CSF concentrations of the following purines: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine, inosine monophosphate (IMP), inosine, hypoxanthine, xanthine, and uric acid. Analyses were performed with Shimadzu Class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto injector valve with 50 µl loop, and an UV detector. Separations were achieved on a Supelco C18 250 mm x 4.6 mm, 5 µm particle size column. The mobile phase flowed at a rate of 1.2 ml/min and the column temperature was 24 ^{o}C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of

buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 10 µl were injected into the injection valve loop. Absorbance was read at 254 nm. CSF concentrations of purines are expressed as mean ± SEM in µM.

Glutamate Uptake: Mice were treated with an i.p. administration of guanosine (60 $mg.kg^{-1}$) or vehicle (0.1 mN NaOH); after 30 min, animals received an i.pl. injection of capsaicin or vehicle (DMSO 5%). Five min thereafter, animals were decapitated, their brains and spinal cords were removed immediately and submerged in a ice-cold Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, adjusted to pH 7.2. Cortices and spinal cords were dissected into a Petri dish filled with ice-cold HBSS. Coronal cortical slices and transversal spinal cord slices (0.4 mm) were obtained using a McIlwain tissue chopper and sections were separated with the help of a magnifying glass. Slices were then transferred to 24-multiwell dishes, containing 500 µL of HBSS solution and pre-incubated for 15 min (cortex) or 120 min (spinal cord) at 35°C. Subsequently, slices were washed with 1 ml HBSS, and the total glutamate uptake was assessed by addition of 0.33 µCi/ml L-(^{3}H)glutamate with 100 µM unlabeled glutamate in HBSS solution at 35°C. Incubation was stopped after 7 min by two ice-cold washes with 1 ml HBSS immediately followed by the addition of 0.5 N NaOH, which was kept overnight. Lysates were taken for determination of intracellular content of L-(^{3}H)glutamate through scintillation counting. To determine the sodium-independent glutamate uptake, parallel assays were done under ice using *N*-methyl-D-glucamine instead of sodium chloride in the incubation medium. Sodium-dependent

glutamate uptake was obtained by subtracting the sodium-independent uptake from the total in order to obtain the specific uptake. Protein was measured using the method of Peterson et al. [1977] using bovine albumin as standard. The experiments were done in triplicate.

Statistical analysis: Data are expressed as mean ± standard error of the mean (SEM), except the ID_{50} values (i.e., the dose of guanosine necessary to reduce the nociceptive response by 50% relative to the control value) and LD_{50} (i.e., the dose of guanosine necessary to cause mortality in 50% of mice), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} and LD_{50} values were determined by linear regression from individual experiments using linear regression GraphPad software (GraphPad software, San Diego, CA, USA). Data were submitted to Kolmogorov-Smirnov test for normality evaluation. Statistical analysis among groups was performed using one-way analysis of variance (ANOVA) plus the *post-hoc* Student-Newman-Keuls test when necessary. All results with $P < 0.05$ were considered statistically significant.

Results:

Effects of guanosine in pain models: The results depicted in Figs. 1 – 3 show that a single systemic (i.p. or p.o.) administration of guanosine produces antinociception against i.pl. capsaicin- (Fig. 1), hot-plate- (2B and 3B), i.pl. glutamate- (2C and 3C), i.p. acetic acid- (2D and 3D) and i.pl. formalin (2E/F and 3E/F)-induced pain. However, guanosine did not affect nociception in the tail-flick test (Fig. 1A and 2A). Neither i.p. nor p.o. administration of 0.1 mN NaOH (vehicle) affected nociception as compared to

control (sham) animals (data not shown). Mean ID_{50} values (95% confidence limits) for i.p. guanosine against capsaicin, glutamate, acetic acid and formalin (1st and 2nd phases) tests were 84 (31 – 230), 107 (83 – 137), 48 (13 – 173), 76 (22 – 262) and 66 (33 – 133) $mg.kg^{-1}$, respectively; maximal inhibitions of 58±14%, 48±15%, 56±10%, 40±8% and 57±17%, respectively. Mean ID_{50} values (95% confidence limits) for p.o. guanosine against capsaicin, glutamate, acetic acid and formalin (1st and 2nd phases) tests were 88 (29 – 267), 85 (64 – 112), 42 (21 – 83), 119 (103 – 138) and 83 (49 – 139) $mg.kg^{-1}$, respectively; maximal inhibitions of 52±12%, 58±13%, 72±6%, 29±11% and 43±20%, respectively.

Effects of guanosine against spinal algogen-induced nociception-related behavior: Fig. 4 shows that i.p. administration of guanosine significantly inhibited the nociceptive response induced by i.t. injection of glutamate (4A), AMPA (4C), kainate (4D), and trans-ACPD (4E), when compared to the control group (0.1 mN NaOH). The calculated mean ID_{50} values for the antinociceptive effect of guanosine against glutamate, AMPA, kainate, and trans-ACPD were 65 (53 – 81), 152 (122 – 188), 122 (106 – 140), and 126 (90 – 177) $mg.kg^{-1}$ and maximal inhibitions of 81±8, 47±15, 36±20, and 45±4%, respectively. In contrast, systemic administration of guanosine produced no significant effect against NMDA, substance P and capsaicin mediated biting response in mice.

Analysis of the mechanisms involved in guanosine action on the glutamate test: The results presented in Fig. 5A show that the pretreatment of mice with the nitric oxide precursor L-arginine (600 $mg.kg^{-1}$, i.p.), given 20 min earlier, completely reversed the antinociception caused by L-NOARG (75 $mg.kg^{-1}$, i.p.), but not by guanosine (60 $mg.kg^{-1}$, i.p.), when analyzed against i.pl. glutamate-induced licking. D-arginine did not affect

antinociception produced by either L-NOARG or guanosine (data not shown). Fig. 5B shows that the injection of a non-specific inhibitor of NO/guanylyl cyclase, methylene blue (1 $mg.kg^{-1}$, i.p.), did not inhibit guanosine-induced antinociceptive effect when analyzed against glutamate test. Methylene blue by itself did not modify glutamate-induced nociceptive behavior.

Effects of guanosine on glutamate-induced paw oedema: The results presented in Fig. 6 show that i.p. administration of guanosine (60 and 120 $mg.kg^{-1}$) caused a significant inhibition of the paw oedema formation induced by i.pl. injection of glutamate (Fig. 6A). Mean ID_{50} value (95% confidence limits) for i.p. guanosine against glutamate-induced paw oedema was 73 (49 – 107) $mg.kg^{-1}$ and maximal inhibition of 50±10%. However, when co-injected intraplantarly in association with glutamate, guanosine (100, 200 or 400 nmol) did not affect licking behavior (data not shown) or paw oedema formation (Fig. 6B) induced by injection of glutamate.

Effects of guanosine on purine CSF levels: As evidenced in the Fig. 7, systemic administration of guanosine (30, 60 or 120 $mg.kg^{-1}$) produced a significant increase in guanosine CSF levels. Intraperitoneal or p.o. administration of guanosine produced up to a 6.8 and 7.8-fold increase in guanosine CSF levels, respectively. However, guanosine did not affect inosine, xanthine, hypoxanthine, uric acid, adenosine, ATP, ADP, AMP, GTP, GDP, GMP and IMP CSF levels (data not shown).

Effects of guanosine on cortical and spinal cord glutamate uptake: Fig. 8 shows the effects of i.p. guanosine or vehicle followed by i.pl. capsaicin or vehicle on glutamate uptake by mice cortical (8A) and spinal cord (8B) slices. Capsaicin produced a significant increase in spinal cord glutamate uptake, an effect partially prevented by pretreatment

with guanosine. No significant effects on cortical glutamate uptake were observed. Importantly, systemic administration of guanosine did not affect basal cortical or spinal cord glutamate uptake.

Lethal-dose determination and general toxicity related to guanosine: Calculated LD_{50} for guanosine administered by i.p. route was estimated to be > 960 $mg.kg^{-1}$ with an observation period of 72 h. No mortality was observed after the mice exposure to all doses tested. Additionally, no body weight reduction was observed following guanosine exposure as compared to control group (data not shown).

Guanosine (up to 960 $mg.kg^{-1}$) did not cause renal impairment (Fig 9A and B) after a single i.p. administration. However, doses of guanosine higher than 240 $mg.kg^{-1}$ produced a significant increase of serum AST levels (Fig. 9C). This effect was likewise observed on serum ALT levels at the higher dosage (960 $mg.kg^{-1}$ – Fig. 9D). Importantly, guanosine significantly reduced the pentobarbital-induced sleeping time as compared to control group (NaOH – 33.3 ± 6.0 min; guanosine 30 $mg.kg^{-1}$ – 8.9 ± 3.1 min; guanosine 60 $mg.kg^{-1}$ – 10.5 ± 4.4 min; and guanosine 120 $mg.kg^{-1}$ – 20.7 ± 5.9 min; $P = 0.029$), but no significant effects were observed on core temperature (NaOH – 35.2 ± 0.2 ^{o}C; guanosine 30 $mg.kg^{-1}$ – 35.8 ± 0.3 ^{o}C; guanosine 60 $mg.kg^{-1}$ – 35.9 ± 0.4 ^{o}C; and guanosine 120 $mg.kg^{-1}$ – 35.7 ± 0.4 ^{o}C; $P = 0.35$).

In the hole-board model, i.p. guanosine (30, 60 or 120 $mg.kg^{-1}$) did not affect latency to first head-dip, and the number of head-dips, crossings, rearings, groomings and defecations as compared to control (Table 1). Guanosine did not induce motor deficits or ataxia, as evaluated by the performance in the rotarod test and did not affect spontaneous locomotor activity as measured by activity cages as compared to control (Table 1).

Discussion and conclusions:

The results of the present study clearly demonstrate that guanosine caused significant inhibition of pain-related behavior against several pain models. Additionally, guanosine prevented biting behavior induced by i.t. administration of glutamate and non-NMDA agonists, but not against NMDA, substance P or capsaicin. We also demonstrated that these effects may involve spinal cord glutamate uptake and are not related to the nitric oxide-L-arginine-cGMP pathway.

ABPs have been considered important targets for the development of new drugs for treating pain, since the nucleoside adenosine and its analogs induce antinociceptive effects following both systemic and central administration [Sawynok and Liu, 2003]. Considering that guanosine and adenosine closely interact in modulating several functions of the CNS [Dobolyi et al., 2000], guanosine might well play a role on pain transmission. Recently, we demonstrated that i.c.v. GBPs produced consistent antinociceptive effects in several pain models [Schmidt et al., 2008]. We also demonstrated that GMP-induced antinociception was prevented by the 5'-nucleotidase inhibitor AOPCP, suggesting that its effects result from conversion to guanosine. In the present study, only guanosine was used to investigate the role of the systemic administration of GBPs on nociception.

Guanosine was effective against several pain models, including those based on thermal or chemical stimuli. Guanosine produced antinociception in all chemical models and in the hot-plate test, but not in the tail-flick test. Although these animal models are essentially based on short-lasting noxious stimuli, some differences between tests can be found. Tail-flick and hot-plate tests are similar but the former refers predominantly to a

spinal reflex with modest control by supraspinal structures, while hot-plate is a more complex pain model, producing two behavioral components (i.e., paw licking and jumping) considered to be supraspinally integrated responses [Le Bars et al., 2001]. These differences may largely contribute to the lack of efficacy of guanosine in the tail-flick test. Intraplantar injection of algogenic chemical agents (capsaicin, glutamate or formalin) usually produces similar nociceptive responses and represents a longer duration stimulus. Intraperitoneal injection of acetic acid causes a visceral pain and is a very sensitive method to test new molecules whose pharmacodynamic properties are unknown such as guanosine [Le Bars et al., 2001]. Therefore, we may argue that guanosine is a potential new analgesic substance and, considering its effects on the second phase of formalin test, guanosine may produce anti-inflammatory effects as well.

Guanosine may exert antinociceptive effects through one or more mechanisms. These may include reduction of glutamate activity, releasing of trophic factors, modulation of the adenosinergic system and/or peripheral effects which could influence nociception. Since previous studies demonstrated that guanosine directly administered to the CNS is antinociceptive against several pain models, a peripheral mechanism is unlikely. We may speculate that guanosine could influence pain transmission by releasing trophic factors. Purines, including guanosine, stimulate the synthesis and release of several trophic factors by astrocytes [Rathbone et al., 1999]. Although endogenous release of neurotrophic factors may be involved in the induction changes in spinal transmission in various pain states [Jongen et al., 2005], it has been demonstrated that intrathecal administration of neurotrophic factors may be efficient in the treatment of

neuropathic pain [Pezet et al., 2008]. However, an *in vivo* correlation between guanosine, neurotrophic factors and pain transmission remains to be investigated in future research.

Previous studies have suggested involvement of the adenosinergic system in effects of guanosine, since guanosine stimulates the release of adenosine in cultured astrocytes, and both are released under excitotoxic conditions [Ciccarelli et al., 1999]. In contrast, some studies indicate that guanosine-induced enhancement of neurite outgrowth in PC12 cells was not affected by adenosine receptor antagonists [Gysbers and Rathbone, 1996], nor were the effect of guanosine on glutamate uptake [Frizzo et al., 2001], seizures induced by glutamatergic agents [Lara et al., 2001], learning and memory [Roesler et al., 2000; Vinadé et al., 2004], and guanosine-induced antinociception [Schmidt et al., 2008]. Moreover, i.c.v. administration of guanosine [Schmidt et al., 2008] did not increase CSF ABPs levels. In the present study, a single systemic administration of guanosine also failed to increase CSF ABPs levels. Therefore, this study reinforces that guanosine could act independently from adenosinergic system in inhibiting nociception.

Previous studies demonstrated that administration of guanosine produced a significant increase in CSF levels of oxypurines [Schmidt et al., 2008]. The significant production of oxypurines can not be excluded to play a role in the antinociceptive effects of guanosine. However, this study failed to demonstrate an increase in CSF oxypurines levels following a single i.p. or p.o. guanosine administration. Additionally, this study was not accurate to measure CSF guanine levels, but a previous study has demonstrated that after an i.p. administration of guanosine, the amount of both guanosine and guanine at the spinal cord increased, reaching a maximum effect by 30 min [Jiang et al., 2008]. Since extracellular guanine also exerts several biological effects [Rathbone et al., 2008],

the antinociceptive effects of guanosine may likely be regulated by its conversion to guanine by a membrane located purine nucleoside phosphorylase and role for guanine in the antinociceptive effects of guanosine can not be excluded.

Glutamate and its receptors play a crucial role in pain transmission mechanisms and modulation of glutamate receptors may have therapeutic potential for several categories of pain [Millan, 1999]. *In vitro*, guanosine has been shown to prevent ischemic injury [Frizzo et al., 2002] and NMDA-induced excitotoxicity [Ciccarelli et al., 2001]. *In vivo*, guanosine prevents seizures and toxicity induced by drugs that overstimulate the glutamatergic system [Baron et al., 1989; Malcon et al., 1997; Schmidt et al., 2000], is amnesic and anxiolytic in rodents [Vinadé et al., 2003], and is neuroprotective against stroke and spinal cord injury [Jiang et al., 2003; 2007; 2008; Chang et al., 2008]. Although the overall effects of guanosine may be related to attenuating glutamatergic overstimulation, its precise mechanism of action remains unclear. In this study, guanosine produced a significant inhibition of the biting behavior induced by i.t. injection of glutamate or non-NMDA agonists (AMPA, kainate and trans-ACPD), but not against NMDA. Thus, we may suggest that antinociceptive effect caused by guanosine involves a possible interaction with glutamatergic system and its receptors and/or with their signal transduction mechanisms.

Recently, we demonstrated that an i.pl. administration of capsaicin caused a significant decrease in cortical glutamate uptake, an effect prevented by i.c.v. guanosine [Schmidt et al., 2008]. Since guanosine has been shown to stimulate glutamate uptake *in vitro* [Frizzo et al., 2002], it was speculated that the *in vivo* antinociceptive effect of i.c.v. guanosine against capsaicin could result from its effect on glutamate removal from the

synaptic cleft, leading to less activation of glutamatergic receptors. However, in the present study, we showed that an i.pl. capsaicin produced an increase in the spinal cord glutamate uptake, an effect prevented by guanosine as well. Surprisingly, no significant effects were observed on cortical glutamate uptake. Notably, neither i.c.v. nor i.t. guanosine altered basal glutamate uptake at both brain and spinal cord. It is not possible to establish whether the changes in the spinal cord glutamate uptake were responsible for nociceptive behavior or it caused the changes. However, considering our results and previous data [Schmidt et al., 2008], we may argue that these changes were probably produced by nociceptive stimuli and their modulation rather than an underlying mechanism of action.

There is data supporting the possible existence of specific receptor-like binding sites for guanosine on membrane preparations from rat brain [Traversa et al., 2002, 2003]. We may speculate that guanosine, through its specific binding site, may promote its extracellular effect by activating intracellular cAMP-dependent and independent pathways [Tomaselli et al., 2005]. Additionally, guanosine could act as an alternative source of energy for neural cells after further metabolism, as previously demonstrated in spinal cord cultures [Jurkowitz et al., 1998; Litsky et al., 1999]. However, intracellular mechanisms underlying guanosine effects remain to be investigated in future research.

In the present study we also attempted to further characterize some of the mechanisms through which guanosine exerts its antinociceptive action in the glutamate model of nociception. Systemic administration of guanosine prevented pain and produced antioedematogenic effects against i.pl. glutamate pain model. However, our findings revealed that local administration of guanosine failed to affect nociception and paw

oedema induced by glutamate. Additionally, pretreatment with the nitric oxide precursor L-arginine, the non-specific inhibitor of NO/guanylyl cyclase methylene blue, or the inhibitor of nitric oxide synthesis L-NOARG, did not prevent the antinociception caused by guanosine. Altogether, these results indicate that the nitric oxide-L-arginine-cGMP pathway is not involved in the systemic antinociceptive effects of guanosine.

Regarding side effects, our results showed that guanosine did not induce obvious behavioral disturbances, altered coordination or locomotion, consistent with previously [Vinadé et al., 2003]. The minor toxic potential of guanosine was also evidenced by the low index of mortality (no deaths were observed – $LD_{50} > 960$ mg.kg^{-1}) and no alteration in weight body gain or core temperature up to 72 h after guanosine administration. Additionally, no CNS depressant activity of guanosine was observed in the barbiturate-induced sleeping time test. Actually, guanosine produced a CNS excitant effect that remembers some adenosinergic antagonists [El Yacoubi et al., 2003]. Although guanosine did not provide evidence for renal impairment, some hepatic toxicity was observed in doses higher than 240 mg.kg^{-1} reaching a maximum at 960 mg.kg^{-1} when both serum AST and ALT increased. Although these effects were not observed at antinociceptive doses, future studies may focus on potential adverse effects of guanosine including those involved on liver metabolism.

In summary, this is the first study showing antinociceptive effects after systemic administration of guanosine. Because guanosine is an endogenous compound apparently well tolerated and orally active, it could eventually be developed as a drug useful for treating pain. It provides new evidence on the role of extracellular guanosine in the CNS and indicates that guanosine antinociceptive effects probably involve the glutamatergic

system. However, these results do not exclude the involvement of other neurochemical parameters in guanosine effects. We are continuing to investigate the antinociceptive effects of guanosine against chronic pain models and mechanisms underlying these effects.

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Table 1 - Effects of i.p. guanosine or vehicle on the mice hole-board, rotarod and spontaneous locomotor activity tests:

Treatment		Guanosine [mg.kg^{-1}, i.p.]		
	0.1 mN NaOH	30	60	120
Latency to head-dip (s)	6.7 (1.9)	5.6 (1.3)	7.1 (1.3)	6.3 (1.5)
Head-dips (n)	81.8 (6.1)	87.5 (5.9)	88.7 (4.6)	76.8 (5.5)
Squares crossed (n)	44.8 (5.4)	51.5 (7.9)	39.8 (6.7)	48.5 (6.6)
Rearings (n)	1.4 (0.8)	2.0 (0.9)	1.3 (0.6)	2.2 (0.8)
Groomings (n)	1.6 (0.5)	1.4 (0.5)	1.0 (0.5)	1.3 (0.3)
Fecal boli (n)	0.8 (0.4)	1.3 (0.5)	1.6 (0.6)	1.1 (0.5)
Latency to fall (s)	59.0 (0.8)	60 (0)	57.7 (2.4)	55.6 (3.7)
Crossings (n)	152 (17)	165 (25)	153 (11)	159 (24)

Legends:

Table 1: Vehicle (0.1 mN NaOH) or guanosine (30, 60 or 120 $mg.kg^{-1}$) were i.p. administered 30 min prior to the behavior measurements: latency to the first head-dip; head-dips; squares crossed; rearings; groomings; fecal boli; latency to fall (rotarod); number of crossings (spontaneous locomotor activity). Data are mean ± SEM. N = 7 – 8 animals per group; one-way ANOVA.

Figure 1: Effects of i.p. (A) or p.o. (B) administration of vehicle (0.1 mN NaOH), morphine (Mor – 6 $mg.kg^{-1}$) or guanosine (7.5 to 240 $mg.kg^{-1}$) against i.pl. capsaicin test in mice. Columns represent mean time spent licking the injected hindpaw and vertical bars represent SEM. N = 8 – 10 animals per group. * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$ compared to vehicle, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 2: Effects of i.p. administration of vehicle (0.1 mN NaOH), morphine (Mor – 6 $mg.kg^{-1}$) or guanosine (30, 60 or 120 $mg.kg^{-1}$) against tail-flick (A), hot-plate (B), i.pl. glutamate (C), i.p. acetic acid (D), formalin tests (E-neurogenic phase and F-inflammatory phase) in mice. Dexamethasone (Dexa – 30 $mg.kg^{-1}$) was also administered in the formalin test. (A and B) Columns represent mean percent of maximum possible effect (%MPE) and vertical bars represent SEM. (C – F) Columns represent mean time spent licking the injected hindpaw and vertical bars represent SEM. N = 8 – 10 animals per group. * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$ compared to vehicle, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 3: Effects of p.o. administration of vehicle (0.1 mN NaOH), morphine (Mor – 6 $mg.kg^{-1}$) or guanosine (30, 60 or 120 $mg.kg^{-1}$) against tail-flick (A), hot-plate (B), i.pl. glutamate (C), i.p. acetic acid (D), formalin tests (E-neurogenic phase and F-inflammatory phase) in mice. Dexamethasone (Dexa – 30 $mg.kg^{-1}$) was also administered in the formalin test. (A and B) Columns represent mean percent of maximum possible effect (%MPE) and vertical bars represent SEM. (C – F) Columns represent mean time spent licking the injected hindpaw and vertical bars represent SEM. N = 8 – 10 animals per group. * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$ compared to vehicle, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 4: Effects of i.p. vehicle (0.1 mN NaOH) or guanosine (30, 60 or 120 $mg.kg^{-1}$) against glutamate (175 nmol/site, i.t., A)-, NMDA (450 pmol/site, i.t., B)-, AMPA (135 pmol/site, i.t., C)-, kainate (110 pmol/site, i.t., D)-, trans-ACPD (50 nmol/site, i.t., E)-, substance P (135 ng/site, i.t., F)-, or capsaicin (30 ng/site, i.t., G)-induced biting in mice. Columns represent mean and vertical bars represent SEM. N = 8 – 10 animals per group.* = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$ compared to vehicle, one-way ANOVA followed by Newman–Keuls test.

Figure 5: Effect of pretreatment of animals with L-arginine (600 $mg.kg^{-1}$, i.p.) 20 min before injection of L-NOARG (75 $mg.kg^{-1}$, i.p.), vehicle (0.1 mN NaOH) or guanosine (60 $mg.kg^{-1}$, i.p.) against the i.pl. glutamate-induced nociception (panel A). Effect of pretreatment of animals with L-NOARG (30 $mg.kg^{-1}$, i.p.), methylene blue (1 $mg.kg^{-1}$,

i.p.), or vehicle (saline, i.p.) 15 min before injection of guanosine (60 $mg.kg^{-1}$, i.p.) or vehicle (0.1 mN NaOH, i.p.) (panel B). The total time spent licking the hindpaw was measured for 15 min after i.pl. injection of glutamate. Columns represent mean and vertical bars represent SEM. N = 8 – 10 animals per group.* = $P < 0.05$ and ** = $P < 0.01$ compared to vehicle, one-way ANOVA followed by Newman–Keuls test.

Figure 6: Effects of i.p. administration of vehicle (0.1 mN NaOH) or guanosine (30, 60 or 120 $mg.kg^{-1}$) against i.pl. glutamate-induced paw oedema in mice (panel A). Effects of i.pl. administration of vehicle (0.1 mN NaOH) or guanosine (100, 200, 400 nmol) against i.pl. glutamate-induced paw oedema in mice (panel B). Columns represent mean weight difference (injected - non injected paw) and vertical bars represent SEM. N = 8 animals per group. ** = $P < 0.01$ compared to vehicle, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 7: Effects of i.p. (panel A) or p.o. (panel B) administration of vehicle (0.1 mN NaOH) or guanosine (30, 60 or 120 $mg.kg^{-1}$) on CSF concentration of purines. The columns represent mean (μM) and vertical bars represent SEM. N = 8 animals per group. ** = $P < 0.01$ and *** = $P < 0.001$ compared to vehicle, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 8: Effects of i.p. guanosine and i.pl. capsaicin on glutamate uptake by cortical (panel A) and spinal cord (panel B) slices from mice. Mice were treated with an i.p. injection of vehicle (0.1 mN NaOH) or guanosine (60 $mg.kg^{-1}$); after 30 min, animals

received an i.pl. injection of vehicle (DMSO 5%) or capsaicin. After behavioral evaluation, the mice were sacrificed and the cortical and spinal cord slices processed for glutamate uptake assay. Data are mean ± SEM. N = 12 animals per group. * = $P < 0.05$ compared to vehicle, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 9: Effects of i.p. vehicle (0.1 mN NaOH) or guanosine (60 to 960 $mg.kg^{-1}$) on serum levels of creatinine (panel A), urea (panel B), aspartate aminotransferase (AST – panel C), and alanine aminotransferase (ALT – panel D) in mice. Mice received an i.p. injection of vehicle or guanosine 72 h before blood sampling. Data are mean ± SEM. N = 8 animals per group. ** = $P < 0.01$ and *** = $P < 0.001$ compared to vehicle, one-way ANOVA followed by Student-Newman-Keuls test.

Statement of conflicts of interest:

None.

Figure 1

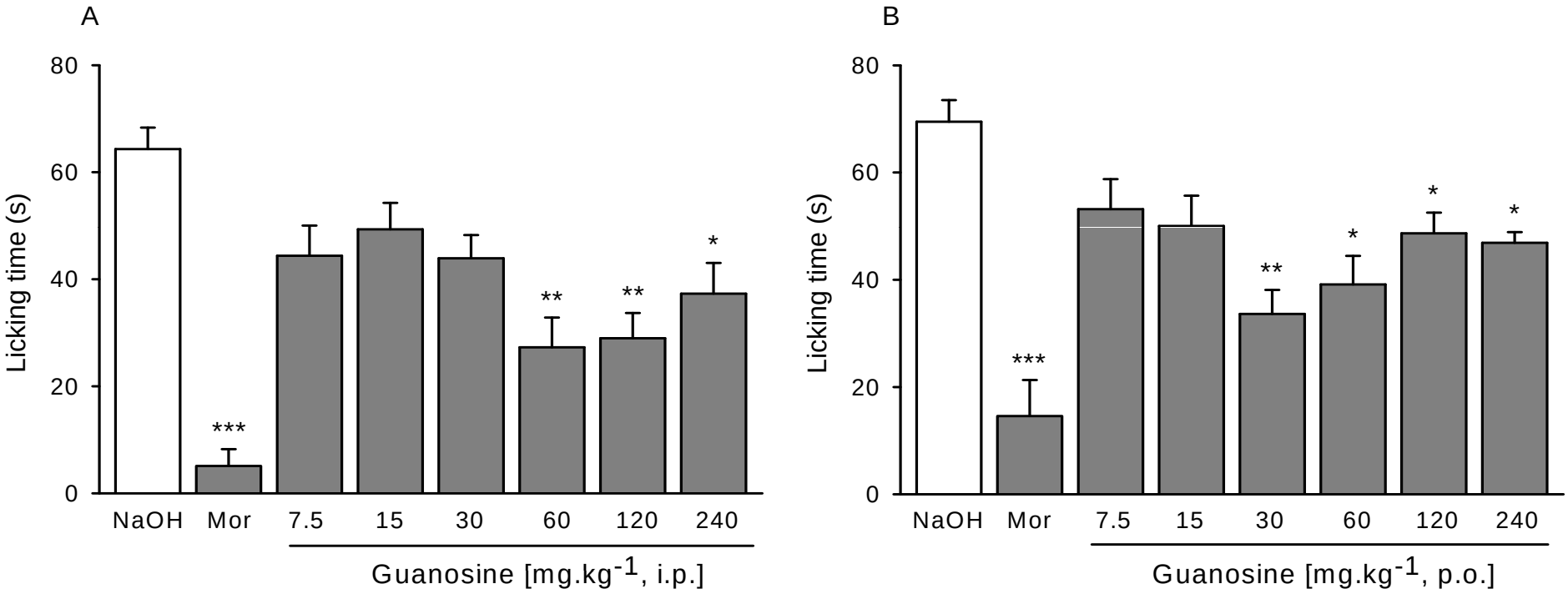

A
B
Licking time (s)
80
60
40
20
0
***
**
**
*
NaOH
Mor
7.5
15
30
60
120
240
Guanosine [mg.kg-1, i.p.]
Guanosine [mg.kg-1, p.o.]

Figure 2

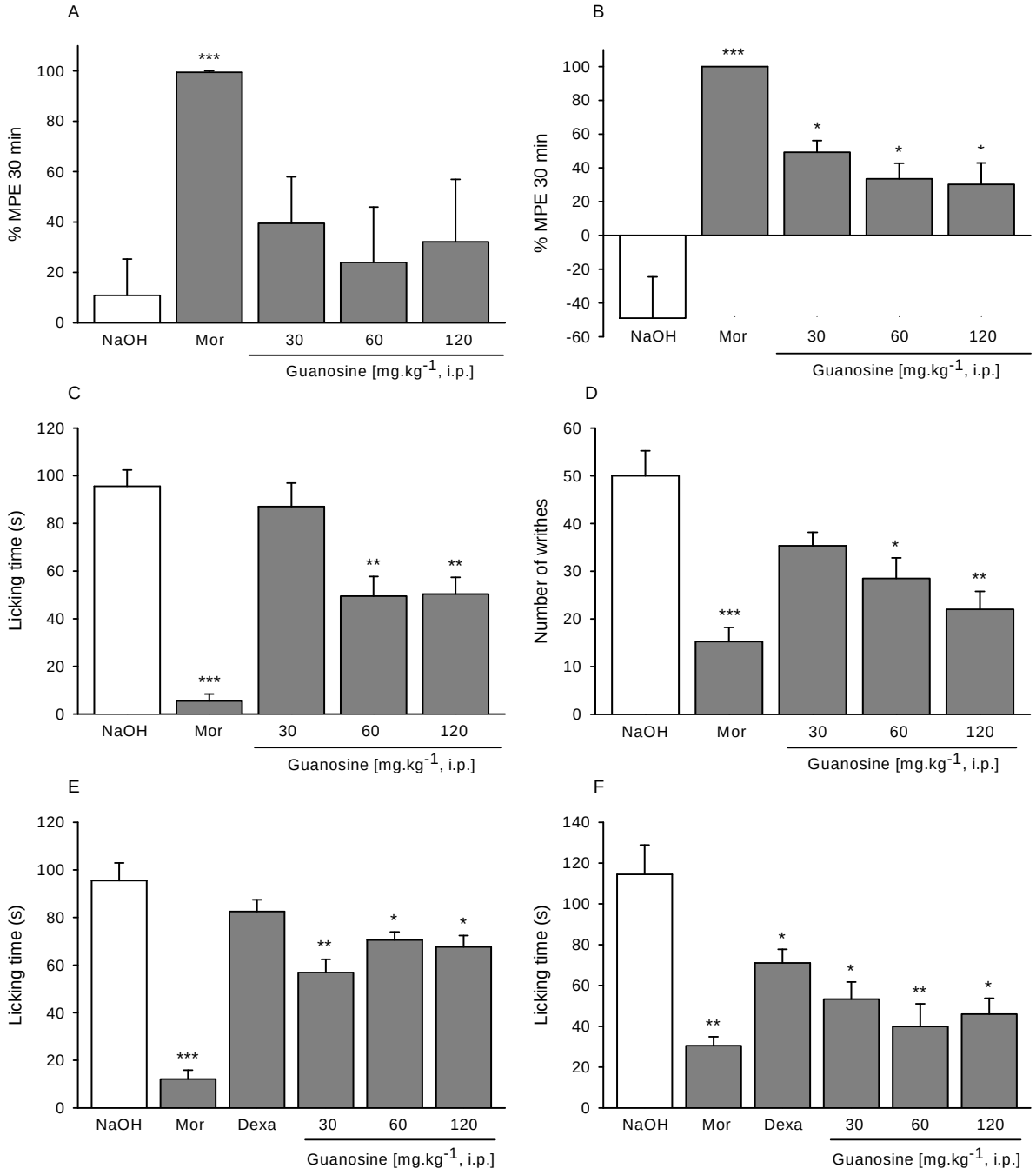

A
% MPE 30 min
NaOH
Mor
30
60
120
Guanosine [mg.kg-1, i.p.]
B
% MPE 30 min
NaOH
Mor
30
60
120
Guanosine [mg.kg-1, i.p.]
C
Licking time (s)
NaOH
Mor
30
60
120
Guanosine [mg.kg-1, i.p.]
D
Number of writhes
NaOH
Mor
30
60
120
Guanosine [mg.kg-1, i.p.]
E
Licking time (s)
NaOH
Mor
Dexa
30
60
120
Guanosine [mg.kg-1, i.p.]
F
Licking time (s)
NaOH
Mor
Dexa
30
60
120
Guanosine [mg.kg-1, i.p.]

Figure 3

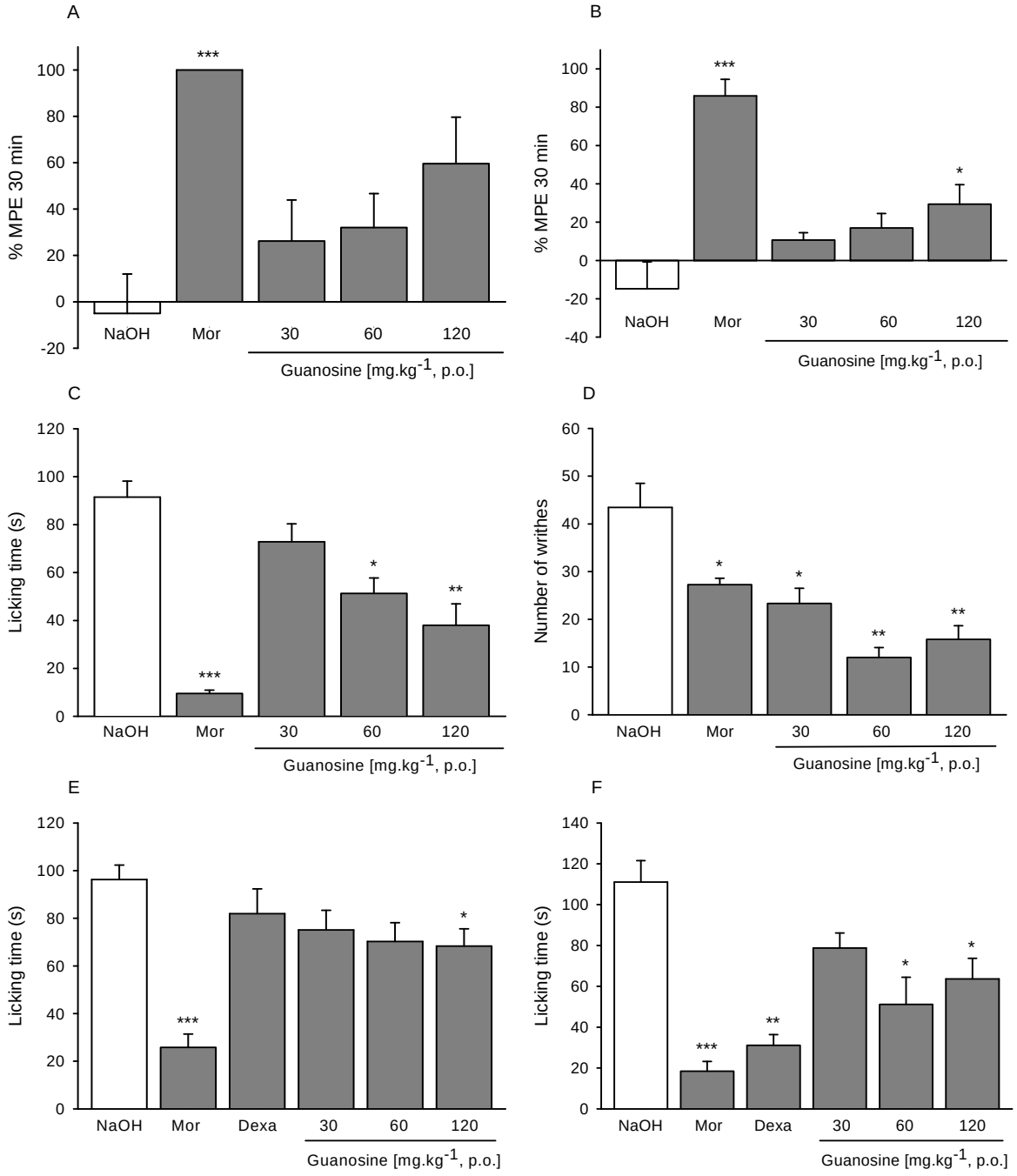

A
% MPE 30 min
100
80
60
40
20
0
-20
***
NaOH
Mor
30
60
120
Guanosine [mg.kg-1, p.o.]
B
% MPE 30 min
100
80
60
40
20
0
-20
-40
***
*
NaOH
Mor
30
60
120
Guanosine [mg.kg-1, p.o.]
C
Licking time (s)
120
100
80
60
40
20
0
***
*
**
NaOH
Mor
30
60
120
Guanosine [mg.kg-1, p.o.]
D
Number of writhes
60
50
40
30
20
10
0
*
*
**
**
NaOH
Mor
30
60
120
Guanosine [mg.kg-1, p.o.]
E
Licking time (s)
120
100
80
60
40
20
0
***
*
NaOH
Mor
Dexa
30
60
120
Guanosine [mg.kg-1, p.o.]
F
Licking time (s)
140
120
100
80
60
40
20
0
***
**
*
*
NaOH
Mor
Dexa
30
60
120
Guanosine [mg.kg-1, p.o.]

Figure 4

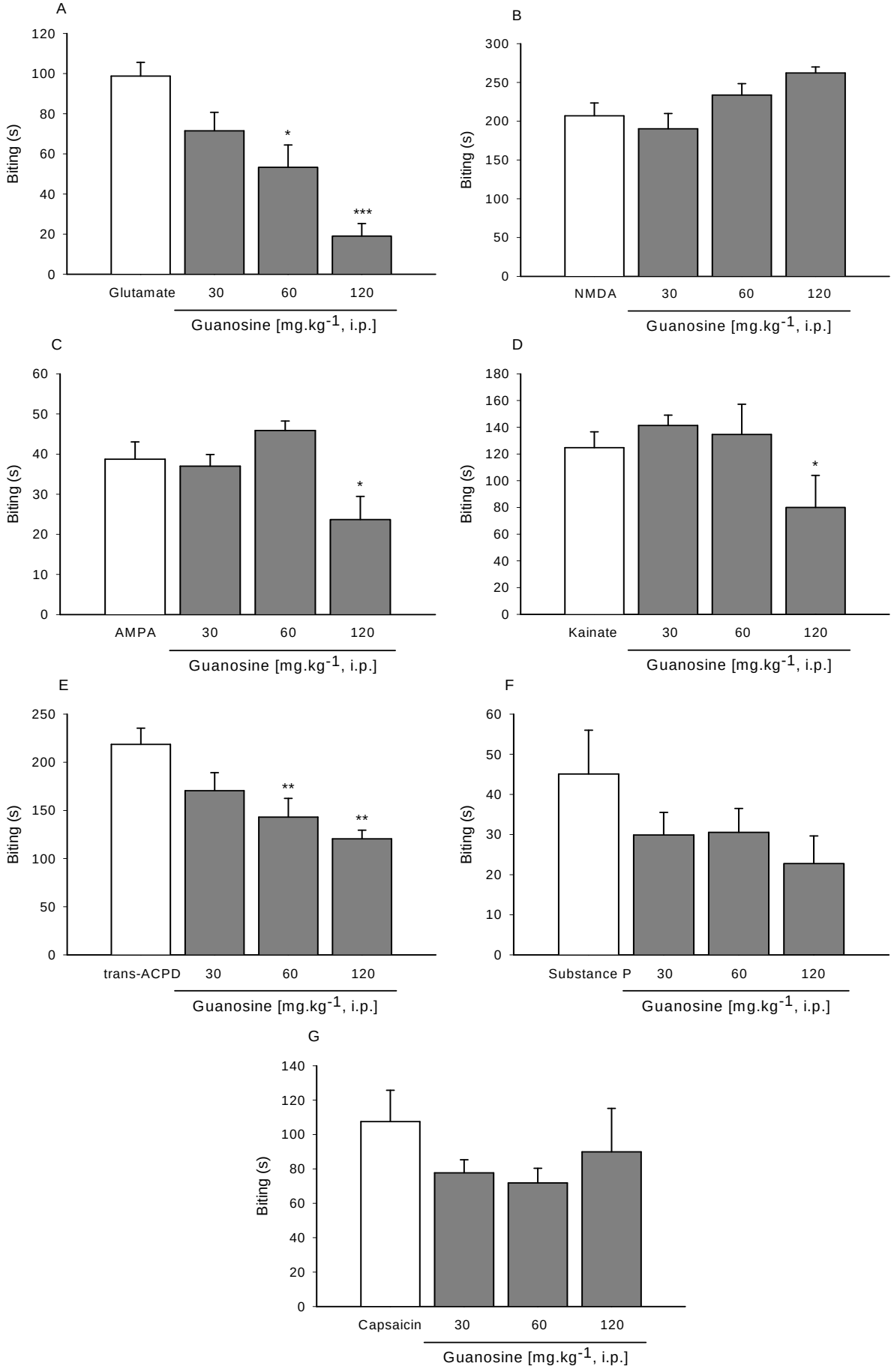

A
Biting (s)
Glutamate
30
60
120
Guanosine [mg.kg-1, i.p.]
B
NMDA
C
AMPA
D
Kainate
E
trans-ACPD
F
Substance P
G
Capsaicin

Figure 5

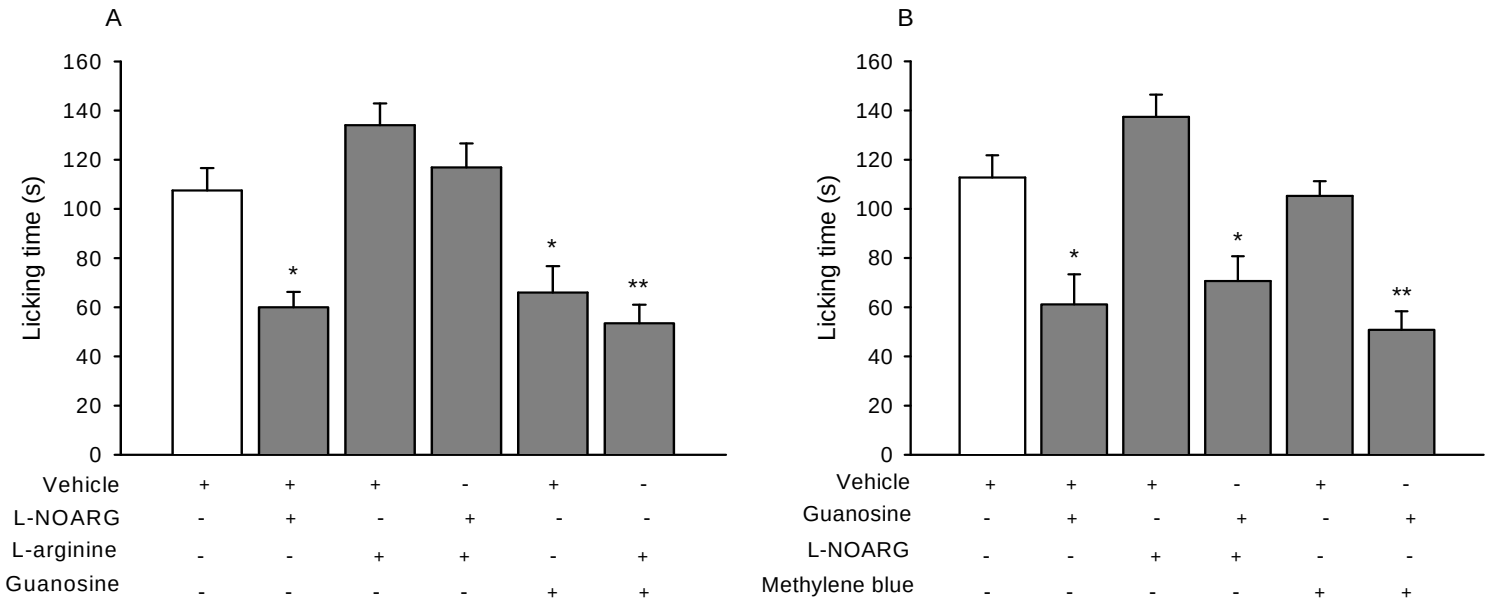

A
B
Licking time (s)
160
140
120
100
80
60
40
20
0
*
**
Vehicle
L-NOARG
L-arginine
Guanosine
Methylene blue
+
-

Figure 6

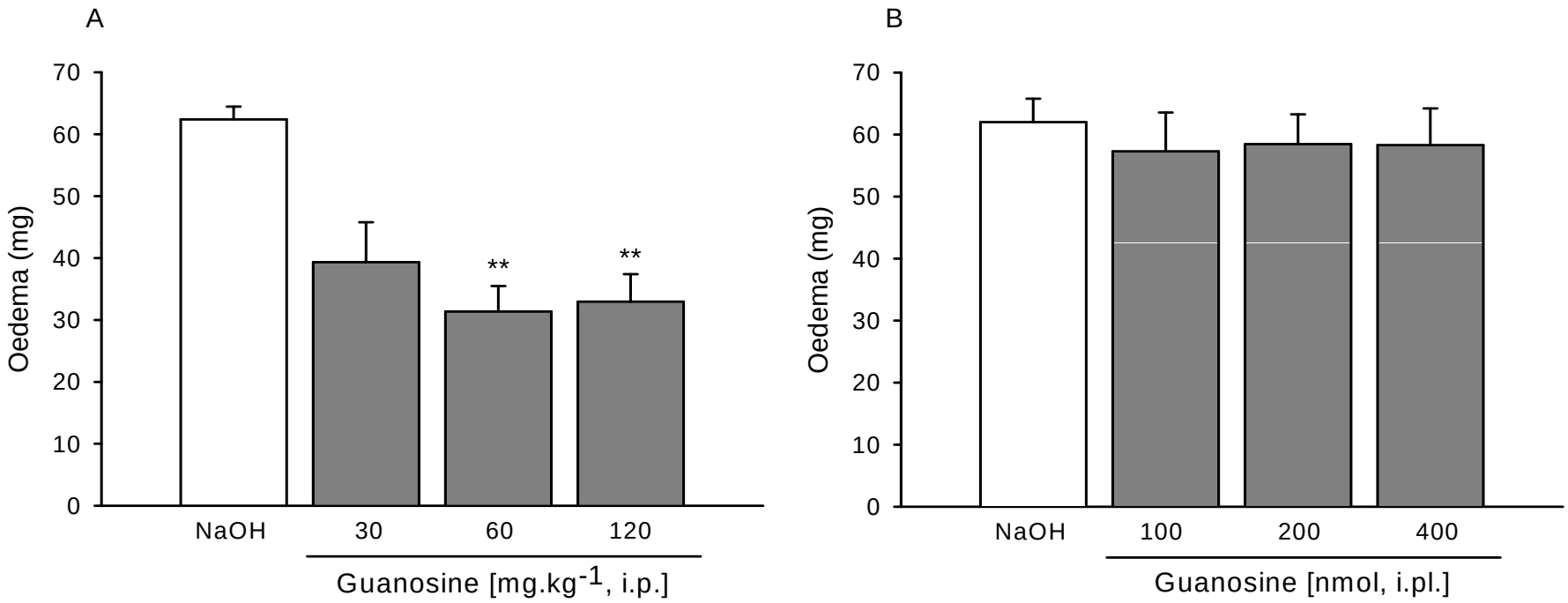

A
B
Oedema (mg)
70
60
50
40
30
20
10
0
**
**
NaOH
30
60
120
Guanosine [mg.kg-1, i.p.]
NaOH
100
200
400
Guanosine [nmol, i.pl.]

Figure 7

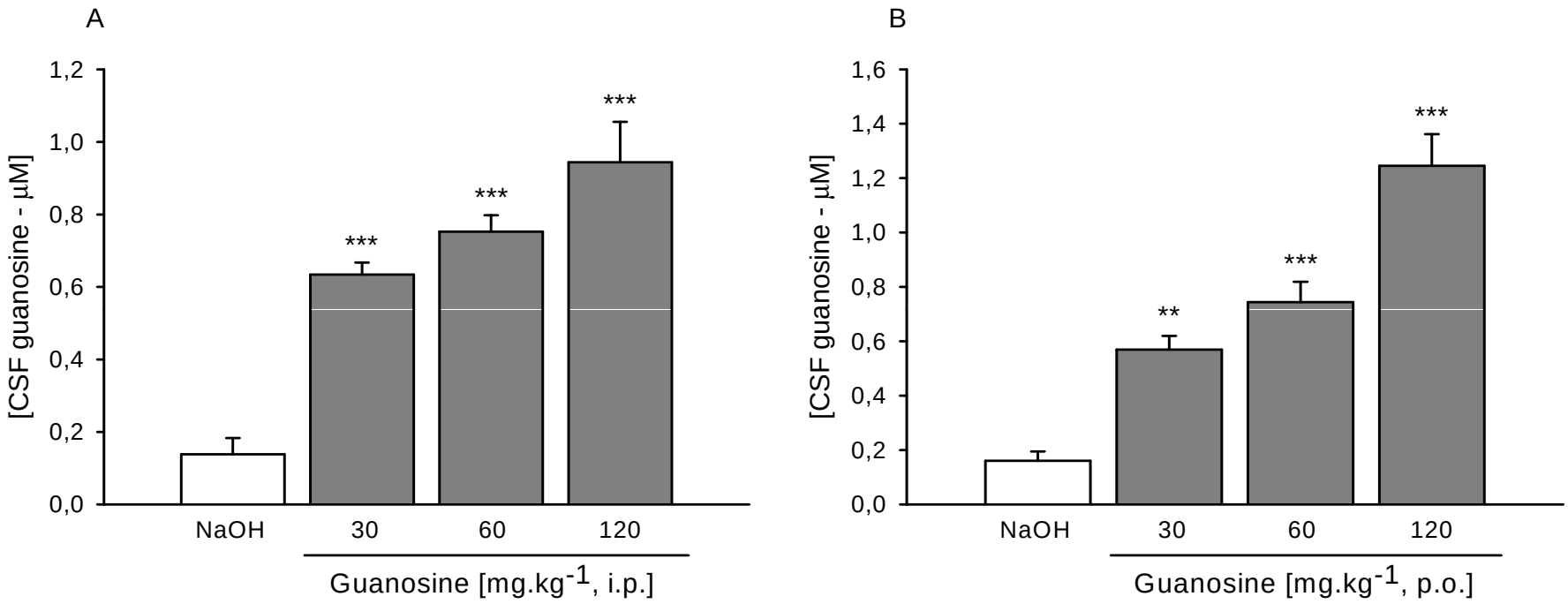

A
[CSF guanosine - µM]
1,2
1,0
0,8
0,6
0,4
0,2
0,0
***
***
***
NaOH
30
60
120
Guanosine [mg.kg-1, i.p.]
B
[CSF guanosine - µM]
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
**
***
***
NaOH
30
60
120
Guanosine [mg.kg-1, p.o.]

Figure 8

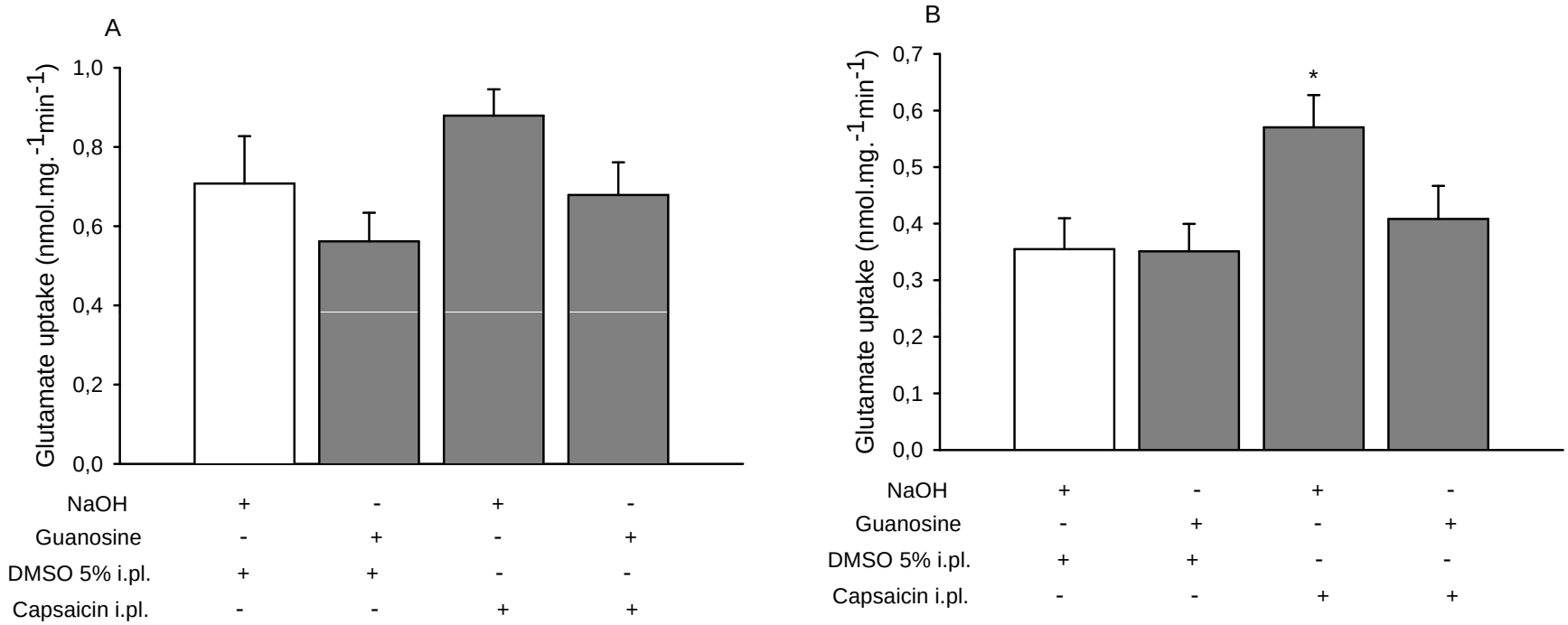
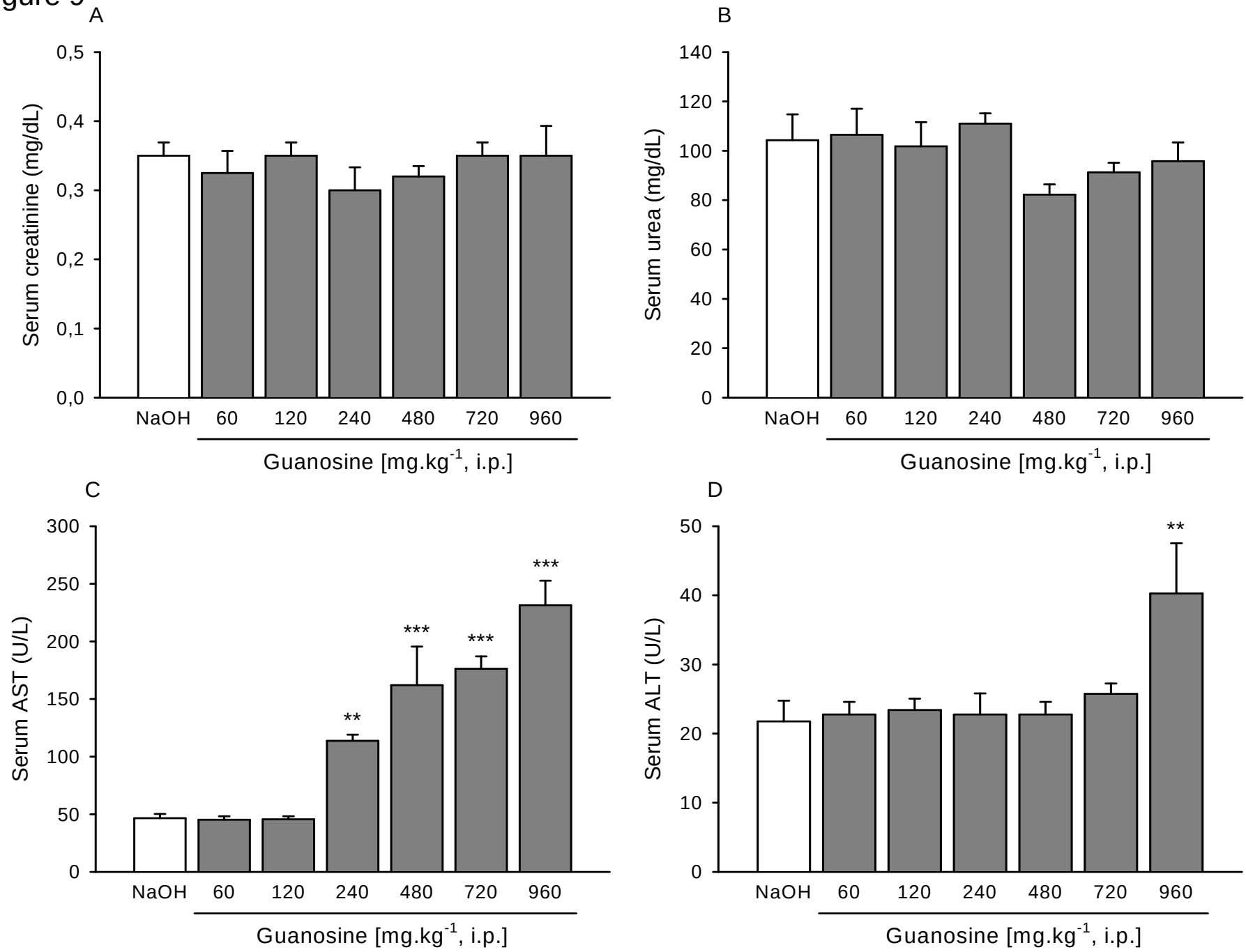

A
Glutamate uptake (nmol.mg.-1min-1)
1,0
0,8
0,6
0,4
0,2
0,0
NaOH + - + -
Guanosine - + - +
DMSO 5% i.pl. + + - -
Capsaicin i.pl. - - + +
B
Glutamate uptake (nmol.mg.-1min-1)
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
*
NaOH + - + -
Guanosine - + - +
DMSO 5% i.pl. + + - -
Capsaicin i.pl. - - + +

Figure 9


A
Serum creatinine (mg/dL)
0,5
0,4
0,3
0,2
0,1
0,0
NaOH
60
120
240
480
720
960
Guanosine [mg.kg-1, i.p.]
B
Serum urea (mg/dL)
140
120
100
80
60
40
20
0
NaOH
60
120
240
480
720
960
Guanosine [mg.kg-1, i.p.]
C
Serum AST (U/L)
300
250
200
150
100
50
0
**
***
***
***
NaOH
60
120
240
480
720
960
Guanosine [mg.kg-1, i.p.]
D
Serum ALT (U/L)
50
40
30
20
10
0
**
NaOH
60
120
240
480
720
960
Guanosine [mg.kg-1, i.p.]

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Groomings (n)	1.6 (0.5)	1.4 (0.5)	1.0 (0.5)	1.3 (0.3)
Fecal boli (n)	0.8 (0.4)	1.3 (0.5)	1.6 (0.6)	1.1 (0.5)
Latency to fall (s)	59.0 (0.8)	60 (0)	57.7 (2.4)	55.6 (3.7)
Crossings (n)	152 (17)	165 (25)	153 (11)	159 (24)

II.2.h. Guanosine prevents thermal hyperalgesia in a rat model of peripheral mononeuropathy: investigation of the mechanism of action

Artigo submetido ao periódico The Journal of Pain.

Guanosine prevents thermal hyperalgesia in a rat model of peripheral mononeuropathy: investigation of the mechanism of action.

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Running title: Guanosine prevents thermal hyperalgesia in rats.

Keywords: Guanosine; Neuropathic Pain; Glutamate; Antinociception; Purines; Plantar Test.

Abstract:

It is well known that adenine-based purines exert multiple effects on pain transmission. However, less attention has been given to the antinociceptive effects of guanine-based purines (GBPs). The aim of this study was to investigate the effects of intraperitoneal (i.p.) administration of guanosine on a rat model of peripheral mononeuropathy. Additionally, investigation of the mechanism of action of guanosine, its general toxicity and measurements of CSF purine levels were performed. Rats received an i.p. administration of vehicle (0.1 mN NaOH) or guanosine (up to 120 $mg.kg^{-1}$) in an acute or chronic regimen. Guanosine significantly decreased the paw withdrawal response on the ipsilateral side of the partial sciatic nerve ligation. Additionally, guanosine prevented locomotor deficits and weight body loss induced by the mononeuropathy. Acute systemic administration of guanosine caused an approximately 11-fold increase on CSF guanosine levels, but this effect was not observed after chronic treatment. Chronic guanosine prevented the increase on cortical glutamate uptake, but not the decrease in spinal cord glutamate uptake induced by the mononeuropathy. No significant general toxicity was observed after chronic exposure to guanosine. This study provides new evidence on the mechanism of action of GBPs, with guanosine presenting antinociceptive effects against a chronic pain model.

Perspective:

This study provides a new role for guanosine: chronic pain modulation. Guanosine presents as a new target for future drug development and might be useful for treat pain related to overstimulation of the glutamatergic system.

Introduction:

It is well known that extracellular adenosine 5-triphosphate (ATP) and adenosine have an important role in pain signaling at both the periphery and in the central nervous system (CNS)[24,43,44]. ATP has been reported to stimulate sensory nerve endings causing pain and a marked increase in the discharge from sensory neurons and is involved in the initiation of different types of nociception and pain[6]. Adenosine regulates pain transmission by actions at spinal, supraspinal and peripheral sites[43,45], and may play a important role in inflammatory and neuropathic pain[11]. Adenosine can alter pain transmission by actions on both nociceptive afferent and transmission neurons, and these actions are mediated primarily by adenosine A_1 receptors[44]. Additional actions on inflammatory cells at peripheral sites[16] and on glia in the CNS[20] mediated by adenosine A_{2A}, A_{2B} and A_3 receptors also occur, and these potentially can produce indirect effects on pain transmission. Endogenous adenosine can also be released from both the spinal cord and from peripheral tissues, and the regulation of such release by various pharmacological agents can alter pain processing through activation of adenosine A_1 receptors on neurons, and perhaps other receptors on adjacent structures[44].

Although the nucleotide ATP and the nucleoside adenosine are usually considered the main effectors of the purinergic system[6], extracellular guanine-based purines (GBPs) exert biological effects unrelated to the direct G-proteins modulation; these include modulation of glutamatergic activity[49,51], trophic effects on neural cells[8], and behavioral effects[30,46,48]. Concerning *in vitro* effects on the glutamatergic system, GBPs inhibit the binding of glutamate and analogs[1,5,40], prevent cell responses to excitatory amino acids[51], present neuroprotective effects in cultured neurons submitted to hypoxia, and increase

glutamate uptake in cultured astrocytes[17-19]. *In vivo*, GBPs prevent glutamate-induced seizures, are anxiolytic and amnesic in mice and rats[30,41,42,48,56-58]; these effects seem to be related to conversion to guanosine[46,50]. Recently, we have demonstrated that intracerebroventricular (i.c.v.) injection of guanosine or GMP is antinociceptive against several chemical and thermal pain models in mice[47]. Additionally, we have shown that spinal and systemic administration of guanosine produces significant inhibition of glutamate and non-NMDA agonists-induced biting behavior (personal communication). Importantly, most of these effects seem to be related, at least partially, to guanosine-induced modulation of the glutamatergic pathways[47,49].

This study was designed to further investigate acute and chronic antinociceptive effects of intraperitoneal (i.p.) administration of guanosine in a rat model of peripheral mononeuropathy. Attempts have been made to investigate some of the possible mechanisms that underlie the antinociceptive action of guanosine and general toxicity induced by its systemic administration.

Material and Methods:

Animals: Male adult Wistar rats (2-3 months of age, 250-350 g) were kept on a 12 hour light/dark cycle (light on at 7:00 am) at temperature of 22 ± 1ºC, housed in plastic cages (five per cage) with tap water and commercial food *ad libitum.* In all nociceptive behavioral experiments, the animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. The ethical guidelines for investigations of experimental pain in conscious animals[61] and our institutional protocols for experiments with animals, designed to avoid suffering and limit

the number of animals sacrificed, were followed throughout. This study was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Drugs: Guanosine and glutamate were purchased from Sigma Chemicals (St Louis, MO, USA). The anesthetic sodium thiopental was obtained from Cristália (SP, Brazil). Guanosine was dissolved in 0.1 mN NaOH. The amount of NaOH caused no detectable effect. All other solutions were dissolved in saline (NaCl 0.9%) and buffered with 0.1 N NaOH or 0.1 N HCl to pH 7.4 when necessary.

Drugs administration: Animals were submitted to three different protocols for guanosine treatment: i. protocol 1 – single i.p. administration of guanosine; ii. protocol 2 – 7-day i.p. guanosine treatment; iii. protocol 3 – 14-day i.p. guanosine treatment. In the protocol 1, animals were treated with an i.p. injection of guanosine (7.5 to 240 mg/kg) or vehicle (0.1 mN NaOH) and submitted to behavioral evaluation 15, 30, 45, 60, 120, 360 min and 24 h after treatments. In the chronic guanosine treatment, animals were randomly allocated to two different regimens (protocols 2 and 3) of chronic exposure to guanosine. In the protocol 2, animals were operated and maintained in the animal facility for 14 days thereafter. Subsequently, only animals that accomplished criteria for mononeuropathy were randomly assigned to tests. Animals received daily i.p. injections of vehicle (0.1 mN NaOH) or guanosine (60 $mg.kg^{-1}$) during 7 days. In protocol 3, animals were randomly assigned to 2 groups: vehicle (0.1 mN NaOH) or guanosine (60 $mg.kg^{-1}$). Animals received daily i.p. injections of vehicle or guanosine during 14 days following surgical procedure. The doses and time of drug administration were selected on

the basis of literature data and also based in previous results from our laboratory[30,48,56-58]. Separate groups of animals were also included in some behavioral and mechanistic studies: naïve (control), sham operated animals, and non operated animals submitted to vehicle or guanosine chronic exposure.

Surgical procedure: The unilateral peripheral mononeuropathy was induced according to the procedure of Bennett and Xie[3]. Briefly, rats were anaesthetized with sodium thiopental (40 $mg.kg^{-1}$, 1 $ml.kg^{-1}$, i.p.; supplemented as necessary) and the common sciatic nerve was exposed unilaterally at the middle of the right thigh by a dorsolateral incision. Under an operating microscope, the common sciatic nerve was exposed by blunt dissection through biceps femoris. Proximal to the sciatic's trifurcation, about 7 mm of nerve was gently dissected from surrounded connective tissue and 4 ligatures (4.0 chromic gut) were tied loosely around it with 1 mm spacing. Particular care was taken not to block blood flow in superficial epineural vessels. The incision was closed in layers using 3.0 silk suture. In every animal, an identical dissection was performed in the opposite site except that the sciatic nerve was not ligated. Some animals were not operated (naive) and others received bilateral sham procedures (sciatic exposure without ligation).

Plantar Test (thermal hyperalgesia): Thermal hyperalgesia was evaluated by the paw withdrawal test[23]. Operated animals were maintained in the animal facility for at least two weeks before the experiments with food and water ad libitum. On the day of the experiment animals were placed in transparent plastic chambers on an elevated glass floor of the testing apparatus (7370 Plantar Test, Ugo Basile, Italy) and allowed to acclimate to their surroundings for 20 min. Following acclimation, a radiant heat source

(50 W halogen reflector bulb with intensity controlled by a constant voltage source) was aimed at the plantar surface of one of the hindpaws through the glass floor. A photoelectric cell automatically turns the heat source off when the reflected light beam is interrupted (i.e. when the animal withdraws the paw) and records the paw withdrawal latency at the nearest 0.1 s. A limit time of 30 s was used in order to prevent tissue damage. Both paws were tested at random and a one minute interval between consecutive stimulations of the same hindpaw was employed. Testing was performed five times on each side, and the latencies to each side were averaged. Average values were used for statistical analysis. A score was computed by subtracting the average latency of the sham-operated side (control side) from the average latency of the ligated side (experimental side). Negative difference scores indicated a lower threshold on the ligated side. Only animals displaying negative scores < 2 standard deviations were considered neuropathic and included in the tests.

Exploration of an Open Field: 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 cm chamber made of brown polywood with frontal glass wall. The floor of the open field was divided by black lines into 12 equal squares. Animals were placed on the 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 ± SEM.

Cerebrospinal fluid (CSF) sampling: Another group of rats was similarly treated with i.p. administration of guanosine (30, 60 or 120 $mg.kg^{-1}$) or vehicle (0.1 mN NaOH). After 30, 60, 120, 360 min or 24 h, rats were anesthetized with sodium thiopental (40

mg.kg^{-1}, 1 ml.kg^{-1}, i.p.) and placed in a stereotaxic apparatus, where the CSF was drawn (40 - 80 μl per rat) by direct puncture of the *cisterna magna* with an insulin syringe (27 gauge x 1/2 in length)[37]. In order to investigate the effects of chronic guanosine exposure, a separate group of animals were similarly treated with guanosine (60 mg.kg^{-1}, i.p.) or vehicle (0.1 mN NaOH, i.p.) for 7- or 14-day and submitted to the CSF collection 24 h after the last i.p. injection. Individual samples with visible blood contamination were discarded. All samples were centrifuged at 10,000 g at 4^{o}C in an Eppendorf centrifuge during 5 min to obtain cell-free supernatants and stored in single tubes at -70^{o}C.

HPLC procedure: High-performance liquid chromatography (HPLC) was performed with aliquots obtained from the CSF cell-free supernatants in order to measure the concentration of purines. The measurement was done according to previously[15]. It was measured the CSF concentrations of the following purines: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine, inosine monophosphate (IMP), inosine, hypoxanthine, xanthine, and uric acid. Analyses were performed with Shimadzu Class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto injector valve with 50 μL loop, and an UV detector. Separations were achieved on a Supelco C18 250 mm x 4.6 mm, 5 μm particle size column. The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24 ^{o}C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase:

0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 10 μL were injected into the injection valve loop. Absorbance was read at 254 nm. CSF concentrations of purines are expressed as mean ± SEM in μM.

Glutamate Uptake: Animals were divided into six groups for glutamate uptake assay: naïve (animals not submitted to surgery), sham (operated animals without sciatic ligation), vehicle (sham animals that received daily doses of 0.1 mN NaOH, i.p.), guanosine (sham animals that received daily doses of guanosine 60 $mg.kg^{-1}$, i.p.), neuropathic-vehicle (animals displaying unilateral mononeuropathy treated with vehicle), and neuropathic-guanosine (animals displaying unilateral mononeuropathy treated with guanosine). Twenty four hours after last treatments, animals were decapitated, their brain and spinal cords were removed immediately and submerged in ice-cold Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, adjusted to pH 7.2. Cortices and spinal cord were dissected into a Petri dish filled with ice-cold HBSS to obtain the cortical parietal area and the spinal cord thoracic/lumbar segments, respectively. Coronal cortical slices and transversal spinal cord slices (0.4 mm) were obtained using a McIlwain tissue chopper and sections were separated with the help of a magnifying glass. Cortical and spinal cord slices were then transferred to 24-multiwell dishes, containing 500 μl of HBSS solution and pre-incubated for 15 and 120 minutes at 35°C, respectively. Subsequently, slices were washed with 1 ml HBSS, and the total glutamate uptake was assessed by addition of 0.33 μCi/ml L-[^{3}H]glutamate with 100 μM unlabeled glutamate in HBSS solution at 35°C.. Incubation was stopped after 7 min by

two ice-cold washes with 1 ml HBSS immediately followed by the addition of 0.5 N NaOH, which was kept overnight. Lysates were taken for determination of intracellular content of L-[^{3}H]glutamate through scintillation counting. To determine the sodium-independent glutamate uptake, parallel assays were done under ice using *N*-methyl-D-glucamine instead of sodium chloride in the incubation medium. Sodium-dependent glutamate uptake was obtained by subtracting the sodium-independent uptake from the total in order to obtain the specific uptake. Protein was measured using the method of Peterson[34] using bovine albumin as standard. The experiments were done in triplicate.

General toxicity: To investigate the potential toxicity of guanosine, the body weight of animals was recorded every 7 days as a sign of general toxicity. Twenty four hours after the last exposure to guanosine or vehicle, rats were anesthetized for blood collection by heart puncture. Serum was obtained by centrifugation at 5,000 g for 10 min (hemolyzed serum was discarded) and used for biochemical assays. The following biochemical parameters were measured using commercial kits: serum enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used as the biochemical markers for the early acute hepatic damage; renal function was analyzed by determining serum urea and creatinine levels.

Statistical analysis: Data are expressed as mean ± standard error of the mean (SEM), except the ID_{50} values (i.e., the dose of guanosine necessary to reduce the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} value was determined by linear regression from individual experiments using linear regression GraphPad software (GraphPad software, San Diego, CA, USA). Data were submitted to

Kolmogorov-Smirnov test for normality evaluation. Statistical analysis among groups was performed using one-way analysis of variance (ANOVA) plus the *post-hoc* Student-Newman-Keuls test when necessary. All results with $P < 0.05$ were considered statistically significant.

Results:

To evaluate the effect caused by guanosine on neuropathic pain model, we made a partial sciatic nerve ligation in rats. This injury produced a marked development of allodynia and hyperalgesia on the ipsilateral side 2 days after nerve injury procedure (data not shown) as compared to sham and naïve animals. The acute treatment with guanosine (protocol 1 – 30, 60 or 120 $mg.kg^{-1}$) significantly decreased the paw withdrawal response on the ipsilateral side of the partial sciatic nerve ligation 30 and 45 min after drug administration ($P < 0.05$); this effect tended to be kept for up to 120 min after guanosine treatment but not statistically significant (Fig. 1). Administration of 0.1 mN NaOH (vehicle) did not affect nociception as compared to control animals (data not shown). Mean ID_{50} value (95% confidence limit) for i.p. guanosine against thermal hyperalgesia 30 and 45 min after treatments were 18.0 (8.2 – 39.1) $mg.kg^{-1}$ and 17.8 (2.6 – 119.7) $mg.kg^{-1}$, respectively; maximal inhibition of 148±25% and 145±20%.

Fig. 2 shows the effects of a chronic exposure to guanosine or vehicle on the plantar test. Fig. 2A shows that animals submitted to partial sciatic nerve ligation developed hyperalgesia 14 days following surgery ($P < 0.01$). After treatments (protocol 2), guanosine partially reversed hyperalgesia as compared to vehicle (panel A). Fig. 2B shows that guanosine (60 $mg.kg^{-1}$) significantly decreased the paw withdrawal response

on the ipsilateral side of the partial sciatic nerve ligation 7 and 14 days after surgery (protocol 3, $P < 0.05$ as compared to vehicle);

As evidenced in the Fig. 3, systemic administration of guanosine (30, 60 or 120 $mg.kg^{-1}$) produced a significant increase in CSF levels of guanosine, sustained up to 360 min after treatments (panel A, $P < 0.001$). Intraperitoneal administration of guanosine produced up to an 11-fold increase in guanosine CSF levels 30 min following treatments (panel B, $P < 0.001$). Notably, acute treatment with guanosine also produced an increase in oxypurines (hypoxanthine, xanthine and uric acid) CSF levels (panel A and B) 30 min following treatments ($P < 0.05$). As evidenced in Fig. 3 (panels C and D), neuropathic animals show increased CSF levels of adenosine as compared to control animals ($P < 0.05$). Importantly, chronic treatment with guanosine did not affect levels of either guanosine or adenosine in both neuropathic and control animals (panel D). Additionally, chronic exposure to guanosine and/or mononeuropathy did not change inosine, xanthine, hypoxanthine, uric acid, ATP, ADP, AMP, GTP, GDP, GMP, and IMP CSF levels (panels C and D).

Fig. 4 shows the effects of chronic treatment with guanosine or vehicle on glutamate uptake by cortical and spinal cord slices from rats. As evidenced in panel A, no significant effects on cortical and spinal cord glutamate uptake were observed in animals submitted to protocol 2, (guanosine or vehicle treatment during 7 days after mononeuropathy development). However, as evidenced in the protocol 3, the partial sciatic nerve ligation produced a significant increase in cortical glutamate uptake (sham: 0.51 ± 0.08 $nmol.mg^{-1}.min^{-1}$; neuropathic-vehicle: 0.69 ± 0.08 $nmol.mg^{-1}.min^{-1}$; $P < 0.01$) an effect prevented by pretreatment with guanosine (panel A; guanosine-neuropathic:

0.45±0.08 nmol.mg^{-1}.min^{-1}; $P < 0.01$ as compared to neuropathic-vehicle). Additionally, the partial sciatic nerve ligation produced a decrease in spinal cord glutamate uptake, an effect not prevented by pretreatment with guanosine (sham: 0.35±0.05 nmol.mg^{-1}.min^{-1}; neuropathic-vehicle: 0.14±0.03 nmol.mg^{-1}.min^{-1}; neuropathic-guanosine: 0.18±0.06 nmol.mg^{-1}.min^{-1}; $P < 0.05$). Importantly, chronic systemic administration of guanosine did not affect basal cortical or spinal cord glutamate uptake after 14 and 21 days (Fig. 4).

As evidenced by the open field test, the partial sciatic nerve ligation caused a significant decrease in numbers of squares crossed, rearings and groomings and an increase in the latency to start locomotion and defecations as compared to sham animals (Tables 1 and 2). In the protocol 2, guanosine reversed locomotor effects induced by the mononeuropathy (Table 1, $P < 0.05$). In the protocol 3, guanosine also prevented locomotor effects induced by the mononeuropathy after 7 days of treatment ($P < 0.01$), but these effects were not maintained after 14 days of treatment (Table 2). Importantly, neither guanosine nor vehicle per se affected spontaneous locomotor activity as compared to naïve or sham animals (data not shown).

In regard to general toxicity induced by chronic treatment with guanosine, no mortality was observed following acute and chronic exposure to guanosine. Guanosine did not produce acute renal or hepatic damage as evidenced by serum biochemical markers measurement (Table 3). Notably, AST levels were surprisingly increased in all groups, an effect that is probably due to heart puncture for blood collection. Furthermore, the partial sciatic nerve ligation caused body weight loss, an effect prevented by guanosine in both protocols of chronic guanosine administration ($P < 0.05$, Tables 1 and 2).

Discussion:

The results of the present study extend previous data from our group[47] and clearly demonstrate the antinociceptive effects of systemic administration of guanosine against a chronic pain model in rats. Acute and chronic administration of guanosine caused a significant decrease in the paw withdrawal response on the ipsilateral side of the partial sciatic nerve ligation. Additionally, guanosine prevented mononeuropathy-induced locomotor deficits and body weight loss. By using some neurochemical approaches, we also demonstrated that guanosine actively enters in the CNS, remaining significantly increased in the CSF 360 min following i.p. treatments and that guanosine-induced antinociception may involve glutamatergic pathways.

Adenine-based purines (ABPs) have been considered important targets for the development of new drugs for treating pain, since the nucleoside adenosine and its analogs induce antinociceptive effects following both systemic and central administration[44]. Since nucleosides guanosine and adenosine closely interact in modulating several functions of the CNS[14], guanosine might well play a role on pain transmission. Recently, we demonstrated that i.c.v. guanosine or GMP produced dose-dependent antinociceptive effects in several pain models[47]. Interestingly, we also demonstrated that GMP-induced antinociceptive effects were prevented by the 5'-nucleotidase inhibitor AOPCP, suggesting that GMP antinociception results from its conversion to guanosine[46,47,50]. In the present study, only guanosine was used to investigate the role of the systemic administration of GBPs in a chronic neuropathic model of pain.

Guanosine may exert antinociceptive effects through one or more mechanisms. These may include: i. peripheral effects that could influence nociception; ii. increase in production and release of trophic factors; iii. activation of the endogenous adenosinergic system; iv. modulation of glutamatergic activity (glutamate uptake or glutamate receptors). Since a previous study demonstrated that guanosine directly administered to the CNS is antinociceptive against several pain models, a peripheral mechanism is unlikely[47]. We may speculate that guanosine could influence pain transmission by releasing trophic factors. Purines, including guanosine, stimulate the synthesis and release of several protein trophic factors by astrocytes, including nerve growth factor (NGF), neurotrophin-3, S-100β protein and others[38]. Although endogenous release of neurotrophic factors is triggered by nociceptive stimuli and may be involved in the induction changes in spinal transmission in various pain states[28], it has been demonstrated that intrathecal administration of neurotrophic factors may be efficient in the treatment of neuropathic pain[36]. Of note, we have demonstrated that the partial sciatic nerve ligation produces, 14 days after surgery, a significant increase in CSF levels of several neurotrophic factors, including brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), neurotrophin-3, and NGF (personal communication). However, an *in vivo* correlation between guanosine, neurotrophic factors and pain transmission remains to be investigated in future research.

Previous studies have suggested involvement of the adenosinergic system in effects of guanosine, since guanosine stimulates the release of adenosine in cultured astrocytes, and both are released under excitotoxic conditions[8,10]; guanosine induces trophic effects that are partially inhibited by adenosine deaminase and adenosine receptor

antagonists[9]. In contrast, some studies exclude the involvement of adenosine and its receptors to play a role in guanosine effects: guanosine-induced enhancement of neurite outgrowth in PC12 cells was not affected by adenosine receptor antagonists[22], nor were the effect of guanosine on glutamate uptake[17], seizures induced by glutamatergic agents[30], or impairment in the inhibitory avoidance task[41,56]. Additionally, guanosine-induced antinociception was not prevented by adenosine-receptor antagonists[47]. Moreover, i.c.v. guanosine or GMP failed to increase ABPs levels in the CSF[47]. In this study, both single and chronic systemic administration of guanosine also failed to increase CSF ABPs levels. Considering that high affinity binding sites for guanosine have been reported in the rat brain[53,54], this study reinforce the proposal that guanosine could act independently from adenosinergic system in inhibiting nociception, although no pharmacological investigation on adenosinergic system was performed in this study. Notably, the partial sciatic nerve ligation itself caused a significant increase of adenosine CSF levels, but it was not modulated by guanosine administration and probably reflects an endogenous response to pain or extracellular ATP degradation.

Intracerebroventricular administration of guanosine produces a significant increase in CSF levels of oxypurines, which probably indicates an *in vivo* degradation[47]. The significant production of oxypurines can not be excluded to play a role in the antinociceptive effects of guanosine. Although this study failed to demonstrate an increase in CSF oxypurines levels following chronic guanosine administration, a clear increase in oxypurines CSF levels occurred following an acute i.p. administration of guanosine. These effects were significant only 30 min after guanosine administration and might well play a role in guanosine-induced antinociception. Although this study was not

designed to measure CSF guanine levels, a previous study has demonstrated that after an i.p. administration of guanosine, the amount of both guanosine and guanine at the spinal cord increased, reaching a maximum effect by 30 min[26]. Since extracellular guanine also exerts several biological effects[39], the antinociceptive effects of guanosine may likely be regulated by its conversion to guanine by a membrane located purine nucleoside phosphorylase.

The main hypothesis to explain the antinociceptive effects of guanosine is related to its ability to modulate the glutamatergic system[49]. Glutamate and its receptors play a crucial role in pain transmission mechanisms and modulation of glutamate receptors may have therapeutic potential for several categories of pain[33]. *In vitro*, guanosine has been shown to prevent ischemic injury[18] and NMDA-induced excitotoxicity[8]. *In vivo*, acute or chronic administration of guanosine prevents seizures and toxicity induced by drugs that overstimulate the glutamatergic system[1,29,32,46,48,57], is amnesic and anxiolytic in rodents[56-58], and is neuroprotective against stroke and spinal cord injury[7,25-27]. Although the overall effects of guanosine seem to be related to attenuating glutamatergic overstimulation, its precise mechanism of action remains unclear. Guanosine is a poor displacer of glutamate ligands[51], so direct antagonism on glutamatergic receptors seems unlikely. However, we have demonstrated that intrathecal (i.t.) or systemic administration of guanosine prevented biting-behavior induced by i.t. injection of glutamate-receptor agonists (personal communication). These results indicate that the antinociceptive effect caused by guanosine involves a possible interaction with glutamatergic system (AMPA, kainate and metabotropic glutamate receptors) and/or with their signal transduction mechanisms

at the spinal cord, which could also explain some of the beneficial effects of guanosine against neuropathic pain.

Neuropathic pain is thought to be mediated by plasticity of multiple systems within the CNS[21]. There is compelling evidence suggesting that alteration of glutamatergic neurotransmission within the spinal cord contributes to hyperalgesic and allodynic responses following nerve injury[59,60]. In particular, changes in expression and efficacy of glutamate transporters have been reported. A previous study demonstrated that spinal cord glutamate uptake activity was decreased 5 days after a chronic constriction sciatic nerve injury[52]. Recent data have shown that spinal nerve ligation attenuated the glutamate uptake activity extending into the deep dorsal and ventral horn[4]. Therefore, plasticity related to spinal nerve injury produces widespread alteration in glutamate transporter function that may contribute to the pathophysiology of neuropathic pain. Our study reinforces the pivotal role of glutamate in the pathophysiology of neuropathic pain, since the partial sciatic nerve ligation attenuated glutamate uptake by spinal cord slices. In addition, our study indicates that the peripheral nerve injury seems to influence supraspinal structures as well, since it caused significant alterations in cortical glutamate uptake.

Considering these issues, the partial sciatic nerve ligation pain model and glutamate uptake by cortical and spinal cord slices seem to be reliable methods to investigate the mechanisms involved in guanosine-induced antinociception. Recently, we demonstrated that an i.pl. administration of capsaicin modulates cortical and spinal cord glutamate uptake, effects partially prevented by guanosine[47]. Since guanosine has been shown to stimulate glutamate uptake *in vitro*[17-19], we may speculate that the *in vivo*

antinociceptive effects of guanosine may result from its effect on glutamate removal from the synaptic cleft, leading to less activation of glutamatergic receptors. However, in this study, the mononeuropathy caused a decrease in the spinal cord glutamate uptake, an effect not prevented by guanosine. We also showed that the partial sciatic nerve ligation produced an increase in the cortical glutamate uptake, an effect prevented by chronic guanosine treatment. Notably, guanosine did not alter basal glutamate uptake at both sites (brain and spinal cord). It is not possible to establish, through our methodology, whether the changes in the spinal cord glutamate uptake were responsible for nociceptive behavior or it caused the changes. However, considering our results and previous data[47], we may argue that the changes in the glutamate uptake induced by guanosine were probably produced by modulation of nociceptive stimuli rather than an underlying mechanism of action. Studies investigating the interaction between guanosine, glutamate transporters, and nociception may elucidate these issues in the future.

There is increasing evidence that guanosine acts as an intercellular signaling molecule. It is released from cells and has several diverse effects *in vivo* and *in vitro*, particularly trophic effects and glutamatergic modulation[49]. Guanosine is neuroprotective[13,38] and stimulates nerve regeneration[2,27]. It also protects several cell types against apoptosis induced by a variety of agents[12,35] and has been reported to increase intracellular cAMP[22]. Guanosine has a number of effects on various cell types that make it a good candidate to test as an antinociceptive agent against chronic pain conditions since it might potentially interact with several steps of the biochemical and cellular cascade. Therefore, in the present study, we assessed whether guanosine might ameliorate thermal hyperalgesia and enhance functional outcome after a partial sciatic

nerve ligation and propose an underlying mechanism of action. There is data supporting the possible existence of specific receptor-like binding sites for guanosine on membrane preparations from rat brain[53,54]. We may speculate that guanosine, through its specific binding site, may promote its extracellular effects by activating intracellular cAMP-dependent and independent cascades[22,55]. Previous studies have shown that some of guanosine effects are mediated by intracellular activation of the phosphatidylinositol 3-kinase (PI-3-K)/Akt/protein kinase B pathway and the mitogen-activated protein (MAP) kinase pathway[12,33]. These intracellular pathways may mediate guanosine antinociceptive effects as well. Furthermore, receptor-independent mechanisms may mediate guanosine actions. It could also act as an alternative source of energy for neural cells after further metabolism, as previously demonstrated in spinal cord cultures[29,31]. Knowledge about the intracellular mechanisms underlying extracellular guanosine effects are still lacking and remain to be further investigated in the future.

Regarding side effects, a significant point to be considered is that adenosine produces important side effects such as impaired motor function and sedation[44]. It is possible that such alterations could influence antinociceptive effects of guanosine. However, our results showed that chronic administration of guanosine did not induce obvious behavioral disturbances (open field test), consistent with previously[30,47,57]. The minor toxic potential of guanosine was also evidenced by the low index of mortality (no deaths were observed in guanosine doses up to 120 $mg.kg^{-1}$), no alteration in weight body gain and no evidence for renal or hepatic impairment after chronic exposure to guanosine.

Animals submitted to a partial sciatic nerve ligation presented reduced locomotion activity (reduced crossings and rearings), increased signs of anxiety (increased

defecations), and body weight loss, consistent with previously[3]. Interestingly, guanosine prevented these effects, which may be due to its antinociceptive effects. The functional status of guanosine-treated neuropathic rats progressively improved over a chronic exposure to guanosine, indicating that an ongoing process may be involved. It is well known that guanosine stimulates the production and release of several trophic factors[38] that have potentially both neuroprotective and neurorestorative properties. All of these mechanisms may be involved in the regeneration process, but this hypothesis remains to be proved.

In summary, this is the first study showing antinociceptive effects after systemic administration of guanosine against a chronic neuropathic pain model. Because guanosine is an endogenous compound apparently well tolerated with minor toxic potential, it could eventually be developed as a drug useful for treating chronic pain conditions. This study provides additional evidence on the role of extracellular guanosine in the CNS and indicates that its antinociceptive effects probably involve the modulation of glutamatergic system. We are continuing to investigate the antinociceptive effects of guanosine and the mechanisms underlying these effects.

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Legends:

Table 1: Rats were submitted to partial sciatic nerve ligation. After 14 days, animals received daily i.p. injections of either guanosine (GUO – 60 $mg.kg^{-1}$) or vehicle (0.1 mN NaOH) during 7 days; behavior measurements were performed at the baseline (basal), 14 and 21 days after surgery: latency to the first head-dip; head-dips; squares crossed; rearings; groomings; fecal boli; and body weight. Guanosine or vehicle per se did not affect spontaneous locomotor activity and body weight as compared to naïve or sham animals (data not shown). Data are mean ± SEM. N = 8 animals per group. a = $P < 0.05$, b = $P < 0.01$, and c = $P < 0.001$ compared to sham animals (control), one-way ANOVA followed by Student-Newman-Keuls test.

Table 2: Rats were submitted to partial sciatic nerve ligation. Twenty four hours after surgery, animals received daily i.p. injections of either guanosine (GUO – 60 $mg.kg^{-1}$) or vehicle (0.1 mN NaOH) during 14 days; behavior measurements were performed at the baseline (basal), 7 and 14 days after surgery: latency to the first head-dip; head-dips; squares crossed; rearings; groomings; fecal boli; and body weight. Guanosine or vehicle per se did not affect spontaneous locomotor activity and body weight as compared to naïve or sham animals (data not shown). Data are mean ± SEM. N = 8 animals per group. a = $P < 0.05$ and b = $P < 0.01$ compared to sham animals (control), one-way ANOVA followed by Student-Newman-Keuls test.

Table 3: Effects of chronic i.p. guanosine (GUO – 60 $mg.kg^{-1}$) or vehicle (0.1 mN NaOH) on serum levels of creatinine, urea, aspartate aminotransferase (AST), and alanine

aminotransferase (ALT) in rats. Animals received daily i.p. injections of guanosine or vehicle during 7 or 14 days before blood sampling. Data are mean ± SEM. N = 6 – 8 animals per group, one-way ANOVA.

Figure 1: Effects of acute i.p. administration of guanosine (7.5 to 120 $mg.kg^{-1}$) or vehicle (0.1 mN NaOH) against thermal hyperalgesia in rats (plantar test). Animals were submitted to a partial sciatic nerve ligation. Symbols represent mean difference scores [i.e., latency to right hindpaw withdrawn (experimental side) – latency to left hindpaw withdrawn (control side), in seconds] and vertical bars represent SEM. Zero time represents the baseline before treatments. Sham operated animals were submitted to bilateral sciatic exposure without nerve ligation (mean difference score ± SEM = 0.15 ± 0.25). N = 10 animals per group. * = $P < 0.05$ as compared to vehicle, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 2: Effects of chronic i.p. administration of guanosine (60 $mg.kg^{-1}$) or vehicle (0.1 mN NaOH) against thermal hyperalgesia in rats (plantar test). Animals were submitted to a partial sciatic nerve ligation. Panel A: animals were submitted to plantar test 14 days after surgery; during 7 days thereafter, animals received daily injections of guanosine or vehicle. Panel B: animals were operated and during 14 days thereafter, animals received daily injections of guanosine or vehicle. Sham operated animals represent bilateral sciatic exposure without nerve ligation. Symbols represent mean difference scores [i.e., latency to right hindpaw withdrawn (experimental side) – latency to left hindpaw withdrawn (control side), in seconds] and vertical bars represent SEM. N = 10 animals per group. *

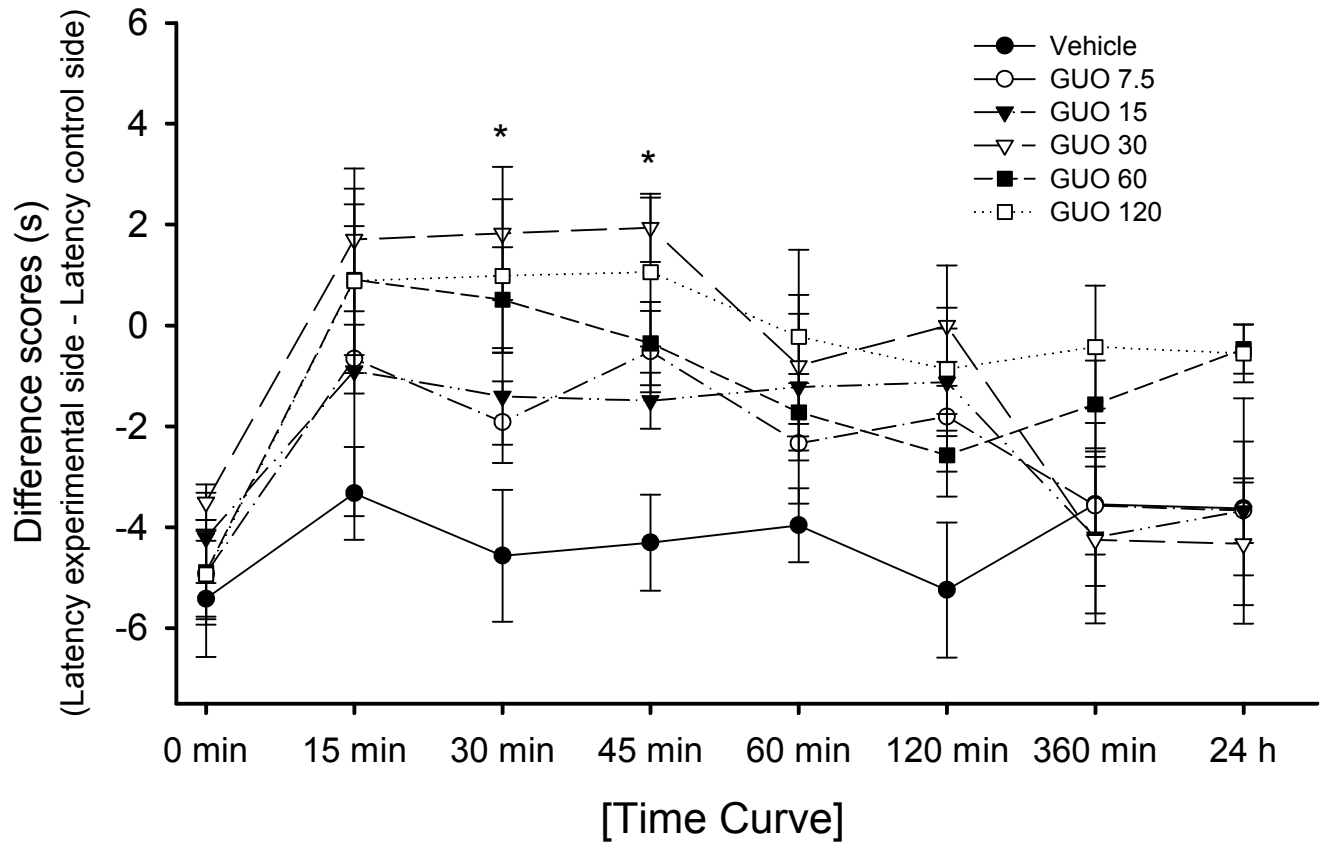
= $P < 0.05$ and ** = $P < 0.01$ as compared to vehicle, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 3: Effects of i.p. administration of guanosine on CSF concentration of purines. Panel A: animals received an i.p. injection of guanosine (60 $mg.kg^{-1}$) and CSF was collected at the baseline, 30, 60, 120, 360 min and 24 h thereafter; Panel B: animals received an i.p. injection of guanosine (30, 60 or 120 $mg.kg^{-1}$) or vehicle (0.1 mN NaOH) and CSF was collected 30 min thereafter; Panel C: animals were submitted to a partial sciatic nerve ligation or were operated bilaterally without nerve ligation (sham) and CSF was collected 14 days thereafter; Panel D: animals received daily i.p. injections of guanosine (60 $mg.kg^{-1}$) or vehicle (0.1 mN NaOH) and CSF was collected 7 and 14 days thereafter (protocol 2 and 3, respectively). The columns represent mean (μM) and vertical bars represent SEM. N = 8 animals per group. * = $P < 0.05$, ** = $P < 0.01$ and # = $P < 0.001$ as compared to baseline (panel A), vehicle (panel B) or sham (panels C and D), one-way ANOVA followed by Student-Newman-Keuls test.

Figure 4: Effects of chronic administration of guanosine and the partial sciatic nerve ligation on glutamate uptake by cortical and spinal cord slices from rats. Panel A: animals were submitted to a partial sciatic nerve ligation; after 14 days, animals displaying thermal hyperalgesia were treated with daily i.p. injections of guanosine (60 $mg.kg^{-1}$) or vehicle (0.1 mN NaOH) during 7 days (protocol 2); after behavioral evaluation, rats were sacrificed and the cortical and spinal cord slices processed for glutamate uptake assay. Panel B: animals were submitted to a partial sciatic nerve ligation; twenty-four hours

thereafter, animals received daily i.p. injections of guanosine (60 $mg.kg^{-1}$) or vehicle (0.1 mN NaOH) during 14 days (protocol 3); after behavioral evaluation, rats were sacrificed and the cortical and spinal cord slices processed for glutamate uptake assay. Data are mean ± SEM. N = 8 – 10 animals per group. C = control (sham operated animals); NP = animals submitted to partial sciatic nerve ligation. * = $P < 0.05$ and ** = $P < 0.01$ as compared to control groups (naïve and sham), one-way ANOVA followed by Student-Newman-Keuls test.

Figure 1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(Latency experimental side - Latency control side)
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4
2
0
-2
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Vehicle
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GUO 30
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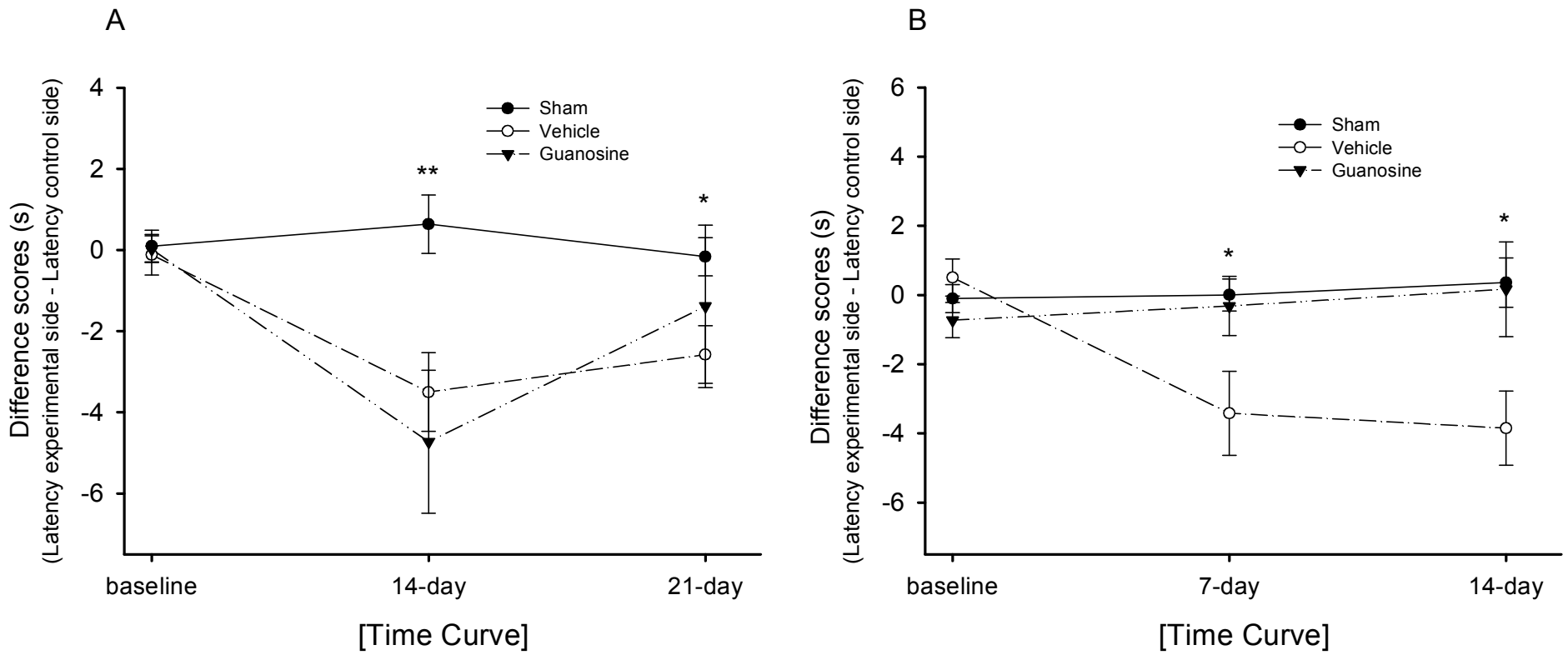
[Time Curve]

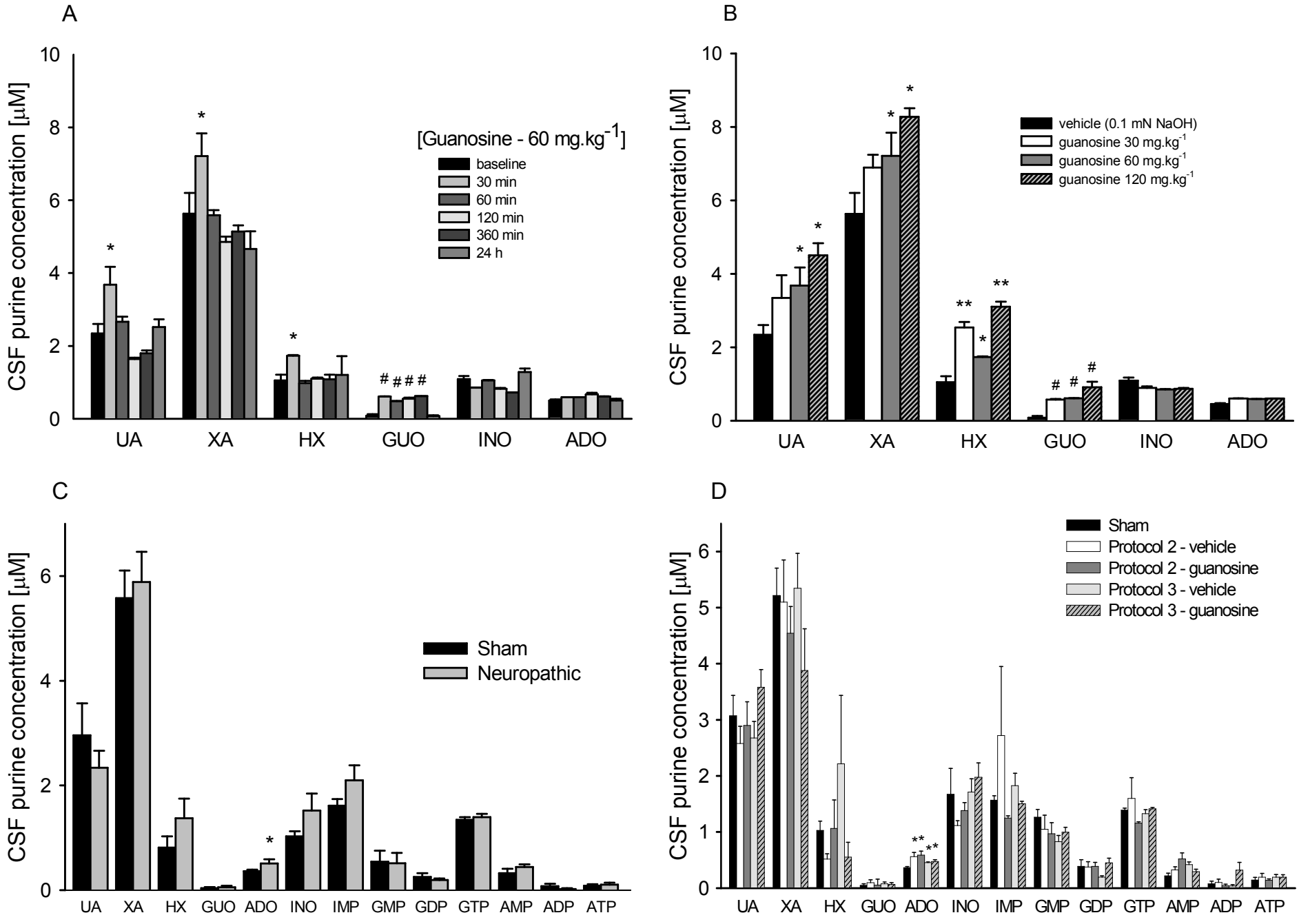
Figure

Figure 2



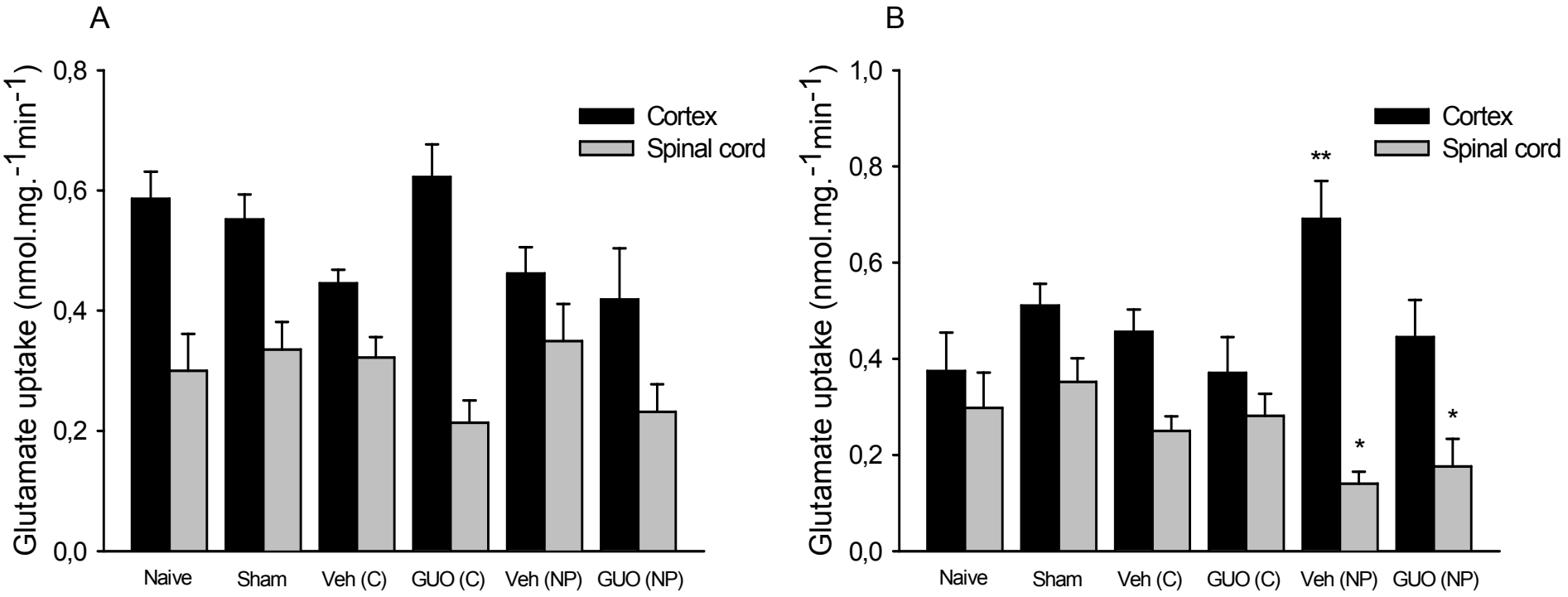
Figure

Figure 3



Figure

Figure 4



Tables

Table 1 - Effects of guanosine on the open field test and body weight gain in rats submitted to a neuropathic pain model (protocol 2 – 7-day guanosine treatment):

Treatment:	Basal			14-day			21-day		
	Sham	Vehicle	GUO	Sham	Vehicle	GUO	Sham	Vehicle	GUO
Latency (s)	2.2 (0.5)	2.8 (0.3)	2.6 (0.3)	2.0 (0.4)	2.2 (0.4)	2.5 (0.6)	2.4 (0.5)	2.7 (0.6)	3.3 (1.0)
Crossings (n)	44 (6)	33 (5)	30 (5)	40 (4)	10 (2)[c]	9 (1)[c]	39 (4)	22 (2)[a]	38 (5)
Rearings (n)	30 (2)	27 (1)	27 (2)	14 (2)	5 (1)[a]	9 (2)[a]	11 (1)	8 (1)[a]	14 (2)
Groomings (n)	2.7 (0.7)	1.9 (0.4)	2.3 (0.5)	2.3 (1.0)	1.0 (0.2)	0.7 (0.3)	1.6 (0.7)	1.4 (0.5)	2.4 (0.6)
Fecal boli (n)	3.8 (0.9)	4.0 (0.7)	3.4 (0.8)	2.9 (0.8)	6.7 (0.3)[c]	6.9 (0.6)[c]	1.6 (0.4)	3.4 (0.7)	3.3 (0.7)
Body weight (g)	362 (4)	344 (9)	363 (6)	387 (3)	325 (13)[b]	352 (10)[a]	394 (3)	329 (14)[c]	362 (10)

Tables

Table 2 - Effects of guanosine on the open field test and body weight gain in rats submitted to a neuropathic pain model (protocol 3 – 14-day guanosine treatment):

Treatment:	Basal			7-day			14-day		
	Sham	Vehicle	GUO	Sham	Vehicle	GUO	Sham	Vehicle	GUO
Latency (s)	1.9 (0.3)	2.6 (0.5)	3.0 (0.5)	1.2 (0.3)	2.1 (0.5)	1.6 (0.3)	2.3 (0.5)	6.1 (1.6)[a]	1.6 (0.4)
Crossings (n)	45 (5)	33 (5)	30 (5)	37 (5)	11 (2)[b]	29 (5)	38 (7)	9 (3)[a]	13 (4)[a]
Rearings (n)	28 (3)	28 (1)	27 (2)	14 (2)	4 (1)[b]	9 (2)	10 (2)	2 (1)[b]	4 (1)[b]
Groomings (n)	3.1 (0.6)	2.7 (0.3)	2.1 (0.3)	3.2 (1.2)	0.3 (0.1)[a]	0.7 (0.2)[a]	2.8 (1.0)	0.1 (0.1)[a]	0.9 (0.4)
Fecal boli (n)	5.4 (0.2)	6.8 (0.8)	4.0 (0.7)	3.2 (0.7)	6.9 (0.8)[b]	7.1 (0.7)[b]	1.6 (0.3)	5.1 (0.7)[b]	4.6 (0.6)[b]
Body weight (g)	387 (2)	371 (7)	395 (10)	394 (3)	363 (7)[b]	390 (8)	397 (3)	371 (7)[a]	400 (9)

Tables

Table 3 - Effects of guanosine (GUO) or vehicle (NaOH) on serum levels of creatinine, urea, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) in rats:

Treatment:	Naïve	Sham	NaOH (7 days)	GUO (7 days)	NaOH (14 days)	GUO (14 days)
Creatinine ($mg.dl^{-1}$)	0.5 (0.04)	0.5 (0.03)	0.6 (0.04)	0.5 (0.02)	0.6 (0.04)	0.5 (0.03)
Urea ($mg.dl^{-1}$)	52 (1.9)	60 (1.4)	53 (1.4)	48 (1.7)	54 (1.7)	50 (2.3)
AST ($U.L^{-1}$)	141 (4)	132 (5)	122 (10)	136 (9)	152 (5)	143 (5)
ALT ($U.L^{-1}$)	32 (1.7)	35 (1.0)	28 (1.6)	34 (2.9)	36 (1.8)	32 (5.8)

II.2.i. Antinociceptive properties of the xanthine oxidase inhibitor allopurinol in mice: role of A_1 adenosine receptors

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RESEARCH PAPER

Anti-nociceptive properties of the xanthine oxidase inhibitor allopurinol in mice: role of A_1 adenosine receptors

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Background and purpose: Allopurinol is a potent inhibitor of the enzyme xanthine oxidase, used primarily in the treatment of hyperuricemia and gout. It is well known that purines exert multiple effects on pain transmission. We hypothesized that the inhibition of xanthine oxidase by allopurinol, thereby reducing purine degradation, could be a valid strategy to enhance purinergic activity. The aim of this study was to investigate the anti-nociceptive profile of allopurinol on chemical and thermal pain models in mice.
Experimental approach: Mice received an intraperitoneal (i.p.) injection of vehicle (Tween 10%) or allopurinol (10–400 mg kg^{-1}). Anti-nociceptive effects were measured with intraplantar capsaicin, intraplantar glutamate, tail-flick or hot-plate tests.
Key results: Allopurinol presented dose-dependent anti-nociceptive effects in all models. The opioid antagonist naloxone did not affect these anti-nociceptive effects. The non-selective adenosine-receptor antagonist caffeine and the selective A_1 adenosine-receptor antagonist, DPCPX, but not the selective A_{2A} adenosine-receptor antagonist, SCH58261, completely prevented allopurinol-induced anti-nociception. No obvious motor deficits were produced by allopurinol, at doses up to 200 mg kg^{-1}. Allopurinol also caused an increase in cerebrospinal fluid levels of purines, including the nucleosides adenosine and guanosine, and decreased cerebrospinal fluid concentration of uric acid.
Conclusions and implications: Allopurinol-induced anti-nociception may be related to adenosine accumulation. Allopurinol is an old and extensively used compound and seems to be well tolerated with no obvious central nervous system toxic effects at high doses. This drug may be useful to treat pain syndromes in humans.
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Keywords: allopurinol; purines; pain; adenosine; guanosine; xanthine oxidase; anti-nociception

Abbreviations: P_1, purinergic receptor type 1; P_2, purinergic receptor type 2; A_1, adenosine receptor type 1; A_{2A}, adenosine receptor type 2A; A_{2B}, adenosine receptor type 2B; A_3, adenosine receptor type 3; DMSO, dimethyl sulphoxide; TFL, tail-flick latency; MPE, maximum possible effect; CSF, cerebrospinal fluid; HPLC, high-performance liquid chromatography; ADA, adenosine deaminase; ROS, reactive oxygen species; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; SCH58261, 5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo-[4,3-e]-1,2,4-triazolo [1,5c]pyrimidine

Introduction

Allopurinol [1,5-dihydro-4*H*-pyrazolo(3,4-*d*)pyrimidin-4-one] is a structural analogue of hypoxanthine and a potent inhibitor of the enzyme xanthine oxidase that catalyses the transformation of hypoxanthine to xanthine and uric acid, reducing both uric acid formation and purine degradation (Pacher *et al.*, 2006; Day *et al.*, 2007). Allopurinol is used primarily in the treatment of hyperuricemia and gout (Rundles, 1982). Besides its hypouricemic effects, allopurinol has been studied for several other indications, including treatment of seizures, psychiatric disorders, ischaemia-reperfusion injury, protozoal diseases and as a measure of liver impairment (Das *et al.*, 1987; Van Waeg *et al.*, 1988; Garcia Garcia *et al.*, 1990; Day *et al.*, 1994; Akhondzadeh *et al.*, 2005).

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Both healthy and hyperuricemic patients exhibit a reduction of uric acid levels after allopurinol, probably leading to accumulation of purines, including the neuromodulator adenosine, which may explain its beneficial anticonvulsant and antipsychotic effects (Tada *et al*., 1991; Wada *et al*., 1992; Zagnoni *et al*., 1994; Lara *et al*., 2000; 2003; Machado-Vieira *et al*., 2001). Of note, positive effects of allopurinol in refractory epilepsy (Tada *et al*., 1991), aggressive behaviour (Lara *et al*., 2000; 2003), mania (Machado-Vieira *et al*., 2001) and schizophrenia (Lara *et al*., 2001a; Brunstein *et al*., 2005) have been suggested to be secondary to its inhibitory effect on purine degradation and thus enhancing activities of adenosine, despite the lack of direct data to support this hypothesis.

The purinergic system involves adenosine and adenosine triphosphate (ATP) as major endogenous effectors, acting on P_1 and P_2 receptors respectively (Ralevic and Burnstock, 1998). Adenosine is mainly an inhibitory neuromodulator, regulating synaptic activity and release of several neurotransmitters, such as noradrenaline, dopamine, serotonin, acetylcholine and glutamate (Brundege and Dunwiddie, 1997; Ralevic and Burnstock, 1998). It is well known that adenosine and its analogues exert multiple effects on pain transmission at peripheral and central sites (Sawynok, 1998; Sawynok and Liu, 2003). Anti-nociceptive effects of adenosine may be related to the inhibition of intrinsic neurons by an increase in K^+ conductance and pre-synaptic inhibition of sensory nerve terminals, decreasing the release of substance P and glutamate (Sollevi, 1997); attenuation by NMDA-induced production of nitric oxide also may be involved (Bhardwaj *et al*., 1995). Adenosine has been shown to mediate opioid analgesia (Bennett, 2000).

Caffeine and theophylline are the classic P_1 adenosine antagonists currently used in humans, but adenosine agonists for human use are still lacking. We hypothesized that the inhibition of xanthine oxidase by allopurinol, thereby reducing purine degradation, could be a valid strategy to enhance purinergic activity, which is in line with the anticonvulsant and neuropsychiatric effects observed with allopurinol treatment (Tada *et al*., 1991; Wada *et al*., 1992; Zagnoni *et al*., 1994; Lara *et al*., 2000; 2001a; 2003; Machado-Vieira *et al*., 2001; Brunstein *et al*., 2005). Based on the considerations above, the aims of the present study were: (i) to investigate the anti-nociceptive activity induced by allopurinol in chemical and thermal pain models in mice; (ii) to identify, by means of pharmacological as well as neurochemical approaches, possible mechanisms by which allopurinol causes anti-nociception in mice; and (iii) to evaluate the acute toxicity induced by allopurinol using behavioural paradigms.

Methods

Animals

All animal procedures and studies followed the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983) and our institutional protocols for experiments with animals, designed to avoid suffering and limit the number of animals used. The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments. Male adult Swiss albino mice (2–3 months of age, 30–40 g) were kept on a 12 h light/dark cycle (light on at 7:00 a.m.) at temperature of 22 ± 1°C, housed in plastic cages (five per cage) with tap water and commercial food pellets *ad libitum*. All behavioural procedures were conducted between 8:00 and 10:00 a.m. In all experiments of nociceptive behavioural, the animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments.

Drug administration

Experiments were performed according to the method described by Schmidt *et al*. (2000): 20 min before the experiment, animals were placed individually in acrylic boxes, which also served as observation chambers. After this adaptation period, treatments were performed. Animals were given an intraperitoneal (i.p.) injection (10 mL kg^{-1}) of vehicle (saline or 10% Tween) or allopurinol (10–400 mg kg^{-1}). In order to investigate the mechanism of action of allopurinol, some animals were also pre-treated (15 min in advance) with an i.p. injection of the non-selective (A_1 and A_{2A}) adenosine receptor antagonist caffeine (30 mg kg^{-1}), the selective A_1 adenosine receptor antagonist DPCPX (0.1 mg kg^{-1}), the selective A_{2A} adenosine receptor antagonist SCH58261 (0.5 mg kg^{-1}) or the non-selective opioid receptor antagonist naloxone (1 mg kg^{-1}). Adenosine (100 mg kg^{-1}) and morphine sulphate (6 mg kg^{-1}) were used as positive controls for those experiments. Caffeine, adenosine, DPCPX and SCH58261 doses were based on earlier work (Lara *et al*., 2001b; Peana *et al*., 2006; Dall'Igna *et al*., 2007).

Capsaicin-induced nociception

The method used for capsaicin-induced licking was similar to that described by Sakurada *et al*. (1993). Thirty minutes after i.p. treatments, 20 µL of capsaicin (1.6 µg per paw) was injected intraplantarly (i.pl.), under the plantar skin of the right hind paw (Hamilton microsyringe with a 26-gauge needle). Animals were observed individually for 5 min after capsaicin administration for the time spent licking the injected paw, which was recorded and considered a measure of nociception.

Glutamate-induced nociception

The procedure used was similar to that described previously (Beirith *et al*., 2002). Thirty minutes after i.p. treatments, a volume of 20 µL of glutamate solution (10 µmol per paw prepared in saline) was injected i.pl., as described above. Mice were observed individually for 15 min following glutamate injection and the amount of time spent licking the injected paw was recorded and considered as indicative of nociception.

Tail-flick test

Nociception was assessed with a tail-flick apparatus (Albrasch Electronic Equipments, Brazil), as described in detail else-

where (D'Amour and Smith, 1941). A source of light was positioned below the tail, focused on a point 2.3 cm rostral to the tip of the tail and the time that the mouse took to withdraw its tail from the noxious stimulus was recorded. Deflection of the tail activates a photocell and automatically terminates the trial. A cut-off time of 10 s was employed in order to prevent tissue damage (a mouse that did not flick its tail by 10 s was considered as fully analgesic). On day one, animals were first habituated with the tail-flick apparatus through three separate measures (data not shown). On day two, baseline tail-flick latency (TFL) was measured for each mouse prior to the treatments. Animals displaying at least two TFL of 10 s on the baseline were excluded from the study. Immediately after the third TFL measurement, animals received i.p. treatments and 30 min thereafter were submitted to the tail-flick apparatus. Data for tail-flick are expressed as mean per cent of maximum possible effect (% MPE) ± SEM, according to the following formula (Calcagnetti *et al.*, 1990): % MPE: 100 × (post-drug latency – baseline latency) × (cut-off time – baseline latency)$^{-1}$.

Hot-plate test

The hot-plate test was used to measure the response latencies according to the method described by Eddy and Leimbach (1953), with minor modifications. In these experiments, the hot-plate apparatus (Ugo Basile, model-DS 37, Italy) was maintained at 55 ± 0.5°C. Animals were placed into a glass cylinder of 24 cm diameter on the heated surface, and the time between placing of the animal on the hot-plate and the occurrence of licking of hind paws or jumping off the surface was recorded as response latency. On day one, the animals were first habituated to the apparatus. On day two, mice were tested and animals displaying baseline latencies of more than 15 s were excluded from the study. An automatic 20 s cut-off was used to prevent tissue damage. Each animal was tested before administration of drugs in order to obtain the baseline. Thirty minutes after i.p. treatments, animals were placed on the heated surface and response latency recorded as described above. Data for hot-plate are expressed as mean % MPE ± SEM.

Hole-board test

The hole-board apparatus (Ugo Basile, Italy) consisted of grey Perspex panels (40 cm × 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 cm × 10 cm with a water-resistant marker. Thirty minutes after i.p. treatments, each animal was placed singly in the centre of the board facing away from the observer and its behaviour 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 (Vinadé *et al.*, 2003).

Measurement of motor performance

In order to evaluate non-specific muscle relaxant or neurotoxic effects, we evaluated the effects of allopurinol in the rotarod test and in spontaneous locomotor activity test. The rotarod apparatus (Ugo Basile, Italy) consists of a rotating (18 r.p.m.) bar (2.5 cm diameter), subdivided by disks into six compartments. As previously described (Leal *et al.*, 2000), mice were initially trained to remain on the rotarod apparatus for 120 s. Those not remaining on the bar for at least two out of three consecutive trials were discarded. On the day after training, the latency to fall from the rotarod (one trial with a maximum of 60 s) was determined 30 min after i.p. treatments. The method to evaluate spontaneous locomotor activity was adapted from Creese *et al.* (1976). Activity cages (45 cm × 25 cm × 20 cm, Albarsch Electronic Equipment, Brazil), equipped with three parallel photocells, automatically recorded the number of crossings. Animals were individually habituated to the activity cage for 10 min before receiving the i.p. treatments. Animals were placed again in the activity cages 30 min after treatments, and the crossings were recorded for 15 min.

Potentiation of barbiturate sleeping time in mice

In order to investigate sedative properties of allopurinol, mice pre-treated with allopurinol (50, 100 or 200 mg kg^{-1}) or vehicle (30 min in advance) received an i.p. injection of sodium pentobarbital (30 mg kg^{-1}). After the barbiturate injection, the sleeping time (time elapsed between loss and recuperation of righting reflex) was recorded. Criterion for recuperation of righting reflex is that animals have to regain their normal posture for three consecutive times when challenged to remain on their backs (Yamamoto *et al.*, 1987).

Rectal temperature

This was measured by using a flexible probe before and 30 min after allopurinol (50, 100 or 200 mg kg^{-1}) or vehicle treatment.

Cerebrospinal fluid sampling

Groups of mice were treated similarly with i.p. administration of allopurinol (200 mg kg^{-1}) or vehicle. After 30 min, mice were anaesthetized with sodium thiopental (60 mg kg^{-1}, 10 mL kg^{-1}, i.p.) and placed in a stereotaxic apparatus, where the cerebrospinal fluid (CSF) was drawn (10–20 μL per mouse) by direct puncture of the cisterna magna, with an insulin syringe (27 gauge × 1/2 in length), with the help of a magnifying glass. All samples were centrifuged at 10 000× *g* in an Eppendorf centrifuge for 5 min to obtain cell-free supernatants and stored in separate tubes in –70°C until analysis.

High-performance liquid chromatography procedure

High-performance liquid chromatography was performed with aliquots obtained from the CSF cell-free supernatants. The following purines were measured according to Domanski *et al.* (2006): ATP, adenosine diphosphate, adenosine monophosphate, adenosine, guanosine triphosphate, guanosine diphosphate, guanosine monophosphate, guanosine, inosine monophosphate (IMP), inosine, hypoxanthine, xanthine and uric acid. Analyses were performed with Shimadzu

Class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto-injector valve with 50 μL loop, and an UV detector. Separations were achieved on a Supelco C18 250 mm × 4.6 mm, 5 μm particle size column. The mobile phase flow rate was 1.2 mL min^{-1} and column temperature was 24°C. Buffer composition remained unchanged (A: 150 mmol L^{-1} phosphate buffer, pH 6.0, containing 150 mmol L^{-1} potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, 100% at 12.30 min and 0% at 12.40 min. Samples of 10 μL were injected every 18 min into the injection valve loop. Absorbance was read at 254 nm.

Statistical analysis

Data are expressed as mean ± SEM, except the ID_{50} values (i.e. the dose of allopurinol necessary to reduce the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} value was determined by linear regression from individual experiments using linear regression GraphPad software (GraphPad software, San Diego, CA, USA). CSF concentrations of purines are expressed as mean ± SEM in μmol·L^{-1}. Data were submitted to Kolmogorov–Smirnov, Levene and Bartlett tests for normality evaluation. Statistical analysis between groups was performed using one-way ANOVA plus the *post hoc* Student–Newman–Keuls multiple comparisons test when necessary. All results with $P < 0.05$ were considered statistically significant.

Drugs

Allopurinol, capsaicin, adenosine, caffeine, naloxone and glutamate were purchased from Sigma Chemicals (St Louis, MO, USA). DPCPX (8-cyclopentyl-1,3-dipropylxanthine) was purchased from Tocris (Northpoint, UK). SCH58261 [5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo-(4,3-e)-1,2,4-triazolo(1,5c)pyrimidine] was provided by S. Weiss (Vernalis, UK). Sodium thiopental and morphine sulphate were obtained from Cristália (SP, Brazil). Allopurinol was dissolved in a 10% Tween solution. The dose of Tween (10%) did not cause any detectable effect. Capsaicin was diluted in 5% DMSO (dimethyl sulphoxide); DPCPX and SCH58261 were diluted in 10% DMSO. All other drugs were dissolved in saline (NaCl 0.9%) and buffered with 0.1 N NaOH or 0.1 N HCl to pH 7.4 when necessary. All other chemicals were purchased from local suppliers. Drug and molecular target nomenclature used in this manuscript conforms to the Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Figures 1–4 (panel A) show that i.p. administration of allopurinol produced anti-nociception in the tail-flick, hot-plate, i.pl. glutamate and i.pl. capsaicin tests in mice. Mean ID_{50} values (and their respective 95% confidence limits) in the glutamate and capsaicin tests were 102.5 (61.9–169.8) and 119.1 (54.0–262.7 mg kg^{-1}) respectively, and maximal inhibitions were 63 ± 12% and 58 ± 8% respectively. Vehicle (10% Tween) did not affect nociception as compared with control (sham) animals (data not shown). Morphine (6 mg kg^{-1} – positive control) produced anti-nociception in all models.

Figure 4B shows that the non-selective opioid-receptor antagonist naloxone completely prevented morphine-induced anti-nociception, without affecting anti-nociception induced by allopurinol. As shown in Figure 4C, i.p. adenosine (100 mg kg^{-1}), as well as allopurinol, produced anti-nociceptive effects against capsaicin-induced pain, an effect

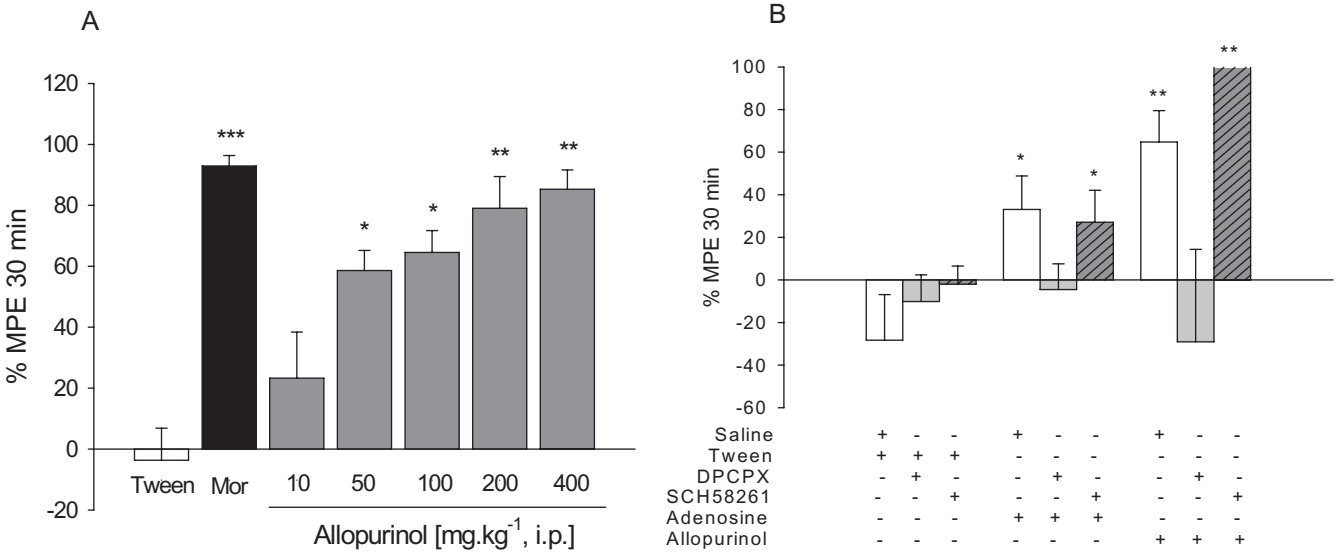

Figure 1 A. Anti-nociceptive effects of allopurinol (10–400 mg kg^{-1}, i.p.) or morphine (6 mg kg^{-1}; Mor) on tail-flick test; mean baseline latencies (s) were: Tween – 6.5 ± 0.4; morphine – 5.4 ± 0.3; allopurinol 10 to 400 mg kg^{-1} – 7.4 ± 0.4, 6.4 ± 0.4, 6.8 ± 0.4, 6.9 ± 0.5 and 6.9 ± 0.5 s respectively. B. Effects of DPCPX (0.1 mg kg^{-1}, i.p.) or SCH58261 (0.5 mg kg^{-1}, i.p.) on anti-nociceptive effects of adenosine (100 mg kg^{-1}, i.p.) or allopurinol (200 mg kg^{-1}, i.p.) on tail-flick test. The columns represent mean values of % of maximum possible effect (% MPE) and vertical bars represent SEM. n = 8–12 animals per group. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with control (10% Tween or saline + Tween), one-way ANOVA followed by Student–Newman–Keuls test.

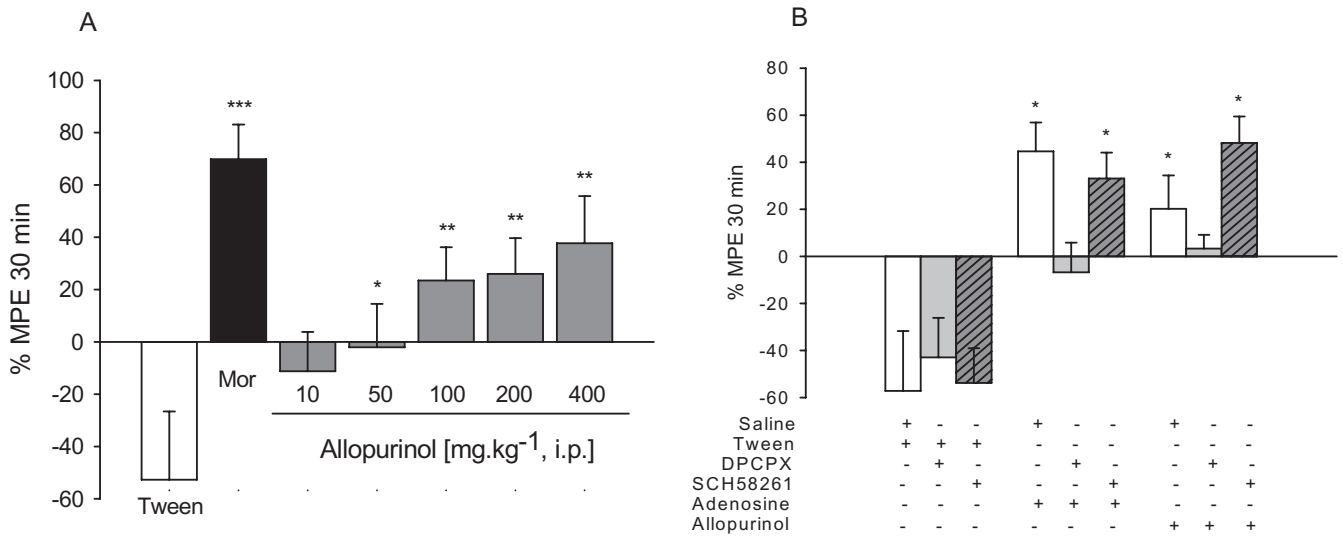

Figure 2 A. Anti-nociceptive effects of allopurinol (10–400 mg kg^{-1}, i.p.) or morphine (6 mg kg^{-1}; Mor) on the hot-plate test; mean baseline latencies (s) were: Tween – 10.4 ± 0.7; morphine – 7.4 ± 0.4; allopurinol 10 to 400 mg kg^{-1} – 8.5 ± 0.4, 9.0 ± 0.7, 10.2 ± 0.6, 8.8 ± 0.7 and 10.2 ± 0.9 s respectively. B. Effects of DPCPX (0.1 mg kg^{-1}, i.p.) or SCH58261 (0.5 mg kg^{-1}, i.p.) on anti-nociceptive effects of adenosine (100 mg kg^{-1}, i.p.) or allopurinol (200 mg kg^{-1}, i.p.) on hot-plate test. The columns represent mean values of % of maximum possible effect (% MPE) and vertical bars represent SEM. n = 8–12 animals per group. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with control (10% Tween or saline + Tween), one-way ANOVA followed by Student–Newman–Keuls test.

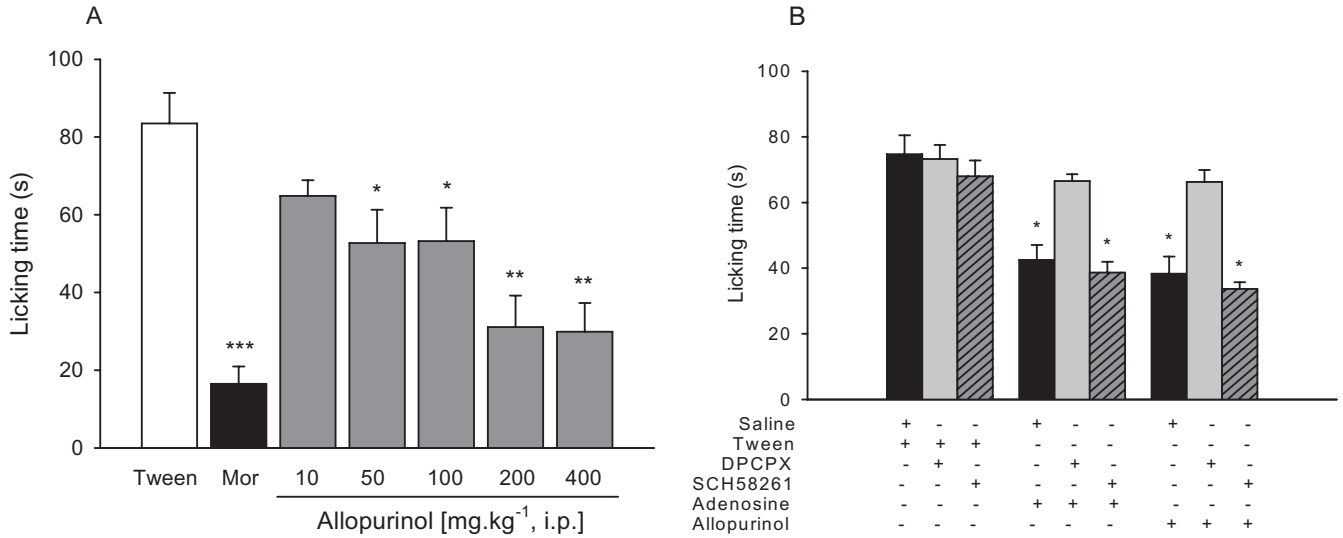

Figure 3 A. Anti-nociceptive effects of allopurinol (10–400 mg kg^{-1}, i.p.) or morphine (6 mg kg^{-1}; Mor) on glutamate-induced pain. B. Effects of DPCPX (0.1 mg kg^{-1}, i.p.) or SCH58261 (0.5 mg kg^{-1}, i.p.) on anti-nociceptive effects of adenosine (100 mg kg^{-1}, i.p.) or allopurinol (200 mg kg^{-1}, i.p.) on glutamate-induced pain. The columns represent mean time spent licking the injected hind paw and vertical bars represent SEM. n = 8–12 animals per group. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with control (10% Tween or saline + Tween), one-way ANOVA followed by Student–Newman–Keuls test.

prevented by pre-treatment with the non-selective adenosine receptor antagonist caffeine (30 mg kg^{-1}). The results depicted in Figures 1–3 (panel B) and Figure 4D show that the selective A_1 adenosine receptor antagonist DPCPX (0.1 mg kg^{-1}), but not the selective A_{2A} adenosine receptor antagonist SCH58261 (0.5 mg kg^{-1}), had no effect *per se*, but prevented anti-nociception induced by allopurinol and adenosine in the tail-flick, hot-plate, i.pl. glutamate and i.pl. capsaicin pain tests.

In the hole-board model (Table 1), neither i.p. allopurinol (10–200 mg kg^{-1}) nor vehicle affected latency to first head-dip, number of head-dips, groomings and defecations. However, allopurinol, at the highest dose (400 mg kg^{-1}), induced a significant decrease in the number of crossings and rearings, compared with vehicle.

Allopurinol (10–200 mg kg^{-1}) did not induce motor deficits, ataxia or affected spontaneous locomotor activity, as evaluated by the performance in the rotarod test and in the activity cages. However, allopurinol (400 mg kg^{-1}) decreased latency to fall in the rotarod test and spontaneous locomotor activity measured by activity cages as shown in Table 1. Allopurinol had no effect on pentobarbital-induced sleeping time (n = 8

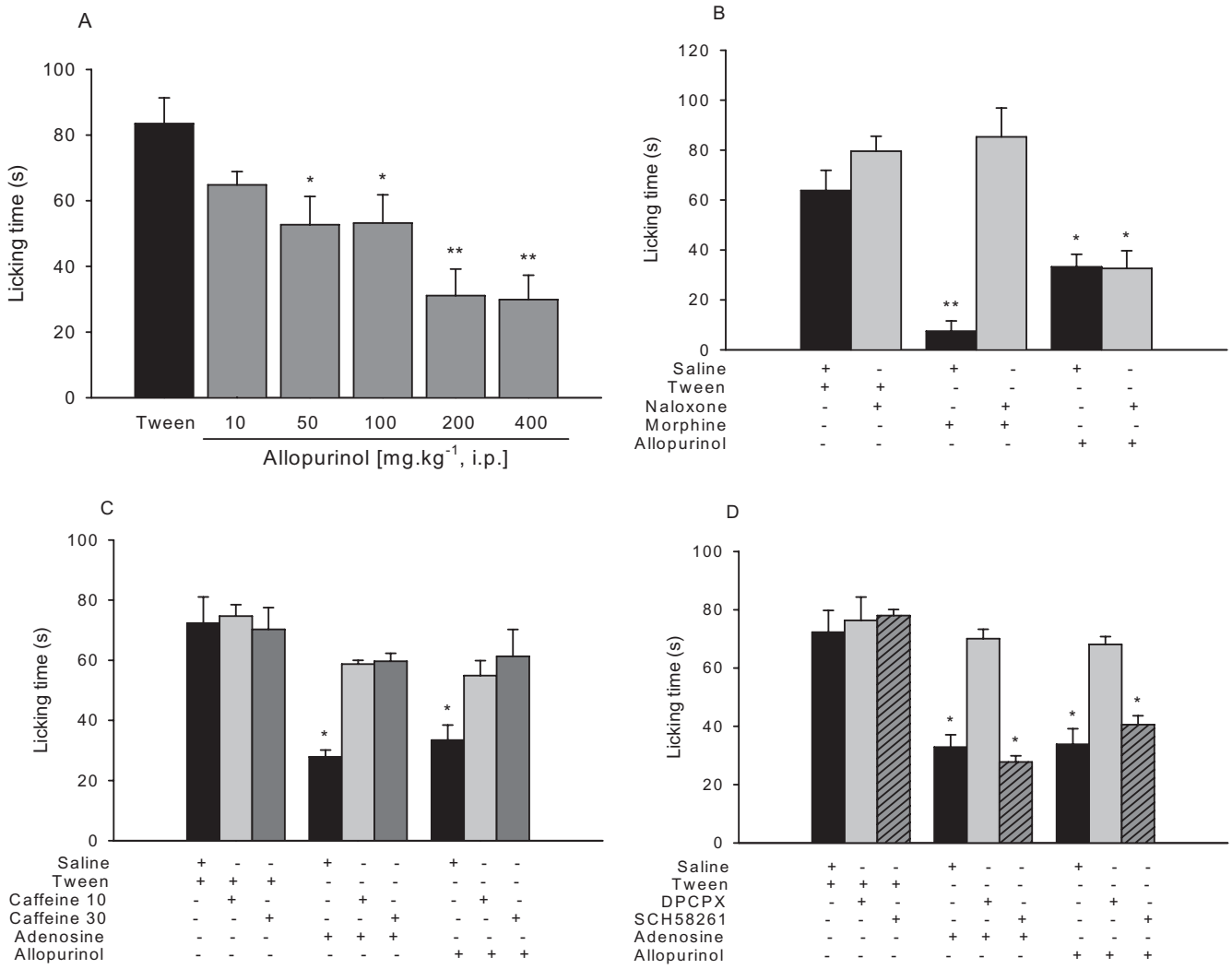

Figure 4 A. Anti-nociceptive effects of allopurinol (10–400 mg kg^{-1}, i.p.) on capsaicin-induced pain. B. Effects of naloxone (1 mg kg^{-1}, i.p.) on the anti-nociceptive effects of morphine (6 mg kg^{-1}, i.p.) or allopurinol (200 mg kg^{-1}, i.p.) on capsaicin-induced pain. C. Effects of caffeine (10 or 30 mg kg^{-1}, i.p.) on adenosine (100 mg kg^{-1}, i.p.) or allopurinol (200 mg kg^{-1}, i.p.) anti-nociception on capsaicin-induced pain. D. Effects of DPCPX (0.1 mg kg^{-1}, i.p.) or SCH58261 (0.5 mg kg^{-1}, i.p.) on anti-nociceptive effects of adenosine (100 mg kg^{-1}, i.p.) or allopurinol (200 mg kg^{-1}, i.p.) on capsaicin-induced pain. The columns represent mean time spent licking the injected hind paw and vertical bars represent SEM. $n = 8$–12 animals per group. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with control (10% Tween or saline + Tween), one-way ANOVA followed by Student–Newman–Keuls test.

Table 1 Effects of allopurinol on the hole-board, rotarod and spontaneous locomotor activity tests, used in mice

Treatment	*Allopurinol (mg kg^{-1})*					
	Tween 10%	*10*	*50*	*100*	*200*	*400*
Latency to head-dip (s)	6.0 (0.9)	6.4 (0.8)	5.5 (0.9)	7.5 (0.2)	5.3 (0.7)	8.5 (0.7)
Head-dips (*n*)	50.1 (5.7)	56.0 (2.2)	49.7 (8.6)	43.5 (6.2)	47.0 (7.5)	35.0 (6.3)
Squares crossed (*n*)	42.1 (5.4)	40.7 (5.9)	44.0 (7.2)	47.7 (5.9)	46.0 (9.4)	21.7 (9.4)*
Rearings (*n*)	2.0 (0.8)	2.3 (0.8)	2.6 (0.9)	1.8 (1.0)	1.7 (0.8)	0.7 (0.3)*
Groomings (*n*)	1.6 (0.3)	1.5 (0.3)	0.8 (0.4)	1.2 (0.4)	1.6 (0.6)	1.3 (0.4)
Fecal boli (*n*)	0.8 (0.4)	1.2 (0.4)	1.2 (0.5)	1.0 (0.3)	1.5 (0.5)	0.8 (0.5)
Latency to fall (s)	56.4 (1.6)	50.6 (7.0)	58.0 (1.5)	50.8 (6.8)	56.2 (2.5)	34.5 (5.8)*
Crossings (*n*)	234 (28)	250 (37)	222 (10)	213 (15)	212 (19)	140 (14)*

Vehicle (10% Tween) or allopurinol was given i.p., 30 min prior to the behaviour measurements: latency to the first head-dip; head-dips; squares crossed; rearings; groomings; fecal boli; latency to fall (rotarod); number of crossings (spontaneous locomotor activity). $n = 8$ animals per group. *$P < 0.05$ compared with control (10% Tween), one-way ANOVA followed by Student–Newman–Keuls test.

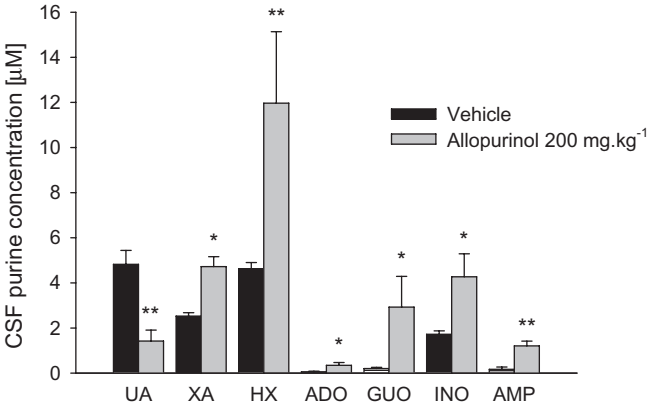

Figure 5 Effects of allopurinol (200 mg kg^{-1}, i.p.) on cerebrospinal fluid (CSF) concentration of purines. The columns represent mean (µmol·L^{-1}) and vertical bars represent SEM. Vehicle was 10% Tween. Vehicle or allopurinol was given i.p. 30 min prior to the CSF sampling. UA, uric acid; XA, xanthine; HX, hypoxanthine; ADO, adenosine; GUO, guanosine; INO, inosine; AMP, adenosine monophosphate. $n = 8$ animals per group. *$P < 0.05$ and **$P < 0.01$ compared with vehicle (control), one-way ANOVA followed by Student–Newman–Keuls test.

for all groups; Tween – 33.5 ± 5.8 min; allopurinol 50 mg kg^{-1} – 33.9 ± 10.4 min; allopurinol 100 mg kg^{-1} – 13.4 ± 8.7 min; and allopurinol 200 mg kg^{-1} – 21.2 ± 8.1 min; $P = 0.36$) and rectal temperature ($n = 8$ for all groups; Tween – 35.8 ± 0.2°C; allopurinol 50 mg kg^{-1} – 36.1 ± 0.3°C; allopurinol 100 mg kg^{-1} – 35.5 ± 0.3°C; and allopurinol 200 mg kg^{-1} – 36.1 ± 0.4°C; $P = 0.72$).

Figure 5 shows that the CSF concentration of uric acid was significantly reduced 30 min after treatment with allopurinol (200 mg kg^{-1}). Conversely, the CSF concentrations of xanthine, hypoxanthine, guanosine, adenosine, inosine and adenosine monophosphate were significantly increased in mice treated with allopurinol compared with mice receiving vehicle. The most significant changes were observed for adenosine (sevenfold increase) and guanosine (14-fold increase). Intraperitoneal administration of allopurinol did not affect CSF levels of ATP, adenosine diphosphate, guanosine triphosphate, guanosine diphosphate, guanosine monophosphate and IMP, compared with vehicle (data not shown). CSF allopurinol concentration was estimated to be 57.7 ± 5.9 µmol·L^{-1}, 30 min after a single i.p. dose of allopurinol (200 mg kg^{-1}). Allopurinol was not detected in the CSF of control animals.

Discussion and conclusions

In this study, i.p. administration of the xanthine oxidase inhibitor, allopurinol, produced dose-dependent anti-nociceptive effects in the i.pl. capsaicin, i.pl. glutamate, tail-flick and hot-plate pain models in mice. The opioid antagonist naloxone did not alter the anti-nociceptive effects of allopurinol. However, the non-selective adenosine receptor antagonist caffeine and the selective A_1 adenosine receptor antagonist DPCPX, but not the selective A_{2A} adenosine receptor antagonist SCH58261, prevented allopurinol-induced anti-nociception. No obvious motor deficits were produced by allopurinol at doses up to 200 mg kg^{-1}. This study also demonstrated that i.p. administration of allopurinol significantly increased CSF levels of purines, including the nucleosides adenosine and guanosine, and decreased CSF concentration of uric acid.

Although allopurinol has been traditionally used in the treatment of gout and its related symptoms (including pain), only anecdotal reports investigating the effects of allopurinol *per se* on pain are found in the literature (Pinelli *et al.*, 1991; Daskalopoulou *et al.*, 2005; Hacimuftuoglu *et al.*, 2006; Inkster *et al.*, 2007). Interestingly, the present study demonstrated that allopurinol produced anti-nociception in four different animal pain models. Although these animal models are essentially based on acute, short-lasting noxious stimuli, some differences between tests can be found. Tail-flick and hot-plate tests are thermal models of pain but the tail-flick refers predominantly to a spinal reflex with modest control by supraspinal structures, while the hot-plate test is a more complex pain model, producing two behavioural components (i.e. paw licking and jumping) considered to be supraspinally integrated responses (Le Bars *et al.*, 2001). Intraplantar injection of algogenic chemical agents (capsaicin or glutamate) usually produces similar nociceptive responses and represents a longer-lasting stimulus (tonic pain). However, i.pl. administration of glutamate produces a nociceptive response and paw oedema that are mainly mediated by non-NMDA receptors (Beirith *et al.*, 2002), while the capsaicin test involves a more complex mechanism, predominantly mediated by tachykinin and NMDA receptors (Sakurada *et al.*, 1993).

The rationale to administer allopurinol for pain is derived from evidence in basic and clinical research on the purinergic system. Purines and their analogues have been considered important targets for the development of new drugs for pain management, as the nucleoside adenosine and its analogues present anti-nociceptive effects at spinal, supraspinal and peripheral sites (Sawynok, 1998; Sawynok and Liu, 2003), and P_1 and P_2 receptors are closely involved in the mechanisms of pain transmission (Sawynok, 1998; Sawynok and Liu, 2003). Adenosine can alter pain transmission by acting on both nociceptive afferent and transmission neurons, and these actions are mediated primarily by adenosine A_1 receptors (Sawynok, 1998). Additional effects on inflammatory cells at peripheral sites (Fredholm, 1997) and on glia in the central nervous system (CNS) (Gebicke-Haerter *et al.*, 1996; Ogata and Schubert, 1996) mediated by adenosine A_{2A}, A_{2B} and A_3 receptors also occur, and these potentially can produce indirect effects on pain transmission. Endogenous adenosine can be released in the CNS and peripheral tissues, and the regulation of its levels by various pharmacological agents can alter pain processing through activation of adenosine A_1 receptors on neurons, and perhaps other receptors on adjacent structures (Sawynok and Liu, 2003). Anti-nociception induced by adenine-derived purines seems to be related to adenosine receptors, probably A_1 receptors, as adenosine antagonists such as caffeine and theophylline block their effect and adenosine uptake blockers and adenosine deaminase inhibitors enhance anti-nociception (McGaraughty *et al.*, 2001; Donnelly-Roberts

et al., 2008). Therefore, allopurinol, by inhibiting xanthine oxidase and production of uric acid, may produce accumulation of other purines (for example adenosine), which may account for its anti-nociceptive properties.

With regard to the mechanism of action of allopurinol, our findings demonstrated that the activation of a analoxone-sensitive opioid pathway is unlikely to be involved in the anti-nociception caused by allopurinol, as naloxone, under conditions where it fully reversed morphine-induced anti-nociception, had no effect against anti-nociception after allopurinol. However, caffeine and DPCPX, but not SCH58261, prevented allopurinol-induced anti-nociception; these results indicate that A_1 adenosine receptors and adenosine are involved in these effects. Importantly, there is no evidence that allopurinol presents any direct agonist or antagonist effect on adenosine receptors (Day *et al.*, 2007).

The basic mechanism of action of allopurinol and its metabolite oxypurinol is inhibition of xanthine oxidase (they bind strongly to the reduced form of xanthine oxidase and inhibit the enzyme). This leads to a decrease in the systemic concentration of uric acid and an increase in the concentration of the precursors, hypoxanthine and xanthine (Day *et al.*, 2007). In addition, hypoxanthine can be converted to inosine, IMP and consequently, to adenosine and guanosine (Day *et al.*, 2007). Thus, the primary effect of both allopurinol and oxypurinol is inhibition of uric acid production, and the overall result is the inhibition of the metabolism of xanthine and hypoxanthine leading to greater salvage of these purines by their conversion to inosine, adenosine and guanosine. These findings, both in CNS and periphery, have been extensively demonstrated after systemic administration of allopurinol in several studies in animals and humans (Kim *et al.*, 1987a,b; Ceballos *et al.*, 1994; Marro *et al.*, 2006). In fact, a significant concentration of allopurinol has been demonstrated in CSF after its systemic administration and a remarkable suppression of CSF uric acid levels has been observed (Kim *et al.*, 1987a; Enrico *et al.*, 1997; Akdemir *et al.*, 2001). Accordingly, in this study, we demonstrated a marked increase in the CSF concentrations of allopurinol (approximately 58 $\mu mol \cdot L^{-1}$) and of the nucleosides adenosine and guanosine and their metabolites, 30 min after an i.p. administration of allopurinol (200 mg kg^{-1}). Therefore, allopurinol-induced CSF adenosine accumulation may play a role in the anti-nociceptive action of allopurinol.

Although our findings indicate a role for adenosine in allopurinol-induced anti-nociception, we cannot rule out the influence of other purines. This study also demonstrated a significant increase in the CSF concentration of the nucleoside guanosine. Our group and others (Dobolyi *et al.*, 2000; Oses *et al.*, 2004; 2007; Cunha, 2005) have demonstrated that the nucleosides guanosine and adenosine closely interact in the CNS. More recently, we have proposed a specific guanine-based purinergic system with relevant physiological and pathological implications to the CNS, in addition to the well-characterized adenine-based purinergic system (Schmidt *et al.*, 2007). Of note, we have demonstrated that guanosine, as well as adenosine, may modulate pain transmission (Schmidt *et al.*, 2008). Therefore, it is not possible to exclude that the nucleoside guanosine may also influence allopurinol-induced anti-nociception. Unfortunately, a limitation of our study is that guanosine receptor antagonists are not available, which would more directly assess the role of guanosine in the anti-nociceptive effects of allopurinol.

Allopurinol was developed and has been extensively used as an inhibitor of the enzyme xanthine oxidase (Day *et al.*, 2007). Xanthine oxidase is a highly versatile flavoprotein enzyme that catalyses the oxidative hydroxylation of purine substrates and generates reactive oxygen species (ROS) (Borges *et al.*, 2002). ROS have been proposed to contribute to and/or maintain conditions of chronic pain (Kim *et al.*, 2006). More recently, some data indicated that ROS may also mediate acute pain transmission (Hacimuftuoglu *et al.*, 2006). Notably, there is overwhelming acceptance that xanthine oxidase activity is significantly increased in various pathological states, including some pain states (Khalil and Khodr, 2001). Therefore, the inhibition of this enzymatic pathway may be beneficial for treating pain (Lee *et al.*, 2007).

The administration of allopurinol has been shown to decrease tissue injury following ischaemia/reperfusion in a variety of *in vitro* and *in vivo* models (Garcia Garcia *et al.*, 1990; Reilly *et al.*, 1991). Recently, Inkster *et al.* (2007) showed that allopurinol treatment (50 and 250 mg kg^{-1}) had marked beneficial effects on nerve and vascular function in diabetic rats. That same study also demonstrated that allopurinol (150 mg kg^{-1}) attenuated diabetes-induced tactile allodynia, thermal and mechanical hyperalgesia. These effects may be related to a reduction on xanthine oxidase activity and consequently on aspects of ROS-mediated nerve dysfunction via adverse vascular effects (Inkster *et al.*, 2007).

Central and systemic administration of the nucleoside adenosine has been traditionally related to significant side effects, such as hypotension, sedation and impaired motor function (Sawynok and Liu, 2003). Therefore, if allopurinol induces anti-nociception by increasing levels of adenosine, the reduction in pain scores could be related to these alterations. However, our results show that allopurinol up to 200 mg kg^{-1} produced no obvious behavioural disturbances (hole-board) and did not alter motor coordination (rotarod) and spontaneous ambulation (activity cages). However, at a higher dose (400 mg kg^{-1}), some CNS side effects were observed (decreased locomotor activity as shown in the hole-board test and activity cages and impaired motor coordination as observed in the rotarod test).

In summary, to the best of our knowledge, this is the first study reporting the anti-nociceptive profile of allopurinol in well-established pain models. Allopurinol-induced anti-nociception may be related to an accumulation of adenosine, and perhaps guanosine, in the CNS. Although it is too early to propose the use of adenine- or guanine-based purines for clinical research, an interesting approach to investigate their role clinically is the investigation of purine derivatives already used in humans, such as allopurinol. Moreover, because allopurinol is a well-known and extensively used compound and seems to be well tolerated, with no obvious CNS toxic effects, except at high doses, this drug may be useful to treat pain syndromes in humans.

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Conflicts of interest

None.

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II.2.j. Physiological modulation of the purinergic system induced by exercise influences nociception in mice: investigation of the mechanism of action

Protocolo experimental em andamento: resultados parciais.

Physiological modulation of the purinergic system induced by exercise influences nociception in mice: investigation of the mechanism of action.

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Abstract

Adenosine and adenosine 5′-trifosphate (ATP) exert a modulatory role in pain conduction already well described. In this direction, several clinical and preclinical studies point the use of adenosine and its analogs in pain treatment. In the same way, exercise appears under great clinical interest. Regular physical activity, an essential component of a healthy lifestyle, recently has been shown to mediate central nervous system adaptations, to protect neurons from various brain insults, to promote neurogenesis and improve the performance in learning tasks. Although exercise effects involve alterations in plasticity, it is not known whether exercise has antinociceptive actions neither if it has a role in the regulation of purinergic system. We hypothesized that exercise, as a physiological intervention, may modulate the purinergic system in the blood-cerebrospinal (CSF) barrier and reduce pain. To address these issues, we evaluated the effects of voluntary exercise in animal models of nociception. Additionally, we sought to determine the mechanisms implicated in the analgesic effects of this training, focusing on the adenosinergic system, largely involved in nociception. We demonstrated that exercise is antinociceptive in the tail-flick, hot-plate and capsaicin tests in mice. Additionally, we demonstrated that exercise produced an increase in the CSF levels of adenosine and in the immunocontent of adenosine A_1 receptors in spinal cord. The administration of A_1 but not A_{2a} adenosine-receptor antagonist prevented the antinociceptive effects of exercise. Altogether, our findings suggest that the analgesic effects of exercise are partially mediated by its modulatory effects on the purinergic system in the central nervous system.

Keywords: Adenosine, Exercise, Purines, Pain, Antinociception.

Introduction

Adenosine and ATP, as part of purinergic system, exert multiple influences on pain transmission at peripheral and spinal sites. Although the ability of adenine-based purines to alter nociceptive transmission peripherally and centrally has been recognized for some time (Collier et al., 1966; Yarbrough and McGuffin-Clineschmidt, 1981) the last decade has seen the development of a particular interest in the role of purines in nociception. At peripheral nerve terminals in rodents, adenosine A_1 receptor activation produces antinociception by decreasing, while adenosine A_2 receptor activation produces pronociceptive or pain enhancing properties by increasing, cyclic AMP levels in the sensory nerve terminal. Adenosine receptor knockout mice, especially A_1R and A_{2A}R mice, have proven very useful to delineate mechanisms underlying adenosinergic effects on pain transmission. The use of such mice has revealed a role for A_1 receptors in mediating antinociception (Johansson et al., 2001; Wu et al., 2005) and of A_{2A} receptors in mediating hyperalgesia (Ledent et al., 1997; Hussey et al., 2007). Endogenous adenosine systems contribute to antinociceptive properties of caffeine, opioids, noradrenaline, 5-hydroxytryptamine, tricyclic antidepressants and transcutaneous electrical nerve stimulation (Keil and DeLander, 1996; Sawynok, 1998). At both peripheral and spinal sites, the manipulation of endogenous adenosine levels by inhibition of adenosine kinase can produce antinociception by activating adenosine A_1 receptor mechanisms (Sawynok, 1998).

Regular physical activity, an essential component of a healthy lifestyle, recently has been shown to mediate central nervous system (CNS) adaptations (Cotman and Berchtold, 2002). Animal studies have demonstrated that exercise protects neurons from various brain insults (Carro et al., 2000; Larsen et al., 2000; Tillerson et al., 2003), promotes neurogenesis

(van Praag et al., 1999a) and improves the performance in learning tasks (Fordyce and Farrar, 1991; van Praag et al., 1999b). Many studies have linked functional improvements, such as those reported for memory and cognition (Fordyce and Farrar, 1991; van Praag et al., 1999b) to changes in the number, structure and function of neuron (van Praag et al., 1999a). Recent studies indicate that physical activity produces these changes by altering genes involved in synaptic plasticity (Farmer et al., 2004; Dishman et al., 2006) and affects the modulation of glutamatergic synapses by increasing glutamate receptors in postsynaptic densities from cortical mice brain (Dietrich et al., 2005).

In parallel with the development of adenosine-based pharmaceuticals, there is an increasing recognition about the role of endogenous adenosine in other therapeutic modalities, such as the regular exercise activity. Studies provide strong support that physical activity beneficially alters several functions in the CNS. Although exercise effects involve alterations in plasticity, it is not known whether exercise has a role in pain transmission neither in the regulation of purinergic system. Therefore, we hypothesized that exercise, as a physiological intervention, may modulate the purinergic system in the CNS, in particular in the blood-(cerebrospinal fluid) CSF barrier, and reduce pain. To address these issues, we evaluated the effects of voluntary exercise in animal models of nociception. Additionally, we sought to determine the mechanisms involved in the analgesic effects of exercise, focusing on the adenosinergic system, largely involved in nociception.

Material and Methods

Animals: Male adult Swiss albino mice (30-40 g) were kept on a 12 hour light/dark cycle (light on at 7:00 am) at temperature of 22 ± 1ºC, housed in plastic cages (five per cage) with tap water and commercial food *ad libitum.* Mice were divided in two groups: sedentary

(Sed) and voluntarily physical active (exercise - Ex) groups. Animals from Ex group had free access, during 45 days, to a running wheel which was located within the living cage; Sed group stayed for the same time in similar conditions without running wheels. In all nociceptive behavioral experiments, the animals were acclimatized to the laboratory for at least 1 h before testing, and used only once throughout the experiments. The ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983) and our institutional protocols for experiments with animals, designed to avoid suffering and limit the number of animals sacrificed, were followed throughout. The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Drugs: Capsaicin, caffeine, naloxone, morphine and purine nucleoside phosphorylase (PNP) were purchased from Sigma Chemicals (St Louis, MO, USA). DPCPX (8-cyclopentyl-1,3-dipropylxanthine) was purchased from Tocris (Northpoint, UK). Immucillin-H and SCH58261 (5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5c]pyrimidine) were provided by S. Weiss (Vernalis, UK). The anesthetic sodium thiopental was obtained from Cristália (SP, Brazil). Capsaicin and Immucillin-H were diluted in DMSO (dimethyl sulfoxide, 5% and 1% respectively). All other solutions were dissolved in saline (NaCl 0.9%) and buffered with 0.1 N NaOH or 0.1 N HCl to pH 7.4 when necessary. All other chemicals were of high grade quality.

Surgical procedure: Surgery and i.c.v. infusion techniques were according to (Schmidt et al., 2000). Mice were anaesthetized with sodium thiopental (60 mg/kg, 10 ml/kg, i.p.). In an stereotaxic apparatus, the skin of the skull was removed and a 27 gauge 7 mm guide cannula was placed at 1 mm posterior to bregma, 1 mm right from de midline and 1 mm above the lateral brain ventricle. Through a 2 mm at the cranial bone, the cannula was

implanted 1.5 mm ventral to the superior surface of the skull, and fixed with jeweler acrylic cement. Experiments were performed 48 hours after surgery.

Tail-flick: Nociception was assessed with a tail-flick apparatus (Albarsch Electronic Equipments, Brazil), as described in detail elsewhere (D´Amour and Smith, 1941). A source of light was positioned below the tail, focused on a point 2.3 cm rostral to the tip of the tail and the time that the mouse took to withdraw its tail from the noxious stimulus was recorded. Deflection of the tail activates a photocell and automatically terminated the trial. The light intensity was adjusted in order to obtain baseline tail flick latency (TFL) of 3-4 s. A cut-off time of 10 s was employed in order to prevent tissue damage (a mouse that did not flick by 10 s was considered as fully analgesic). On day one, the animals were habituated with the tail flick apparatus through three separate measures (data not shown). On day two, baseline tail flick latency was measured for each mouse prior to the treatments. Animals displaying at least two TFL of 10 s on the baseline were excluded from the study. Each animal was tested before treatments in order to obtain the baseline. Data are expressed as the changes in the tail-flick latencies according to the following formula: $\Delta T(s)$ = post-drug latency – pre-drug latency.

Hot-plate: The hot-plate test was used to measure the response latencies according to the method described by (Eddy and Leimbach, 1953), with minor modification. Animals were placed into a glass cylinder of 24 cm diameter on the heated (55 ± 0.5 °C) Hot Plate apparatus (Ugo Basile, model-DS 37, Italy) surface. The time between placement of the animal on the hot-plate and the occurrence of licking of the hindpaws or jumping off the surface was recorded as response latency. On day one, the animals were habituated with the turned off apparatus. On day two, mice were tested and animals displaying baseline latencies

higher than 15 s were excluded; an automatic 20 s cut-off was used to prevent tissue damage. Each animal was tested before treatments in order to obtain the baseline. Data are expressed as the changes in the tail-flick latencies according to the following formula: $\Delta T(s)$ = post-drug latency – pre-drug latency.

Hole-board: The hole-board apparatus (Ugo Basile, Italy) consisted of gray Perspex panels (40 x 40 cm, 2.2 cm thick) with 16 equidistant holes 3 cm in diameter in the floor. Photocells below the surface of the holes automatically recorded the number of head-dips. The board was positioned 15 cm above the table and divided into nine squares of 10 x 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 the locomotion (Vinade et al., 2003).

Measurement of motor performance: In order to evaluate non-specific muscle relaxant or neurotoxic effects, we tested the effects of exercise in the rotarod test and in the spontaneous locomotor activity test. The rotarod apparatus (Ugo Basile, Italy) consists of a rotating (18 rpm) bar (2.5 cm diameter), subdivided by disks into six compartments. As previously described (Leal et al., 2000), mice were initially trained to remain on the rotarod apparatus for 120 sec. Those not remaining on the bar for at least two out of three consecutive trials were discarded. On the day after training, the latency to fall from the rotarod (one trial with a maximum of 60 sec) was determined. The method for the spontaneous locomotor activity was adapted from (Creese et al., 1976). Activity cages (45 x 25 x 20 cm, Albarsch Electronic Equipment, Brazil), equipped with three parallel photocells, automatically record the number of crossings during 15 min.

Spontaneous alternation performance: Spontaneous alternation performance was assessed in a Y-maze. Each of the three arms was 30 cm long, 20 cm high and 6 cm wide, and converged to an equal angle. Each mouse was placed at the end of one arm and allowed to freely move through the maze for 8 min. The series of arm entries was recorded visually. An alternation was defined as entries in all three arms on consecutive occasions. The percentage of alternation was calculated as (total of alternation/total arm entries - 2) X 100, according to (Maurice et al., 1996).

Inhibitory avoidance: Mice are shocked when leaving a platform in a training session making them more prone to remain in the platform during a subsequent test session. The training apparatus was a 50x25x25-cm plastic box with a 2-cm high, 46-cm wide platform at the center of the training apparatus. The floor of the apparatus was made of parallel 0.1-cm caliber stainless-steel bars spaced 1.0-cm apart. In the training session, the animal was placed on a platform and the latency to step down the four paws on the grid was measured with a device; upon stepping down, mice received a 2 s intermittent foot shock (three 0.5s shocks, 0.2mA, with a 0.25s interval between them). Test session step-down latency 10 min and 24 h later was taken as a short- and long-time memory, to a ceiling time of 180 s. No foot shock was given in the test session.

Cerebrospinal fluid (CSF) sampling: Mice were anesthetized with sodium thiopental (60 mg/kg, 10 ml/kg, i.p.) and placed in a stereotaxic apparatus, where the CSF was drawn (10 - 20 μl per mouse) by direct puncture of the *cisterna magna* with an insulin syringe (27 gauge x 1/2 in length), with the help of a magnifying glass. All samples were centrifuged at 10,000g in an Eppendorf centrifuge during 5 min to obtain cell-free supernatants and stored in separate tubes at -70^{o}C until analysis were conducted.

HPLC procedure: High-performance liquid chromatography (HPLC) was performed with CSF cell-free supernatants aliquots for determination of purines concentration, according to (Domanski et al., 2006). CSF concentrations of the following purines were determined: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine, inosine monophosphate (IMP), inosine, hypoxanthine, xanthine, and uric acid. Analyses were performed with Shimadzu Class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto injector valve with 50 μL loop, and an UV detector. Separations were achieved on a Supelco C18 250 mm x 4.6 mm, 5 μm particle size column. The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24 ^{o}C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 10 μL were injected into the injection valve loop. Absorbance was read at 254 nm. CSF concentrations of purines are expressed as mean ± SEM in μM.

Electrophoresis and Western blot analysis: mice were sacrificed by decapitation and the spinal cord from both groups was collect out. Spinal cords were homogenized in 5% SDS solution containing protease inhibitor cocktail (Sigma, São Paulo/Brazil) and kept at -70°C. Protein content was further determined by using Bicinchoninic acid assay using bovine serum albumin (BSA) as standard (Pierce, São Paulo/Brazil). Proteins from spinal cord preparations were separated by 12% SDS-PAGE mini-gels and transferred to

nitrocellulose membrane using a Trans-Blot system (Bio-Rad, Porto Alegre, Brazil). After blocking for 2 h at room temperature with 5% milk in Tris-buffered saline, pH 7.6 containing 0.1% Tween 20 (TBS–T), the membranes were incubated overnight at 4 °C with either a rabbit anti-adenosine A_1 receptor antibody (1:1000 dilution from Affinity Bioreagents) or anti-adenosine A_{2A} receptor antibody (1:5000 dilution from Sigma). After primary antibodies incubation, membranes were washed and incubated with alkaline phosphatase-conjugated secondary antibodies for 2 h at room temperature and developed with ECL (Amersham, São Paulo/Brazil). The autoradiographic films were scanned and densitometric analyses were performed using public domain NIH Image Program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). We used Coomassie blue stain in gels, analyzed by NIH Image, and Ponceau S red stain in membranes to be sure the same quantity of protein was loaded in each lane (5 Ag/lane). The results were presented by A_1 or A_{2A} receptor arbitrary units.

Statistical analysis: Data are expressed as mean ± SEM. Differences among groups were determined by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test when applicable. $P < 0.05$ was considered of statistical significance.

Results

Involvement of adenosine in the antinociceptive effects of exercise

We first explored if exercise could promote analgesia in animal models of nociception. The results presented in Fig. 1A shows that exercise was able to decrease pain sensation against i.pl. capsaicin. Fig. 1B shows that the non-selective opioid-receptor

antagonist naloxone completely prevented morphine- and exercise-induced antinociception. Fig. 1C shows that i.p. administration of adenosine produced antinociception in the i.pl. capsaicin tests in sedentary mice and its effect was prevented by pretreatment with caffeine (10 $mg.kg^{-1}$, i.p.), a non-selective adenosine receptor antagonist, and the same effect was observed in exercise mice. Next, we investigated if the pretreatment with A_1 or A_{2A} adenosine-receptor antagonists would be able to prevent the effect of exercise against capsaicin test. The results depicted in Fig. 1D shows that the selective A_1 adenosine-receptor antagonist DPCPX (0.1 $mg.kg^{-1}$), but not the selective A_{2A} adenosine-receptor antagonist SCH58261 (0.5 $mg.kg^{-1}$) prevented antinociception induced by adenosine and exercise in the capsaicin pain tests.

Effects of exercise in promote analgesia against i.pl. capsaicin was reproduced in tail-flick model. Fig. 2A shows that exercise promoted analgesia either on tail flick test. As expected, morphine 3.0 $mg.kg^{-1}$, which was taken as a positive control, strongly impaired the nociceptive response in tail flick test ($P < 0.001$) when administered to sedentary mice. Naloxone prevented morphine- and exercise-induced antinociception. However, in this pain model, neither caffeine nor DPCPX prevented the antinociceptive effects of exercise (Figs 2C and 2D). Actually, SCH58261 presented a slight increase in antinociceptive effects of exercise.

Exercise was able to reduce pain in a third model of nociception, hot plate-induced pain (Fig. 3A). In addition (Fig. 3B), the non-selective opioid-receptor antagonist naloxone, which had no intrinsic effect over nociception, completely prevented morphine- and exercise-induced antinociception. Fig. 1C shows that i.p. administration of adenosine produced antinociception on hot plate test in sedentary mice and its effect was prevented by pretreatment with caffeine, the same occurring with antinociceptive effects of exercise. In

relation to adenosine-receptor antagonist effects (Fig. 3D), we observed that DPCPX and SCH58261 had a similar effect to that observed in capsaicin test: reversion and maintenance of adenosine and exercise antinociception, respectively.

Spinal cord immunocontent of adenosine receptors and CSF purine levels are modulated by exercise

We investigated the mechanisms implicated in the analgesic effects of exercise, focusing in the adenosinergic system, largely involved in nociception. Since adenosine is widely distributed through the organs, not exclusively in the CNS, we decided to verify adenosine-receptors immunocontent in spinal cord, a critical site for conduction of pain signals to the brain. Western blotting approach was performed and we immunodetected adenosine receptors in the spinal cord. We found out an increased immunocontent of A_1 receptors (fig.4A), but not of A_{2A} receptors (fig.4B**).**

Once the cerebrospinal fluid is rapidly distributed in the up-to-down direction with rapid penetration of the spinal cord tissue, we looked in the CSF levels of adenosine and its metabolites. CSF concentrations of adenosine as well GTP, GDP and guanosine were significantly increased in exercised mice in comparison with sedentary control, in spite of a significant decrease hypoxanthine, xanthine and uric acid (Fig. 5). Exercise did not affect ATP, ADP, AMP, GMP, IMP and inosine CSF levels.

Two interconnected compartments are responsible for the control of extracellular purines and need to be distinguished: the vascular endothelium forming the blood–brain barrier within the brain interstitial compartment, and the choroid epithelium forming the blood–CSF barrier. Thus, we decided to study the purine hydrolysis by *ex vivo* choroid plexus which is implicated in the active clearance of purines from CSF to blood. Fig. 6 (A-D)

shows the nucleotides hydrolysis by *ex vivo* choroid plexus incubated with ATP (100 μM) in an artificial CSF medium. When we analyzed all the purines and its metabolites at the baseline (Fig. 6A), no difference is evident except for uric acid, which is decreased in exercised animals. However, when we look to a time curve, we found that ADP levels are increased in the 5th minute (Fig 6A). No effect was found in a time curve for GTP, GDP, GMP and guanosine (Fig. 6C). When we looked for metabolites, we found an increase in inosine levels as well as a decrease in xanthine and uric acid concentration (Fig. 6D).

Inhibition of purine metabolism causes antinociception

Because the enzyme that converts adenosine in hypoxanthine is PNP, a ubiquitous enzyme essential for purine metabolism which is active in rodent's CSF, we postulated exercise has a PNP-inhibitor-like effect, accumulating adenosine, and decreasing levels of its downstream metabolites. To confirm our hypothesis, we injected mice i.c.v. with the PNP inhibitor immucillin-H and checked if it mimics the effects of exercise in nociception. Indeed, we used immucillin-H 12.5 μM injected into the CSF to achieve a final CSF concentration of 1 μM. Immucillin-H promoted notable analgesia in a similar fashion as did exercise (Fig. 7). In other group, we injected mice i.c.v. with PNP (2 units/mouse) and no effect was observed.

Exercise and antinociception memory (neuroplasticity)

Considering that we demonstrated that exercise is able to reduce pain in three different models of pain, we decided to investigate if these effects would be maintained with the interruption of training. Thus, we let mice in running wheels for 45 days and after 30

days of detraining, animals returned to exercise for 15 days. Pain tests were performed in these three moments: training, detraining and retraining. Analgesic effects were observed in capsaicin and tail-flick tests during training from the 7th day through the 45th day (Fig. 8). However, on hot-plate model, analgesic effects were observed only in the 7th day of training. On detraining period, any effect was observed on capsaicin, tail-flick and hot-plate models. When animals returned to exercise, significant analgesia was observed in capsaicin model (during 3rd, 7th and 15th) session tests and tail-flick model (during 7th and 15th session tests), but no difference was observed in hot plate-induce pain.

Exercise and behavioral tests

In the hole-board model, exercise did not affect latency to first head-dip, and the number of head-dips, rearings, groomings and defecations, but decreased the number of crossings (Fig. 9). No effect on motor performance in the rotarod test was observed. Additionally, exercise reduced spontaneous locomotor activity on the 3rd and 30th day locomotion as measured by activity cages as shown in Fig. 10A, but did not affect the time exploring the center (Fig. 10B) neither the spontaneous alternation performance in the Y-maze (Fig. 10C). As we observed an effect memory-like in analgesic properties of exercise, it was necessary to analyze memory *per se.* Then, to evaluate memory, animals were submitted to inhibitory avoidance task. We evaluated short- and long-term memory and no difference was observed between sedentary and exercise groups (Fig. 10D).

Discussion

It has been shown that regular physical activity mediates central nervous system (CNS) adaptations (Cotman and Berchtold, 2002) and is involve in the modulation of

various brain processes (Fordyce and Farrar, 1991; Carro et al., 2000; Larsen et al., 2000; Tillerson et al., 2003). The present results indicate that 45 days of voluntary exercise presents antinociceptive effects against three models of pain, capsaicin, tail-flick and hot-plate. Additionally, we showed that non-selective opioid-receptor antagonist naloxone completely prevented exercise-induced antinociception. These results indicate that exercise effects on antinociception are related to opioid and adenosine (A_1) receptors.

In this work we showed that exercise effects are due, at less in part, to modulation of the purinergic system. Treatment with adenosine showed analgesic effects as already described elsewhere (Gyllenhammar and Nordfors, 2001). The peripheral actions of adenosine in rodents depend very much upon the adenosine receptor subtype activated. Adenosine can alter pain transmission by actions on both nociceptive afferent and transmission neurons, and these actions are mediated primarily by adenosine A_1 receptors (Sawynok, 1998; Sawynok et al., 1999). Thus, the exogenous administration of adenosine A_1 agonists locally to the hindpaw of the rat produces antinociception in a pressure hyperalgesia model (Taiwo and Levine, 1990) and in the formalin model (Karlsten et al., 1992). In contrast, local administration of adenosine A_2 receptor agonists enhances pain responses in both models (Taiwo and Levine, 1990; Karlsten et al., 1992), an action most likely due to adenosine A_{2A} receptor activation (Doak and Sawynok, 1995). In this work, when mice were pretreated with caffeine, a non-selective adenosine receptor antagonist, the antinociceptive effect was abolished either in sedentary control mice that received adenosine after pretreatment and in exercise group. It is important to consider that the local administration of caffeine has not demonstrated any antinociceptive properties, perhaps because of its mixed profile of activity against both adenosine A_1 and A_2 receptors (Doak and Sawynok, 1995). The present results provide the first direct evidence that adenosine A_1

receptors are involved in analgesic effects of exercise in mice. Administration of DPCPX, a selective A_1 adenosine-receptor antagonist entirely prevented the analgesic effect of exercise. This action is not share with A_{2A} receptors once its inhibition had no effect over exercise antinociception. By western blotting approach, we immunodetected adenosine receptor levels in the spinal cord and we found out an increased immunocontent of A_1 receptor but not of A_{2a} receptor. It is additional evidence that A_1 receptors mediate the antinociceptive effects of exercise.

Endogenous adenosine can be released from brain, spinal cord and peripheral tissues, and the regulation of such release by various pharmacological agents can alter pain processing through activation of adenosine A_1 receptors on neurons, and perhaps other receptors on adjacent structures. In the CSF, adenosine, GTP, GDP and guanosine were significantly increased in exercise mice in comparison with sedentary control, in spite of a significant decrease hypoxanthine, xanthine and uric acid. It is an evidence that exercise may modulate the endogenous levels of adenosine probably by decrease its degradation and thus assign antinociceptive benefits.

Following these results, we found that hydrolysis of purines by choroid plexus is affected by exercise. We observed an increase in CSF levels of ADP at 5 min with a concomitant decrease in xanthine at 5 min. It is an indicative of central accumulation of ADP, a precursor of adenosine. It was already described that the concentration of adenosine in the brain interstitial fluid under resting conditions remains low, probably between 120 and 220 nM, despite constant production from both extracellular and intracellular sources (Latini and Pedata, 2001). Here, we observed that adenosine levels did not change with exercise, but ADP levels are significantly increased, an effect that could be an indicative of central accumulation of ADP, a precursor of adenosine.

In physiological conditions, adenosine may enter brain interstitial fluid from the blood through the blood–brain barrier and may be removed by brain endothelial cells, followed by either an efflux to blood or metabolic degradation. *In vivo* this system probably serves to excrete the purine metabolites from cerebrospinal fluid into the blood (Berlin, 1969). Thus, the choroid plexus may serve as an excretory mechanism for endogenous purine metabolites from brain, thereby regulating their levels in the CSF.

Besides the modulation of purinergic system in the blood-CSF barrier by exercise and its antinociceptive effects, evidence to the participation of this system in the exercise effects appears from experiments with Immucillin-H, a PNP-inhibitor. While PNP is an enzyme that converts adenosine in hypoxanthine, its inhibition is responsible for accumulate adenosine and decrease levels of its metabolites. Administration of immucillin-H promoted notable analgesia in a similar fashion as did exercise and PNP administration had no effect. It is in accordance with the modulation of CSF levels of purines by exercise. Mice trained in running wheels for 45 days showed antinociception against pain models in parallel with an increased in CSF adenosine levels and a concomitant decrease in its metabolites. In the same way, when we block the degradation of adenosine with Immucillin, increasing its levels, we observed the same effects showed by exercise.

Recently, evidence has accumulated suggesting that physical activity enhances cognition in rodents (Suominen-Troyer et al., 1986; Rogers et al., 1990; van Praag et al., 1999b; Winter et al., 2007) and researchers have shown that in hippocampus, a brain area important for learning and memory, presents a robust increase in new neurons associated with exercise (van Praag et al., 1999b; van Praag et al., 1999a). Here we showed that animals trained during 45 days have an important reduction in pain sensibility, notable already in the 7^{th} day of training. However, after activity has been interrupted, the

antinociception is lost. Interestingly, with a retraining, the antinociception against capsaicin-induced pain returns since the 3rd day and in tail flick model returns in the 7th day, but no effect was detectable on hot plate model. In inhibitory avoidance task no difference was observed between groups. Then, these effects along the time seem to be related to a spinal cord neuroplasticity induced by exercise (memory-like effect at a spinal cord level).

In summary, to the best of our knowledge, this is the first study investigating the antinociceptive effects of voluntary exercise in traditional animal pain models. Exercise-induced antinociception may be related to modulatory effects in the purinergic system, probably due to a decrease in degradation of adenosine with its subsequent accumulation in the CSF and an increase in the immunocontent of adenosine A_1 receptors at the spinal cord. Considering that the clinical use of adenosine and its analogs is not a reality, the non-pharmacological approaches appear as a good option to control pain sensation. Exercise, as a physiological intervention, arises as possible mechanism to control pain and modulate purinergic system besides being able to improve systemic functions and brain health.

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Legends:

Figure 1: Effects of exercise against i.pl. capsaicin test in mice. Columns represent mean time spent licking the injected hindpaw and vertical bars represent SEM. (A) Effects of exercise against capsaicin-induced pain. (B) Effects of pretreatment with naloxone (1 $mg.kg^{-1}$) on morphine-(3 $mg.kg^{-1}$) or exercise-induced antinociception. (C) Effects of pretreatment with caffeine (10 $mg.kg^{-1}$) on adenosine-(100 $mg.kg^{-1}$) or exercise-induced antinociception. (D) Effects of pretreatment with DPCPX (0.1 $mg.kg^{-1}$) or SCH58261 (0.5 $mg.kg^{-1}$) on adenosine-(100 $mg.kg^{-1}$) or exercise-induced antinociception. N = 8 – 10 animals per group. * = $P < 0.05$ and ** = $P < 0.01$ as compared to control, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 2: Effects of exercise against the tail-flick test in mice. Columns represent the changes in the tail-flick latencies according to the following formula: $\Delta T(s)$ = post-drug latency – pre-drug latency and vertical bars represent SEM. (A) Effects of exercise in the tail-flick test. (B) Effects of pretreatment with naloxone (1 $mg.kg^{-1}$) on morphine-(3 $mg.kg^{-1}$) or exercise-induced antinociception. (C) Effects of pretreatment with caffeine (10 $mg.kg^{-1}$) on adenosine-(100 $mg.kg^{-1}$) or exercise-induced antinociception. (D) Effects of pretreatment with DPCPX (0.1 $mg.kg^{-1}$) or SCH58261 (0.5 $mg.kg^{-1}$) on adenosine-(100 $mg.kg^{-1}$) or exercise-induced antinociception. N = 8 – 10 animals per group. * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.01$ as compared to control, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 3: Effects of exercise against the hot-plate test in mice. Columns represent the changes in the tail-flick latencies according to the following formula: ΔT(s) = post-drug latency – pre-drug latency and vertical bars represent SEM. (A) Effects of exercise in the hot-plate test. (B) Effects of pretreatment with naloxone (1 $mg.kg^{-1}$) on morphine-(3 $mg.kg^{-1}$) or exercise-induced antinociception. (C) Effects of pretreatment with caffeine (10 $mg.kg^{-1}$) on adenosine-(100 $mg.kg^{-1}$) or exercise-induced antinociception. (D) Effects of pretreatment with DPCPX (0.1 $mg.kg^{-1}$) or SCH58261 (0.5 $mg.kg^{-1}$) on adenosine-(100 $mg.kg^{-1}$) or exercise-induced antinociception. N = 8 – 10 animals per group. * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.01$ as compared to control, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 4: Effect of 45 days of voluntary exercise on the immunocontent of A_1 e A_{2A} adenosine-receptors (panels A e B, respectively) in spinal cord from mice. Representative Western blot lanes are shown. Histograms represent arbitrary units in sedentary controls and exercise mice through densitometric quantification of immunoblots. Columns represent mean and vertical bars represent SEM. N = 4 – 5. Sed = sedentary group; Ex = voluntary exercise group. * = $P < 0.05$ as compared to control (sedentary), unpaired Student-*t*-test.

Figure 5: Effects of 45 days of voluntary exercise on CSF levels of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine (GUO), inosine monophosphate (IMP), inosine (INO), hypoxanthine (HX), xanthine (XA), and uric acid (UA). The columns represent means (μM) and vertical

bars represent SEM. N = 10 animals per group. * = $P < 0.05$ as compared to control (sedentary), one-way ANOVA followed by Student-Newman-Keuls test.

Figure 6: Effects of 45 days of voluntary exercise on the concentration of purines exposed to the choroid plexus *ex vivo*. (A) Basal secretion of purines from choroid plexus. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine (GUO), inosine monophosphate (IMP), inosine (INO), hypoxanthine (HX), xanthine (XA), and uric acid (UA); (B), (C) and (D) represent medium concentration of purines after exposition to ATP (100 μM). (B) Time curve of ATP, ADP, AMP and adenosine; (C) Time curve of GTP, GDP, GMP and guanosine; (D) Time curve of INO, HX, XA and UA. The columns represent means (μM) and vertical bars represent SEM. N = 10 animals per group. * = $P < 0.05$ compared to control (sedentary), unpaired Student-*t*-test.

Figure 7: Effects of Immucillin-H (ImmH – 12.5 μM) and PNP (2 units per mouse) against i.pl. capsaicin-induced pain. Mice were treated with an i.c.v. injection of Immucillin-H, PNP or vehicle (DMSO 1%). After 5 min, animals received an i.pl. injection of capsaicin. N = 10 animals per group. * = $P < 0.01$ compared to vehicle (control), one-way ANOVA followed by Student-Newman-Keuls test.

Figure 8: Time curve of exercise against i.pl. capsaicin, tail-flick and hot-plate pain tests in mice. Animals were trained for 45 days, detrained for 30 days e retrained for 15 days. Tests

were performed in different times during training, detraining and retraining. (A) Capsaicin-induced pain, (B) tail-flick (C) hot-plate. (A) - columns represent mean time spent licking the injected hindpaw and vertical bars represent SEM. (B) and (C) - Columns represent the changes in the tail-flick latencies according to the following formula: ΔT(s) = post-drug latency – pre-drug latency and vertical bars represent SEM. N = 10 – 15 animals per group. * = $P < 0.05$ as compared to control (sedentary), unpaired Student-*t*-test.

Figure 9: Effects of exercise in the mice hole-board, spontaneous locomotor activity and rotarod tests. (A) head-dips; (B) latency to the first head-dip; (C) squares crossed; (D) rearings; (E) groomings; (F) defecation; (G) Number of crossings (activity test), (H) latency to fall (rotarod test). The columns represent mean and vertical bars represent SEM. N = 8 animals per group. * = $P < 0.05$ as compared to control (sedentary), unpaired Student-*t*-test.

Figure 10: Effects of exercise in memory, anxiety and locomotor parameters in mice. (A) Effects of exercise on locomotor activity; (B) Time exploring the center of a new environment; (C) spontaneous alternation performance in the Y-maze test; (D) Effects of exercise on the inhibitory avoidance task. N = 8 animals per group. * = $P < 0.05$ as compared to control (sedentary), unpaired Student-*t*-test.

Figure 1:

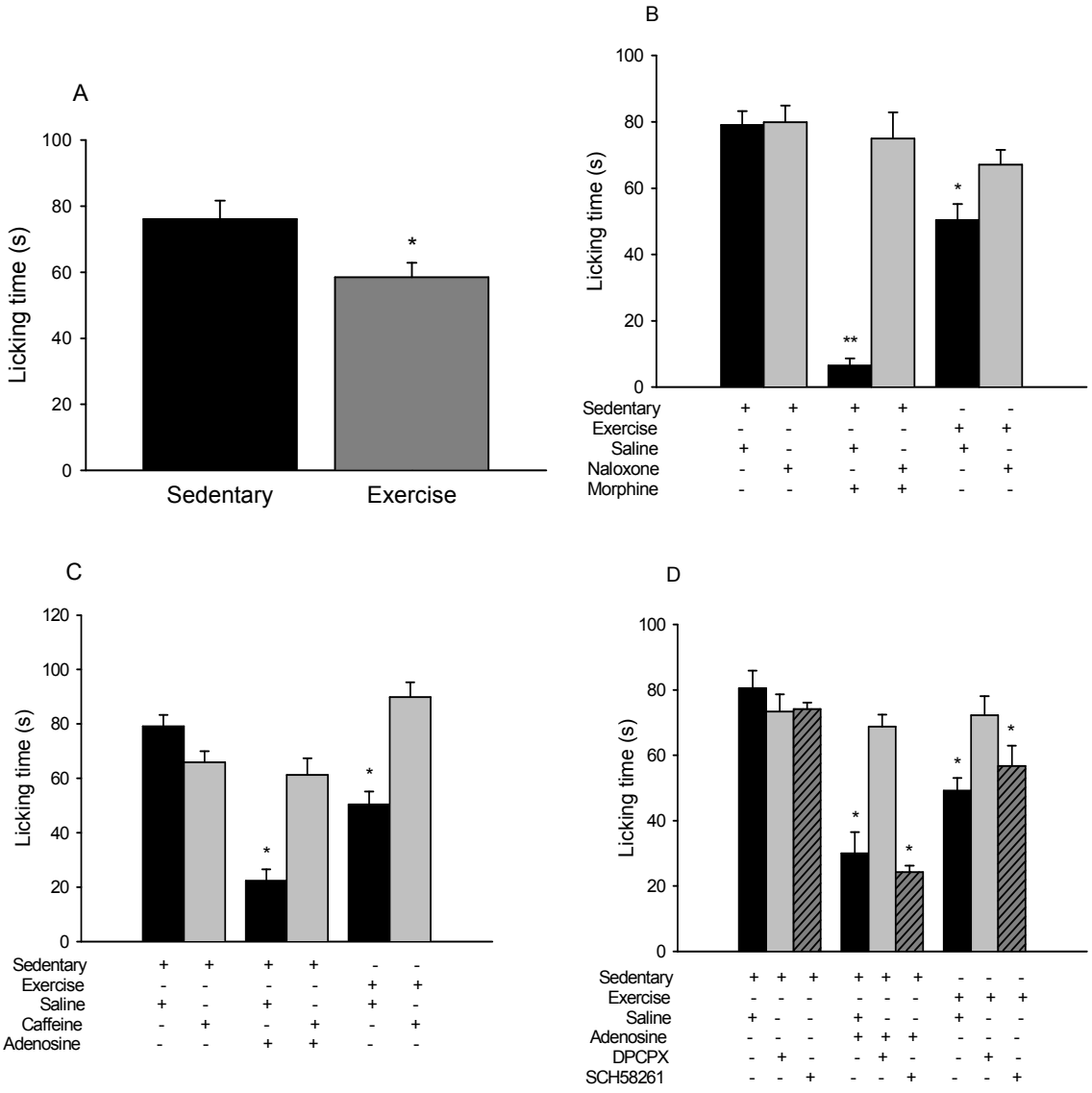

A
Licking time (s)
Sedentary
Exercise
B
Licking time (s)
Sedentary
Exercise
Saline
Naloxone
Morphine
C
Licking time (s)
Sedentary
Exercise
Saline
Caffeine
Adenosine
D
Licking time (s)
Sedentary
Exercise
Saline
Adenosine
DPCPX
SCH58261

Figure 2:

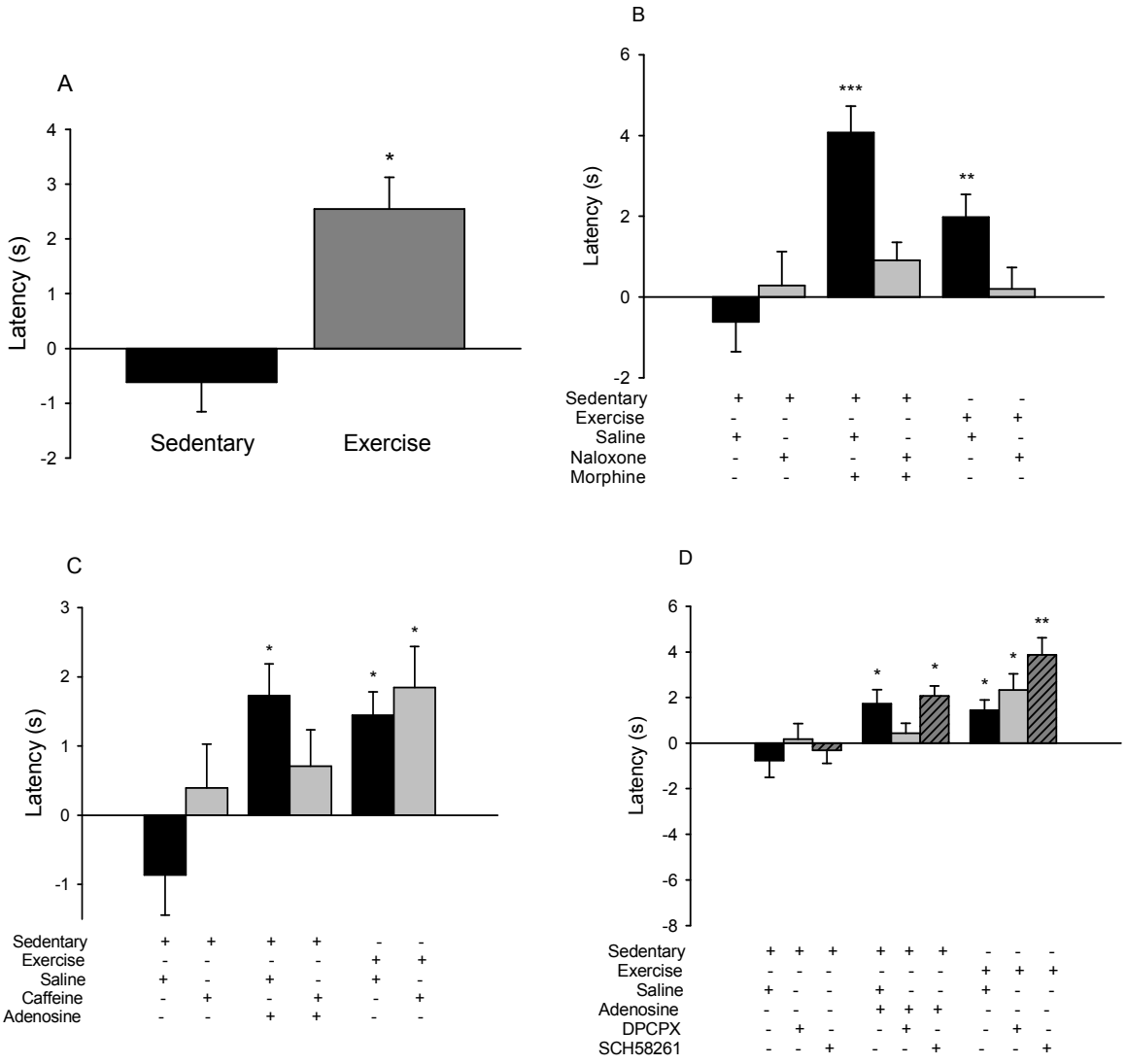

A
Latency (s)
Sedentary
Exercise
B
Latency (s)
Sedentary
Exercise
Saline
Naloxone
Morphine
C
Latency (s)
Sedentary
Exercise
Saline
Caffeine
Adenosine
D
Latency (s)
Sedentary
Exercise
Saline
Adenosine
DPCPX
SCH58261

Figure 3:

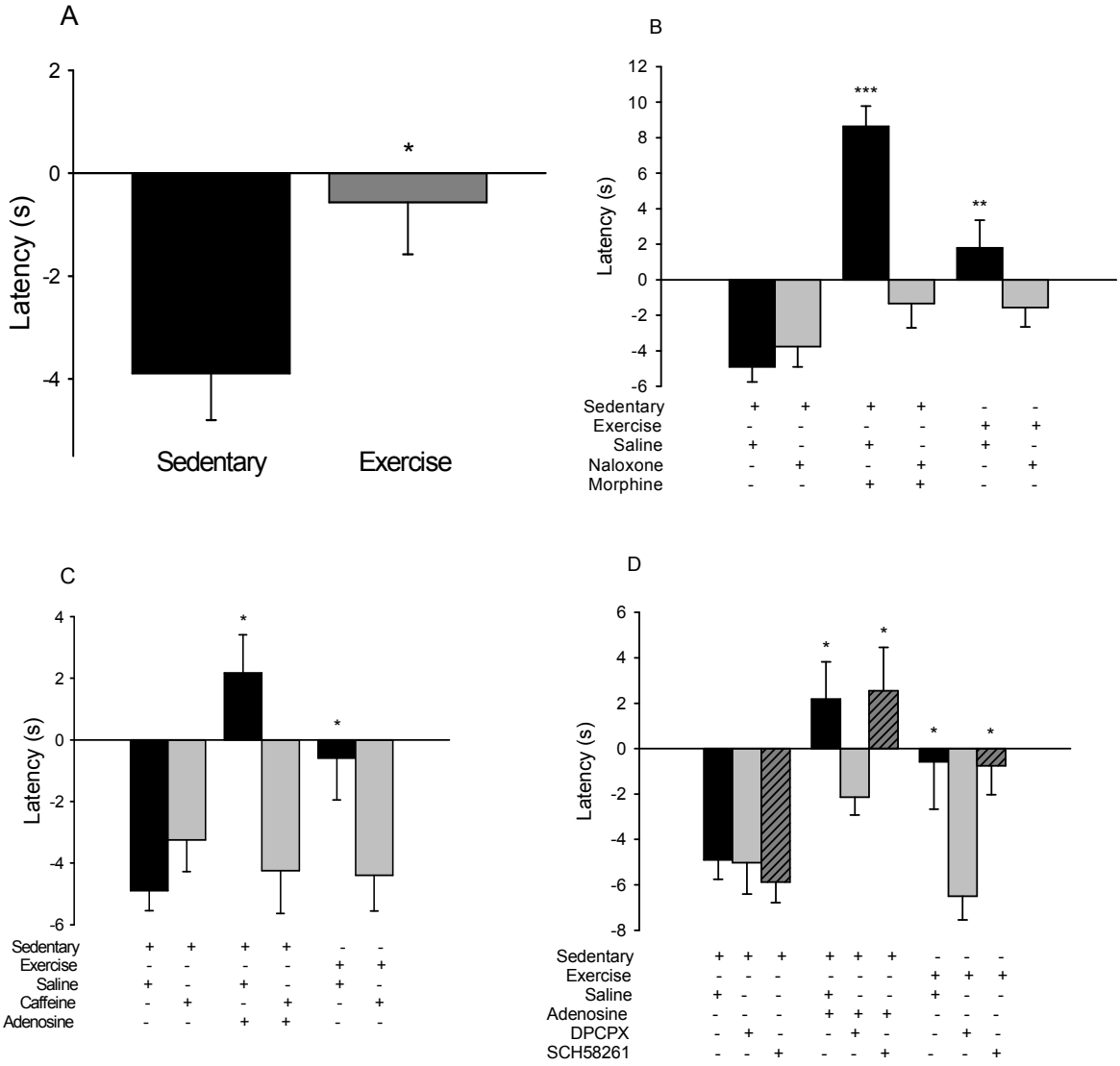

A
Latency (s)
Sedentary
Exercise
B
Latency (s)
Sedentary
Exercise
Saline
Naloxone
Morphine
C
Latency (s)
Sedentary
Exercise
Saline
Caffeine
Adenosine
D
Latency (s)
Sedentary
Exercise
Saline
Adenosine
DPCPX
SCH58261

Figure 4:

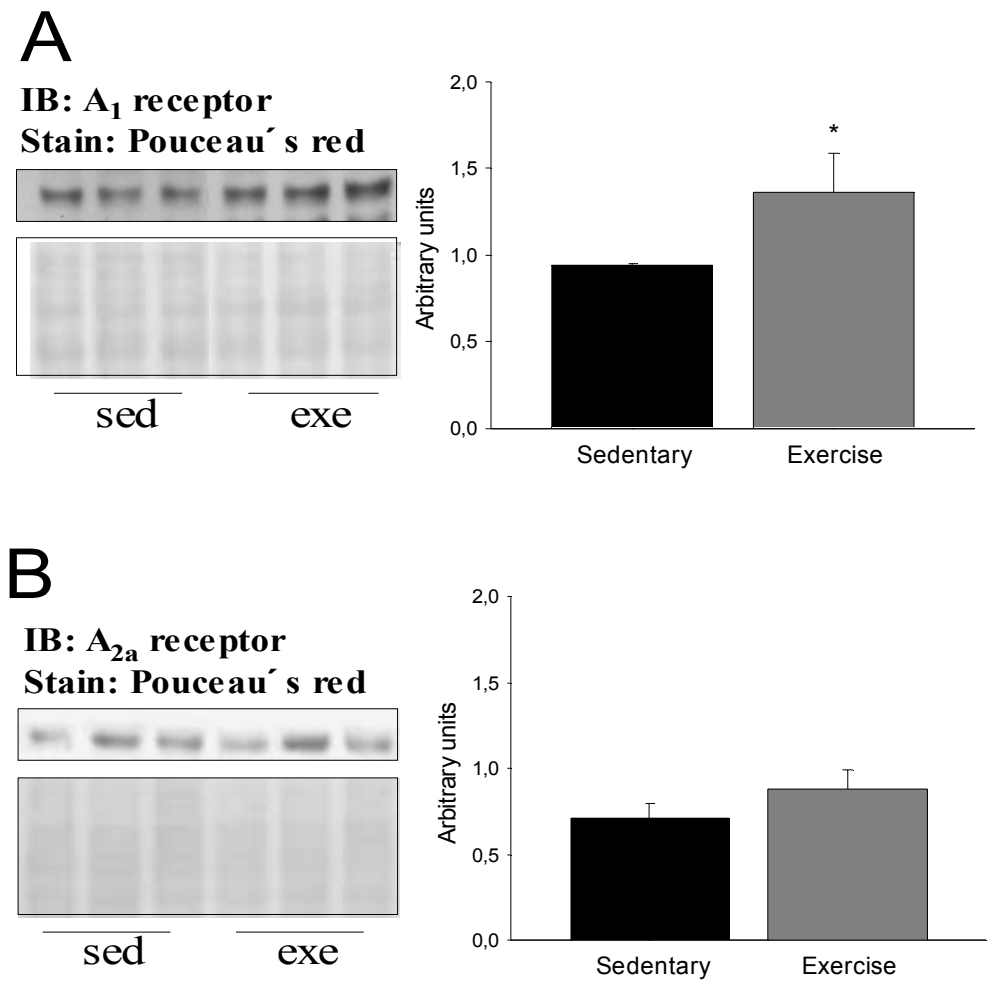

A
IB: A1 receptor
Stain: Pouceau´s red
sed
exe
2,0
1,5
1,0
0,5
0,0
Arbitrary units
*
Sedentary
Exercise
B
IB: A2a receptor
Stain: Pouceau´s red
sed
exe
2,0
1,5
1,0
0,5
0,0
Arbitrary units
Sedentary
Exercise

Figure 5:

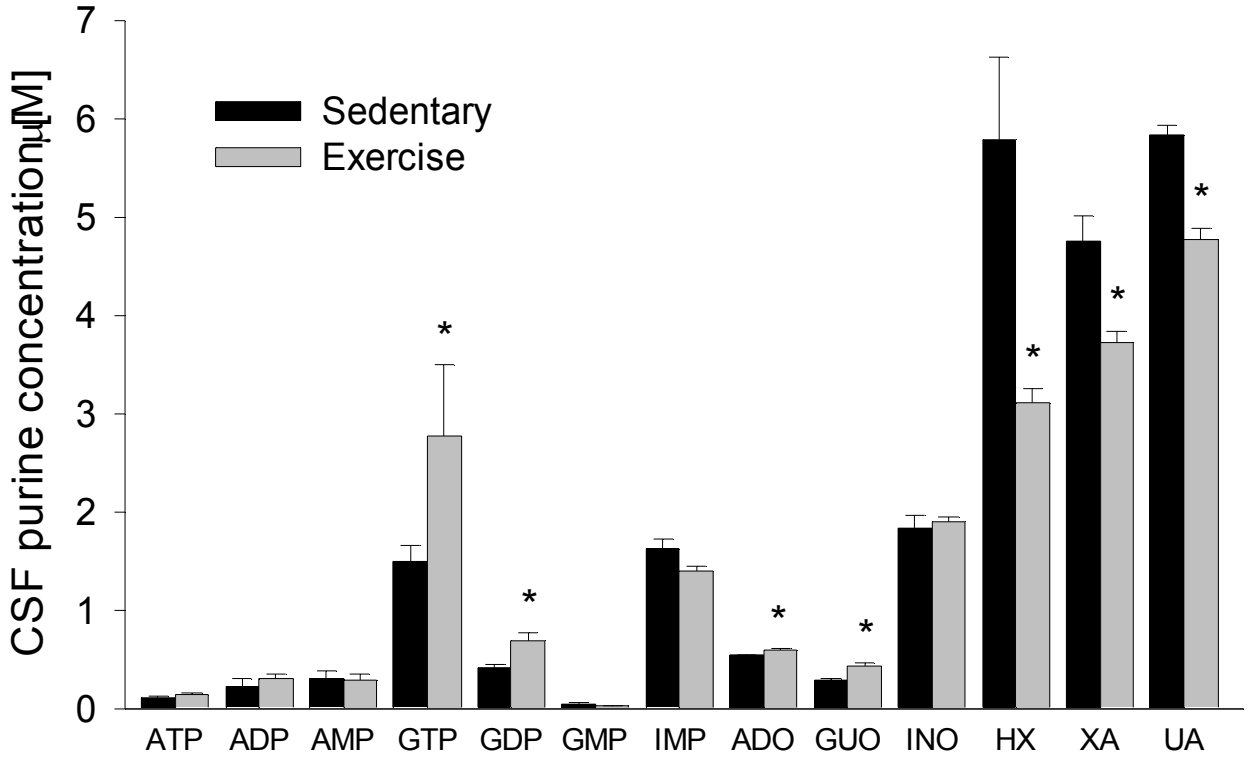

CSF purine concentration [μM]
Sedentary
Exercise
0
1
2
3
4
5
6
7
ATP
ADP
AMP
GTP
GDP
GMP
IMP
ADO
GUO
INO
HX
XA
UA
*

Figure 6:

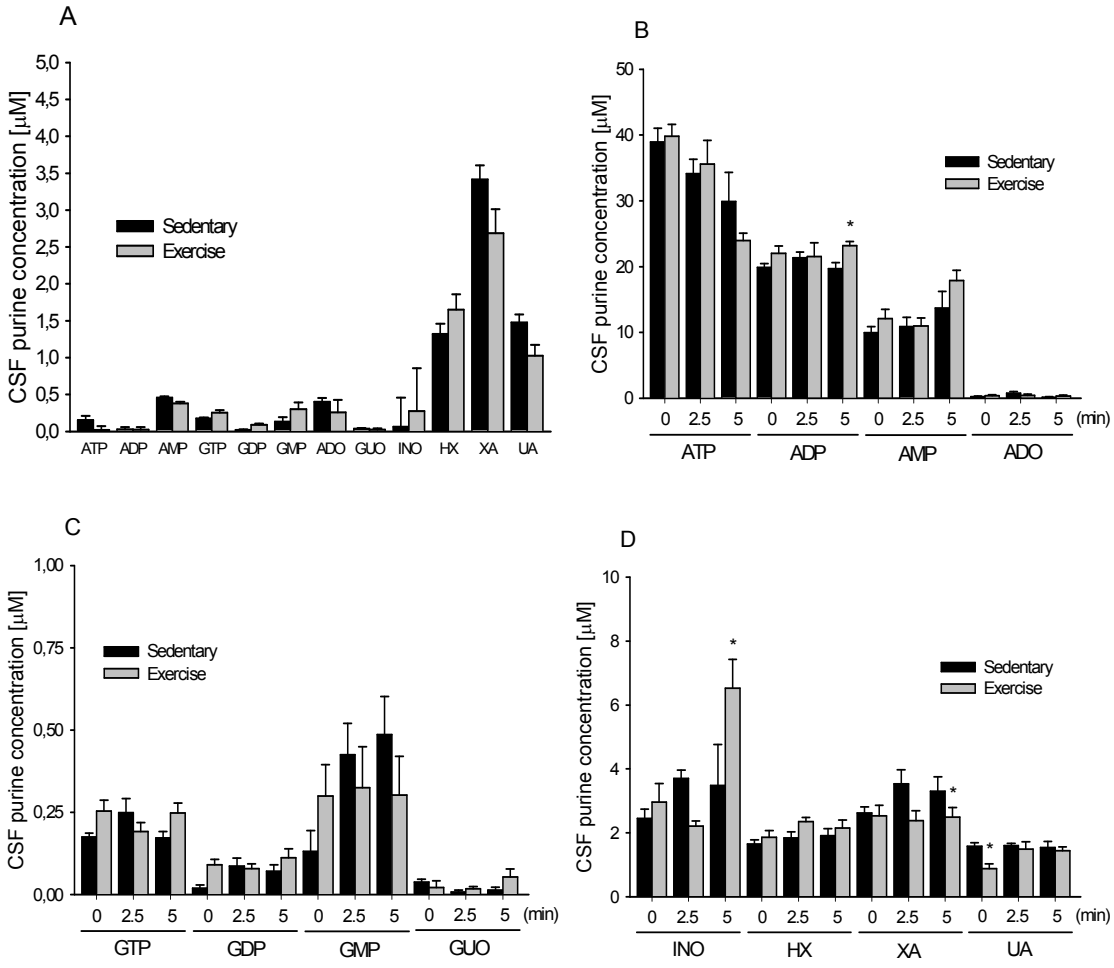
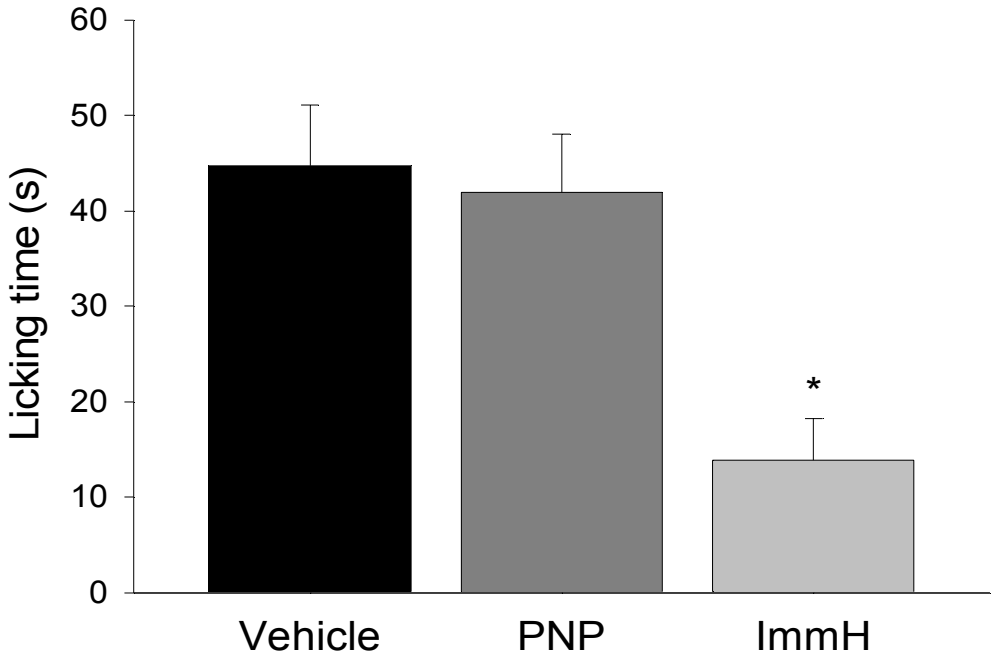

A
CSF purine concentration [µM]
Sedentary
Exercise
ATP ADP AMP GTP GDP GMP ADO GUO INO HX XA UA
B
CSF purine concentration [µM]
Sedentary
Exercise
0 2.5 5 (min)
ATP ADP AMP ADO
C
CSF purine concentration [µM]
Sedentary
Exercise
0 2.5 5 (min)
GTP GDP GMP GUO
D
CSF purine concentration [µM]
Sedentary
Exercise
0 2.5 5 (min)
INO HX XA UA

Figure 7:



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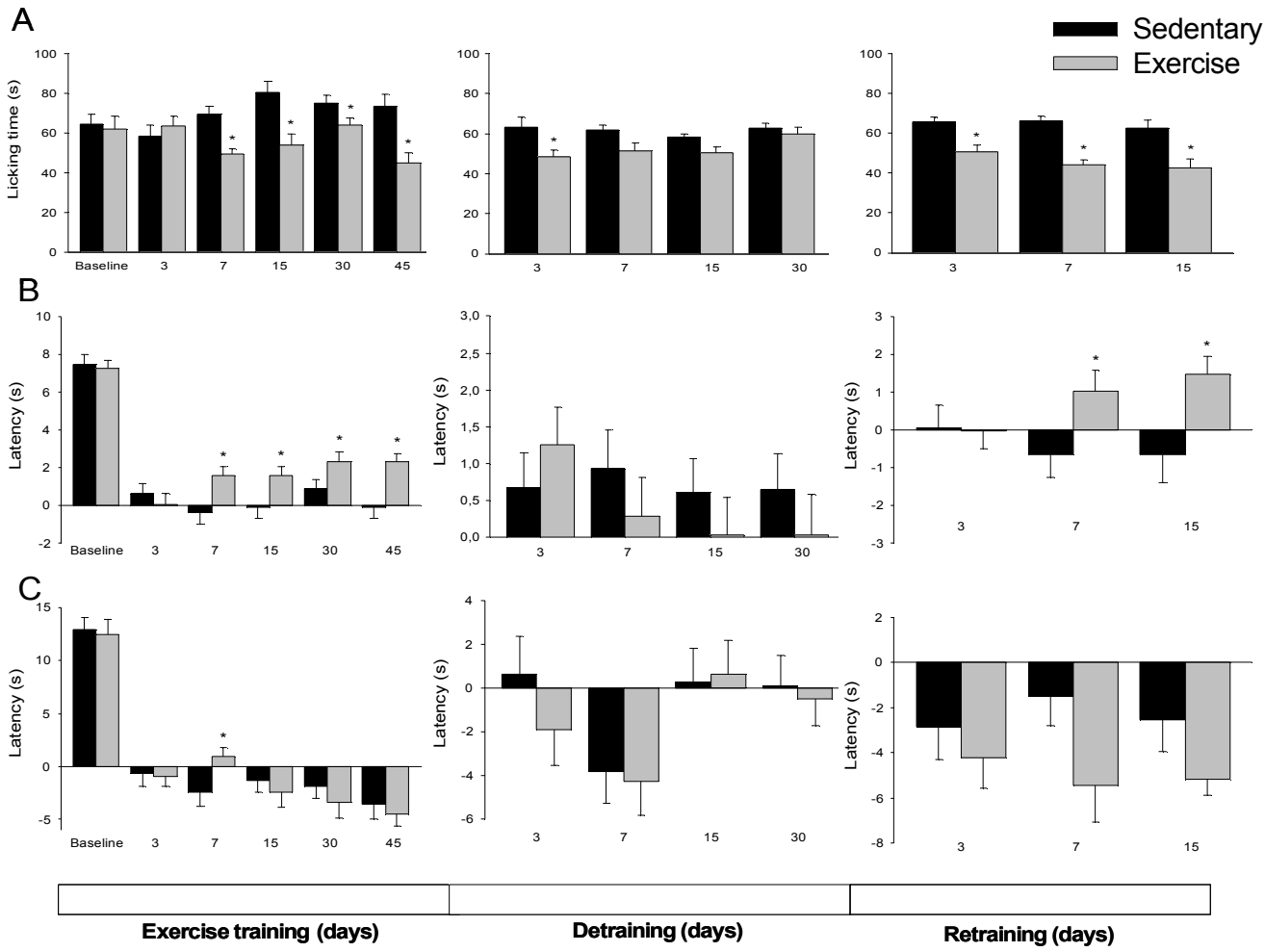

A
Sedentary
Exercise
Licking time (s)
Baseline
3
7
15
30
45
B
Latency (s)
C
Exercise training (days)
Detraining (days)
Retraining (days)

Figure 9:

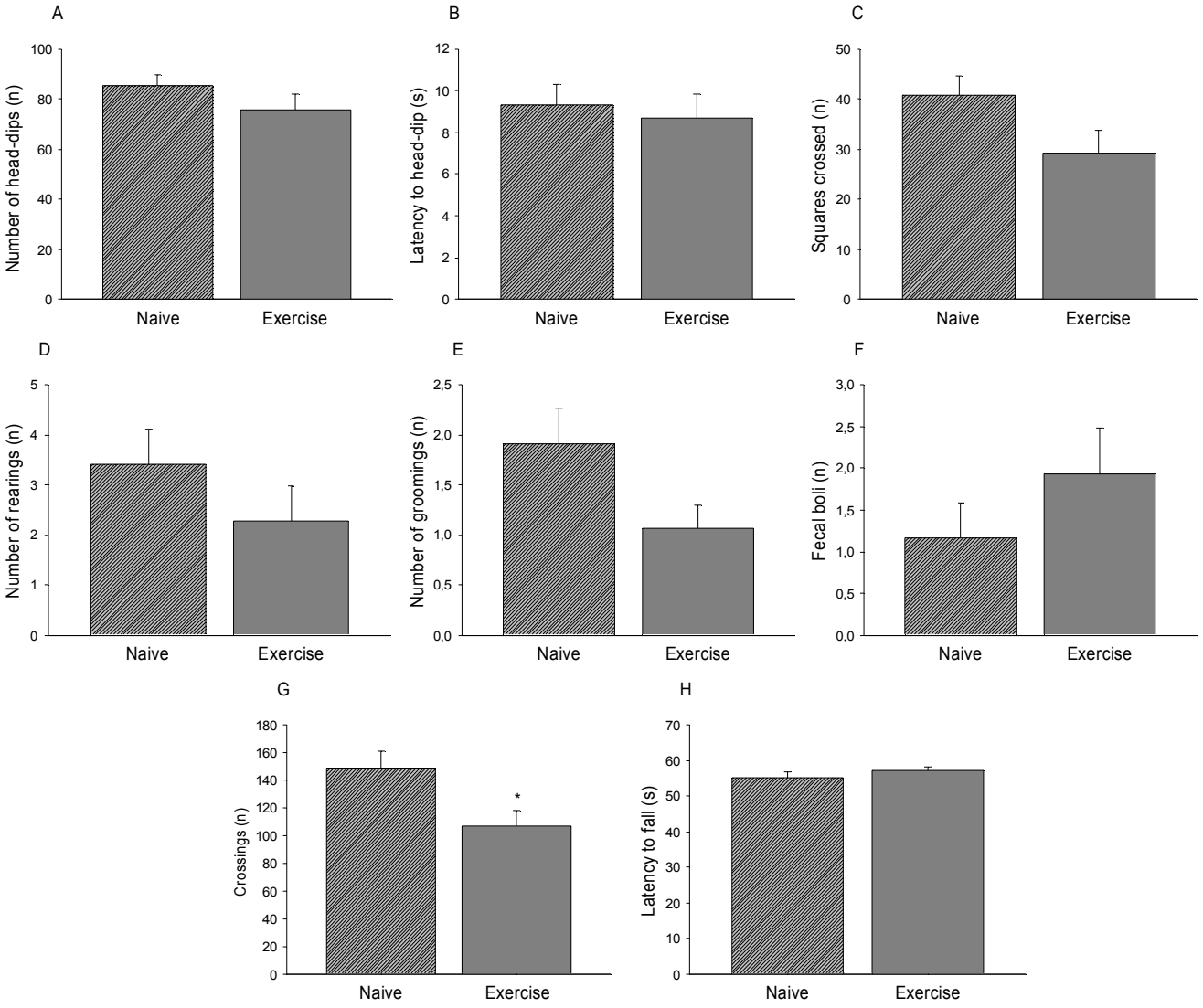
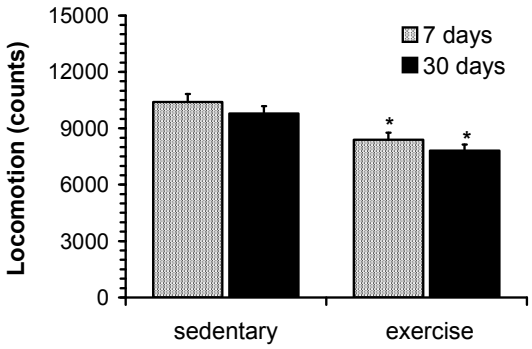
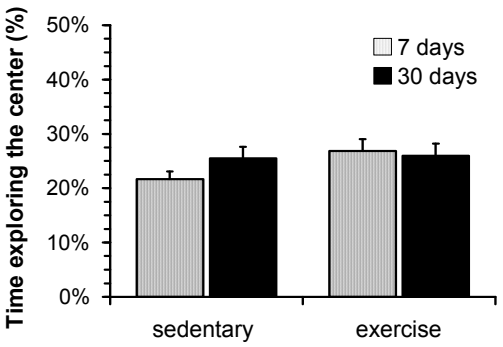

A
Number of head-dips (n)
100
80
60
40
20
0
Naive
Exercise
B
Latency to head-dip (s)
12
10
8
6
4
2
0
Naive
Exercise
C
Squares crossed (n)
50
40
30
20
10
0
Naive
Exercise
D
Number of rearings (n)
5
4
3
2
1
0
Naive
Exercise
E
Number of groomings (n)
2,5
2,0
1,5
1,0
0,5
0,0
Naive
Exercise
F
Fecal boli (n)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Naive
Exercise
G
Crossings (n)
180
160
140
120
100
80
60
40
20
0
*
Naive
Exercise
H
Latency to fall (s)
70
60
50
40
30
20
10
0
Naive
Exercise

Figure 10:

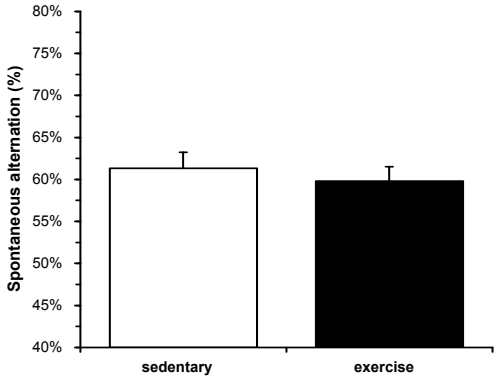
A



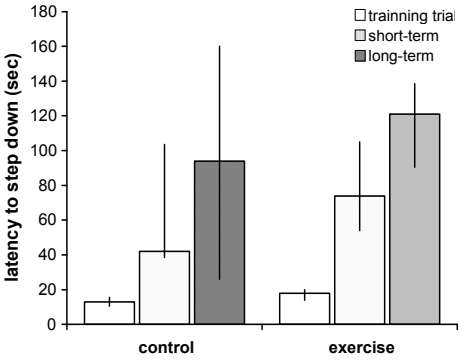
B



C



D



II.3. RESULTADOS EXPERIMENTAIS COM MODELOS HUMANOS

II.3.a. Changes in purines concentration in the cerebrospinal fluid of pregnant women experiencing pain during active labor

Artigo submetido ao periódico Anesthesia and Analgesia.

Changes in purines concentration in the cerebrospinal fluid of pregnant women experiencing pain during active labor.

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Short title: CSF purines in pregnant women.

Financial Support: FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" # 01.06.0842-00, CNPq, CAPES, FAPERGS, UFRGS.

Conflict of Interest: None.

Implications Statement:

This study demonstrated that the CSF levels of adenosine were significantly lower in pregnant women experiencing pain during active labor. These findings suggest that purines, in special the nucleoside adenosine, are associated with pregnancy and labor pain.

Abstract:

Background: Labor pain has been reported as a severe pain and can be considered as a model of acute visceral pain. It is well known that extracellular purines have an important role in pain signaling in the central nervous system (CNS). This study analyzes the relationship between extracellular purines and pain perception during active labor.

Methods: Cerebrospinal fluid (CSF) levels of the purines and their metabolites were compared between women at term pregnancy with labor pain (n = 49) and without labor pain (Caesarian section; n = 47). Control groups (healthy men and women without chronic or acute pain – n = 40 and 32, respectively) were also investigated.

Results: The CSF levels of adenosine were significantly lower in the labor pain group (P = 0.026) and negatively correlated with pain intensity measured by a visual analogue scale (r = -0.48, P = 0.0005). Interestingly, CSF levels of uric acid were significantly higher in healthy men as compared to women. Additionally, pregnant women showed increased CSF levels of ADP, GDP, adenosine and guanosine and reduced CSF levels of AMP, GTP, and uric acid as compared to non-pregnant women ($P < 0.05$).

Conclusions: These findings suggest that purines, in special the nucleoside adenosine, are associated with pregnancy and labor pain.

Word Count: 208.

Keywords: Purines; Adenosine; Acute pain; Cerebrospinal fluid; Labor pain; Visceral pain.

Introduction:

Labor pain has been reported as a severe pain and can be considered as a model of acute visceral pain[1]. It is well known that extracellular purines, mainly the nucleotide adenosine 5-triphosphate (ATP) and the nucleoside adenosine have an important role in pain signaling at both the periphery and in the central nervous system (CNS)[2-4]. ATP has been reported to stimulate sensory nerve endings causing pain and a marked increase in the discharge from sensory neurons[5]. Additionally, ATP is involved in the initiation of different types of nociception and pain[5]. Adenosine regulates pain transmission by actions at spinal, supraspinal and peripheral sites[3,6], and may play a particularly important role in inflammatory and neuropathic pain[7]. Endogenous adenosine can also be released from both the spinal cord and from peripheral tissues, and the regulation of such release by various pharmacological agents can alter pain processing through activation of adenosine A_1 receptors on neurons, and perhaps other receptors on adjacent structures[3].

Cerebrospinal fluid (CSF) surrounds the brain and spinal cord and fills up the ventricles as well as the subarachnoid space. It is a secretion product of various CNS structures and obtains its ultimate composition through exchange with the blood and the adjacent brain tissue. Changes in composition of CSF may accurately reflect changes in the brain and spinal cord tissue[8-11]. There are several studies investigating the role of potential biochemical markers of pain transmission in the CSF; these include excitatory amino acids, nitric oxide, substance P, and others[11-16]. The presence of purines and their metabolites in the CSF has been extensively demonstrated[8,9,17]. Previous studies have investigated the role of extracellular purines in the CSF in several conditions, such as traumatic brain injury and epilepsy[8,9]. Although extracellular purines play a pivotal role on pain transmission[3], to date, there are no studies correlating CSF purine levels and pain conditions.

Given the pivotal role of purines and their receptors in the mechanisms underlying pain transmission, this study was designed to evaluate the CSF levels of purines (adenine-based purines, guanine-based purines and their metabolites) in labor pain. Additionally, we also investigated the physiological concentration of purines in CSF of healthy subjects.

Subjects and methods:

Subjects: A prospective study of adult patients was performed in two tertiary care hospitals in Brazil. The protocol was evaluated and approved by the Institution's Research and Ethics Committee. Written informed consent was obtained from all patients. In this study, 168 healthy patients were enrolled with ages ranging from 18 to 65 years old. This study involved four groups of patients: i. pregnant women who were in established labor (labor pain group, n = 49); ii. pregnant women scheduled for elective caesarean delivery (no labor pain group, n = 47); iii. healthy women scheduled for elective gynecological surgery (n = 32); iv. healthy men scheduled for elective urologic or general surgery (n = 40). Exclusion criteria incorporated patients who presented chronic or acute pain (except for pregnant women in active labor), cognitive impairment or difficulty in understanding verbal commands, using analgesics and anticonvulsants during the preoperative period or preanesthetic medications before evaluation. None of the patients enrolled in the study received oral premedication or analgesics before evaluation.

All patients were asked to report any pain in two self-assessment instruments – a verbal scale (VPS) and a visual analogue scale (VAS). In the first one, the reported pain was graded from 1 to 4, according to intensity: (1) none, (2) slight, (3) moderate, or (4) severe[18]. The VAS[19] is widely used as a measure of self-reported pain assessment. The scale consists of a 100-mm line, that pictorially represents a continuum between two

extremes: no pain (score of 0) and extreme pain (score of 100). The use of the VAS was explained to all patients before evaluation. In order to stratify the data of VAS, cutoff points were established from percentiles 25, 50 and 75 of the measures, corresponding to 0.1, 1.0 and 6.95 cm. Based on these cutoff points, the clinical significance of the values and the methodological strategy used by Collins *et al.*[20], absence of pain corresponded to the range from zero to percentile 25 (0.1 cm); mild pain corresponded to the range from the first percentile to the median (0.2 to 1.0 cm); moderate pain corresponded to the range from the median to percentile 75 (1.0 to 6.95 cm), and intense pain corresponded to the scores above the second percentile (6.95 to 10 cm).

A power analysis was performed using CSF purine levels as the primary outcome measure. The sample size was calculated so that a mean difference of 10 % between groups would permit a type 1 error probability of $\alpha = 0.05$ (two-tailed test) with altered CSF purine levels in the labor pain group, and the null hypothesis would be retained with $\beta = 0.10$. This indicated that 15 patients would have to be included in each group (total = 60 patients). A higher number of patients were included to allow more adequate control of the potential confounding effect of variables.

Collection of CSF samples: Patients in the labor pain group received a combined spinal epidural (CSE) technique for pain relief while patients in the control groups received spinal anesthesia for their operative delivery or elective surgery. All the CSF was collected by experienced anesthesiologists. The first 0.5 ml of CSF aspirated was discarded to reduce contamination. The CSF samples were inspected visually and discarded if blood contamination was present. A total of 0.5 ml of CSF was collected from the patients after successful subarachnoid puncture before the intrathecal injection of anesthetics or

analgesics. All samples were centrifuged at 10,000 g in an Eppendorf centrifuge during 5 min to obtain cell-free supernatants, stored at -70 ^{o}C within 30 min of collection and not thawed until laboratory evaluations.

HPLC procedure: High-performance liquid chromatography (HPLC) was performed with CSF cell-free supernatants aliquots for determination of purines concentration, according to Domanski *et al.*[21] CSF concentrations of the following purines were determined: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine, inosine monophosphate (IMP), inosine, hypoxanthine, xanthine, and uric acid. Analyses were performed with Shimadzu Class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto injector valve with 50 μL loop, and an UV detector. Separations were achieved on a Supelco C18 250 mm x 4.6 mm, 5 μm particle size column. The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24 ^{o}C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 10 μL were injected into the injection valve loop. Absorbance was read at 254 nm. CSF concentrations of purines are expressed as mean ± SEM in μM.

Statistical analysis: Data were stored in EPI-INFO software version 6.0 and analyzed by Statistical Package for the Social Sciences (SPSS) for Windows version 15.0

(registered trademark). Data are expressed as mean ± standard deviation (SD). Data were submitted to Kolmogorov-Smirnov, Levene and Bartlett tests for normality evaluation. Statistical analysis between groups was performed using student *t* test or one-way ANOVA followed by the *post-hoc* Tukey-Kramer multiple comparisons test when applicable for parametric data. Non-parametric data were analyzed by using Mann-Whitney *U* test or Kruskal-Wallis test followed by Dunn test as a *post hoc* when appropriate. Correlations are presented as linear Pearson's or nonparametric Spearman's coefficient. $P < 0.05$ was considered for statistically significant differences.

Results:

The average age of the pregnant women assessed was 30.0 ± 1.2 and 28.5 ± 0.9 years old for patients with and without pain, respectively ($P = 0.31$). However, there was a significant difference between control groups and pregnant women, since the average age of the control women and men was 43.9 ± 3.6 and 46.9 ± 2.2 years old ($P < 0.001$). There was no other difference on demographic characteristics between groups (data not shown) and no patients presented significant diseases preoperatively. Types of surgery performed during this study included: abdominal or vaginal hysterectomy, inguinal or umbilical herniorrhaphy, excision of inguinal lymph nodes, and open or trans-urethral prostatectomy.

As shown in Fig. 1, a significant difference was found between men and women regarding CSF uric acid levels (men = 28.2 ± 1.8 μM vs. women = 18.6 ± 4.0 μM, $P = 0.014$). No other differences were found between control groups regarding CSF purine levels (Fig. 1A) or demographic characteristics (data not shown). Fig. 1B shows that pregnant women present increased CSF levels of ADP, GDP, adenosine and guanosine and

reduced CSF levels of AMP, GTP, and uric acid as compared to non-pregnant women ($P < 0.05$). Since there were significant differences regarding age between control and pregnant groups, a subgroup analysis was performed in order to control for age. Importantly, differences in CSF levels of purines were maintained (GTP – control = 1.99 ± 0.14 μM vs. pregnant women = 1.25 ± 0.15 μM, $P = 0.03$; GDP – control = 0.21 ± 0.05 μM vs. pregnant women = 0.80 ± 0.13 μM, $P = 0.02$; ADP – control = 0.20 ± 4 μM vs. pregnant women = 0.37 ± 0.04 μM, $P = 0.03$; AMP – control = 1.5 ± 0.20 μM vs. pregnant women = 0.30 ± 0.08 μM, $P = 0.0004$; guanosine – control = 0.015 ± 0.005 μM vs. pregnant women = 0.35 ± 0.03 μM, $P = 0.0007$; adenosine – control = 0.09 ± 0.03 μM vs. pregnant women = 0.49 ± 0.08 μM, $P = 0.002$; uric acid – control = 22.6 ± 2.2 μM vs. pregnant women = 11.64 ± 1.45 μM, $P = 0.0001$) .

As depicted in Fig. 2, an active labor with pain produced a significant decrease in the CSF levels of adenosine [pregnant women without pain (control) = 0.58 ± 0.09 μM vs. labor pain patients = 0.33 ± 0.06 μM, $P = 0.02$]. Notably, labor pain did not affect CSF levels of ATP, ADP, AMP, GTP, GDP, GMP, IMP, guanosine, inosine, xanthine, hypoxanthine, and uric acid (Fig. 2).

As shown in Fig. 3, the CSF levels of adenosine were significantly correlated with labor pain intensity (r = -0.48, $P = 0.0005$ for VAS; r = -0.27, $P = 0.06$ for VPS), but not with duration of active labor (r = -0.12, $P = 0.39$).

Discussion:

In this study, we demonstrated that pregnant women present increased CSF levels of ADP, GDP, adenosine and guanosine and reduced CSF levels of AMP, GTP, and uric acid

as compared to non-pregnant women. Additionally, the CSF uric acid levels were higher in men than women. Active labor associated to pain produced a significant decrease in the CSF levels of adenosine and which was significantly correlated with pain intensity, but not with labor pain duration.

The population sample studied was homogenous since the preanesthetic characteristics of patients, except for age, were quite similar and only healthy patients were enrolled. A subgroup analysis indicated that similar CSF purine concentration changes were found between groups when controlling for age, reducing the risk of selection bias.

There is general acceptance of widespread physiological and pathological roles of purines acting via extracellular receptors and a number of recent studies implicate the purinergic system as essential in nociceptive pathways[3,22,23]. Adenine-based purines have been considered important targets for the development of new drugs for treating pain, since the nucleoside adenosine and its analogs induce antinociceptive effects following both systemic and central administration[3]. Adenosine can alter pain transmission by actions on both nociceptive afferent and transmission neurons, and these actions are mediated primarily by adenosine A_1 receptors[3]. Additional actions on inflammatory cells at peripheral sites and on glia in the CNS[24] mediated by adenosine A_{2A}, A_{2B} and A_3 receptors also occur, and these potentially can produce indirect effects on pain transmission.

In a recent study, we demonstrated that rats submitted to a partial sciatic nerve ligation which caused thermal hyperalgesia produced a significant increase in CSF adenosine levels as compared to control animals [personal communication]. However, in the present study, a negative correlation between CSF adenosine levels and acute pain was found. Notably, no significant modulation of CSF adenine or guanine nucleotides levels was observed in humans displaying an acute visceral pain. We may argue that changes in

purine metabolism in the CSF may be partially compensated by homeostasis, since no significant effects in CSF levels of ATP, guanine-based purines and purine metabolites (oxypurines) were produced by acute labor pain.

The physiopathological implications of a decrease in the CSF levels of adenosine are difficult to interpret. It is not possible to determine whether the changes in the CSF adenosine content were responsible for labor-related acute pain or it, at least partially, caused the changes. However, considering previous data regarding purine metabolism and pain mechanisms, we suggest that the decrease in CSF adenosine levels were probably produced by nociceptive stimulus modulation rather than an underlying mechanism of action. There is an overwhelming acceptance that ATP plays a crucial role in pain processes and large amounts of ATP are released from vascular endothelial cells after noxious stimuli[5]. ATP acts on its specific receptors resulting in glial activation and release of several algogenic and inflammatory mediators, including reactive oxygen species, nitric oxide, arachidonic acid, and cytokines[5,25-27]. These processes are largely involved in the mechanisms underlying acute and chronic pain generation[28]. Considering the pivotal role of ATP as a pro-nociceptive substance in the CNS and adenosine as an endogenous antinociceptive purine[5,25], our results may reflect breakdown of ATP and adenosine from CNS cells following sustained noxious (visceral) stimulation. It is tempting to propose that our findings are related to the breakdown of ATP to AMP by ecto-nucleotide-diphosphohydrolases, AMP to adenosine by ecto-5'-nucleotidases, and consequently to oxypurines by purine nucleoside phosphorylases (PNP). Therefore, decreased CSF levels of adenosine may be a biochemical marker of adenine-based purine breakdown following sustained noxious stimuli.

Since nucleosides guanosine and adenosine closely interact in modulating several functions of the CNS[29], it is tempting to investigate the potential role of guanosine and other guanine-based purines (GBPs) on pain transmission and nociception. There is increasing evidence that guanosine acts as an intercellular signaling molecule. It is released from cells and has several diverse effects *in vivo* and *in vitro*, particularly trophic effects and glutamatergic modulation[30]. Recently, we demonstrated that guanosine produced consistent and dose-dependent antinociceptive effects in several pain models in mice[30-33]. Although guanosine and its analogs may be of potential interest in the development of new drugs for managing pain, we did not find any correlation between endogenous levels of guanine-based purines and labor pain.

In summary, this study provides additional evidence on the role of extracellular purines in the CNS and suggests that the breakdown of adenosine may play a role in acute visceral pain related to active labor. These findings suggest that purines, in special the nucleoside adenosine, are associated with the mechanisms underlying acute visceral pain transmission during active labor.

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Figure legends:

Figure 1: Panel A represents CSF levels of purines in men and women without pain (control group); Panel B represents the comparison between pregnant and non pregnant women without pain regarding CSF purines levels; ADO = adenosine, GUO = guanosine, INO = inosine, HX = hypoxanthine, XA = xanthine, and UA = uric acid. The columns represent mean (μM) and vertical bars represent SEM. N = 40 (men), 32 (non pregnant women), and 47 (pregnant women); * = $P < 0.05$, ** = $P < 0.01$, and # = $P < 0.001$, Student *t* test.

Figure 2: CSF levels of purines in pregnant women without pain (control) and patients displaying acute visceral pain (active labor pain group). ADO = adenosine, GUO = guanosine, INO = inosine, HX = hypoxanthine, XA = xanthine, and UA = uric acid. The columns represent mean (μM) and vertical bars represent SEM. N = 47 (control) and 49 (labor pain); * = $P < 0.05$, Student *t* test.

Figure 3: Correlation between CSF levels of adenosine and labor duration (panel A), visual analogue scale (VAS) scores (panel B), or verbal pain scale (VPS) scores (panel C). (A) Pearson's coefficient $r = -0.12$; $P = 0.39$; (B) Pearson's coefficient $r = -0.48$; $P = 0.0005$; (C) Spearman's coefficient $r = -0.27$; $P = 0.06$).

Figure 1:

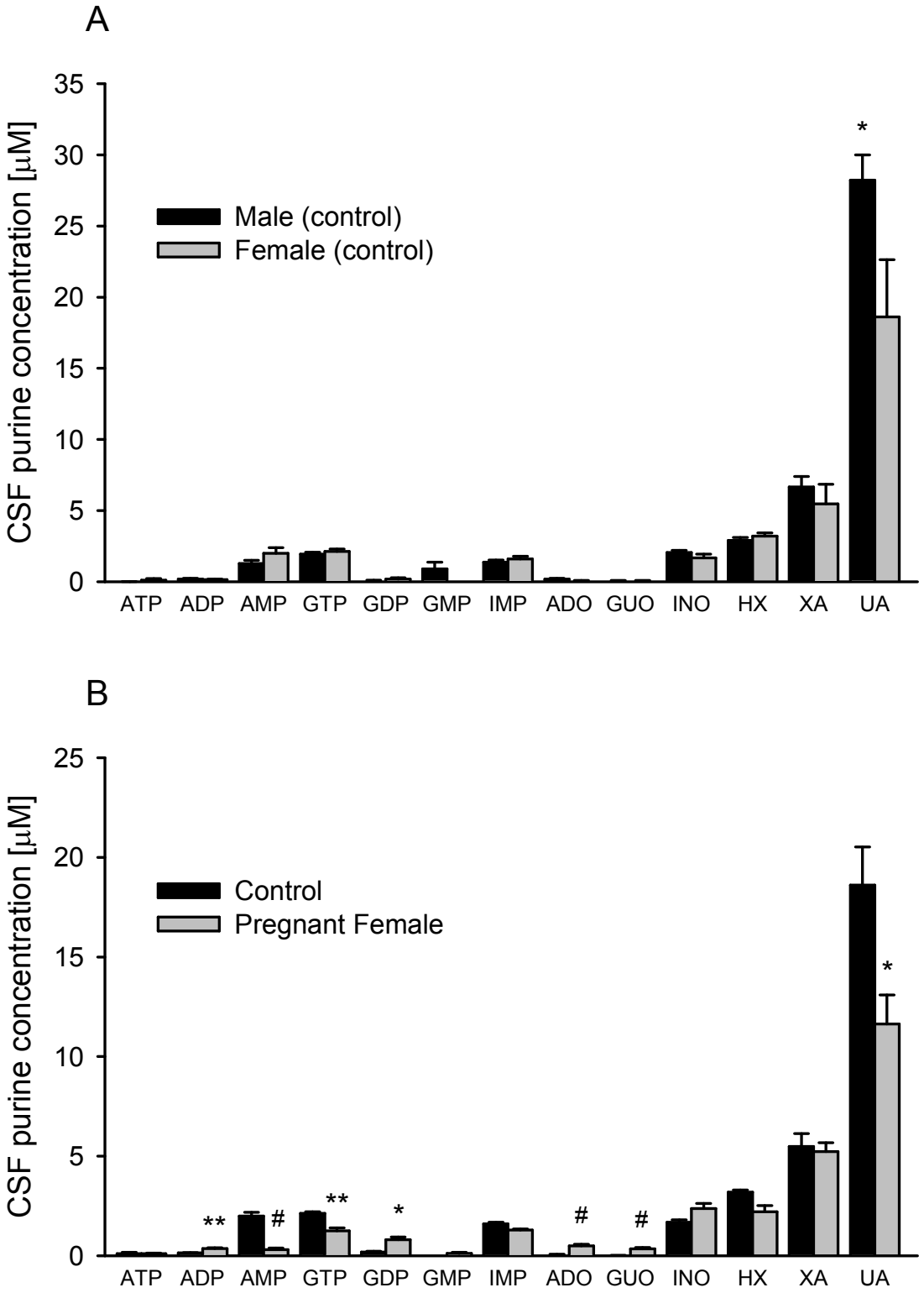

A
CSF purine concentration [μM]
35
30
25
20
15
10
5
0
Male (control)
Female (control)
*
ATP ADP AMP GTP GDP GMP IMP ADO GUO INO HX XA UA
B
CSF purine concentration [μM]
25
20
15
10
5
0
Control
Pregnant Female
**
#
**
*
#
#
*
ATP ADP AMP GTP GDP GMP IMP ADO GUO INO HX XA UA

Figure 2:

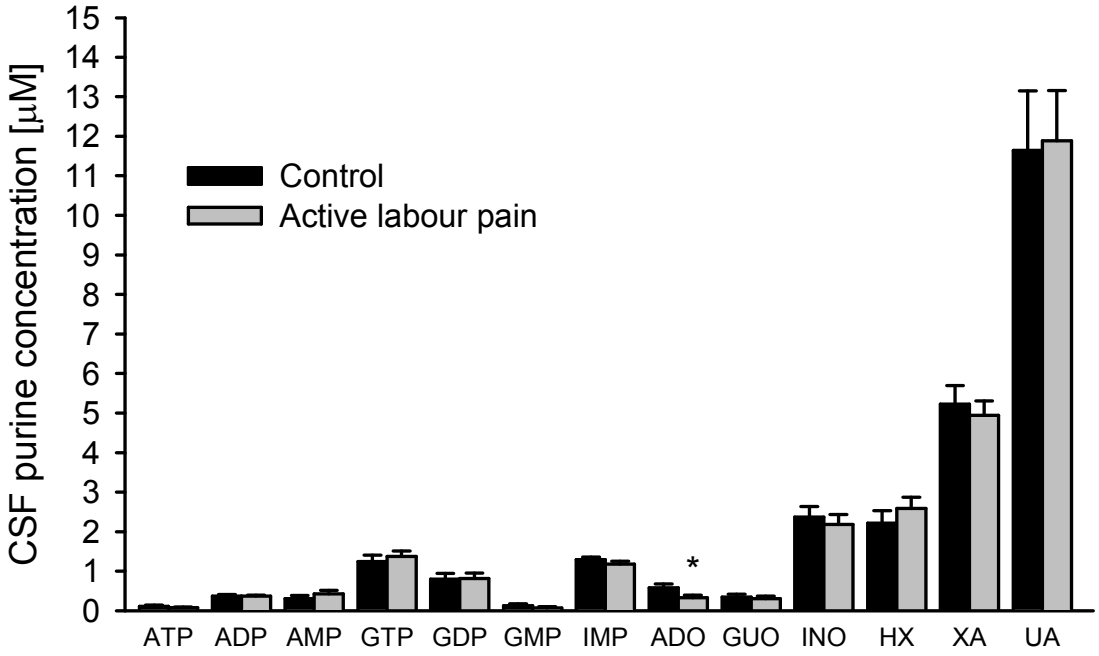
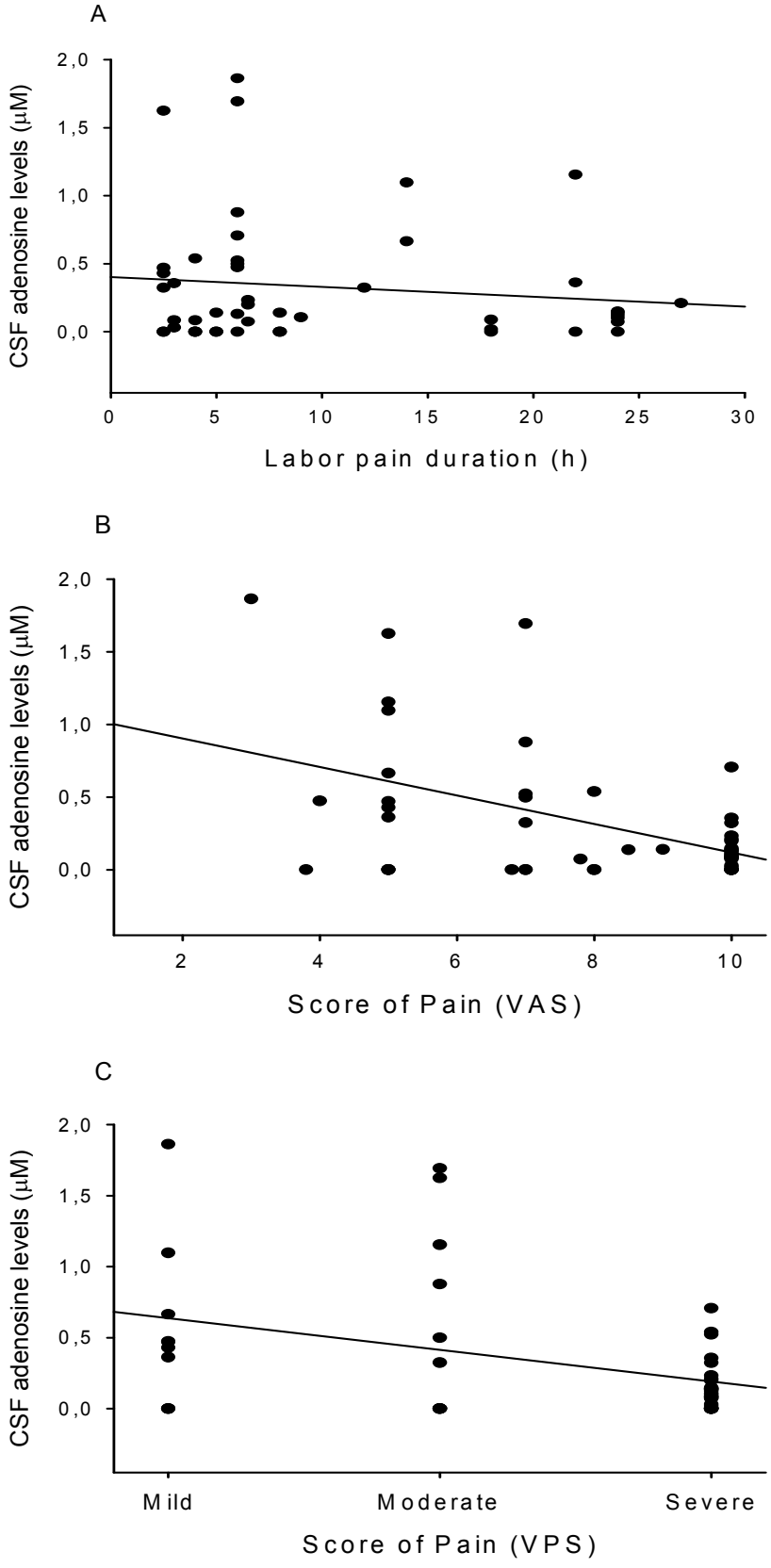

CSF purine concentration [μM]
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Control
Active labour pain
*
ATP
ADP
AMP
GTP
GDP
GMP
IMP
ADO
GUO
INO
HX
XA
UA

Figure 3:


A
CSF adenosine levels (µM)
2,0
1,5
1,0
0,5
0,0
0
5
10
15
20
25
30
Labor pain duration (h)
B
CSF adenosine levels (µM)
2,0
1,5
1,0
0,5
0,0
2
4
6
8
10
Score of Pain (VAS)
C
CSF adenosine levels (µM)
2,0
1,5
1,0
0,5
0,0
Mild
Moderate
Severe
Score of Pain (VPS)

II.3.b. Changes in purines concentration in the cerebrospinal fluid of patients experiencing chronic pain

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Changes in purines concentration in the cerebrospinal fluid of patients experiencing chronic pain.

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Abstract:

Chronic pain usually outlasts tissue or nerve damage and is generally characterized by its spontaneous nature and by the presence of hyperalgesia and/or allodynia. It is well known that extracellular purines have an important role in pain signaling in the central nervous system (CNS). This study analyzes the relationship between extracellular purines and pain perception during chronic pain conditions. Cerebrospinal fluid (CSF) levels of the purines and their metabolites were compared between patients displaying chronic pain of vascular or orthopedic origin (n = 16) and patients without pain (n = 52). Groups displaying chronic and/or acute pain (n = 20 and 16, respectively) were also investigated. The CSF levels of IMP, inosine, guanosine and uric acid were significantly higher in the chronic pain group ($P < 0.05$) and significantly correlated with pain intensity measured by a visual analogue scale. Interestingly, patients presenting chronic and acute pain presented similar changes in the CSF purines concentration as compared to control group ($P < 0.05$). However, when comparing the acute pain group, this change was only significant in CSF inosine and uric acid levels ($P < 0.05$). Chronic and/or acute pain did not affect the CSF levels of ATP, ADP, AMP, GTP, GDP, GMP, adenosine, xanthine, hypoxanthine, and uric acid. These findings suggest that purines, in special inosine and guanosine, are associated with the mechanisms underlying pain transmission.

Keywords: Purines; Guanosine; Inosine; Chronic pain; Cerebrospinal fluid; Somatic pain.

Introduction:

Pain is one of the most prevalent problems in our society and has high social costs due to significant impairment or permanent disabling of millions of people. Chronic or persistent pain which accompanies, and can outlast, tissue or nerve damage is generally characterized by its spontaneous nature and by the presence of hyperalgesia and/or allodynia [Millan, 1999; Julius and Basbaum, 2001]. Therefore, prolonged or chronic pain is regarded as fulfilling no physiological purpose. Recent years have seen a progressive unraveling of the neuroanatomical circuits and cellular mechanisms underlying the induction and maintenance of pain. In addition to the traditional inflammatory mediators, several new roles for a variety of substances were discovered, including the nitric oxide, protons, cytokines, excitatory amino acids, neuropeptides, neurotrophins and purines [Millan, 1999]. Further, in the central nervous system (CNS), non-neuronal glial cells have been shown to play a pivotal modulatory role in the response to inflammation and injury, and in processes modifying nociception [Fields and Burnstock, 2006; Scholz and Woolf, 2007].

In this context, the purinergic system, mainly represented by the nucleotide adenosine 5-triphosphate (ATP) and the nucleoside adenosine, have an important role in pain signaling at both the periphery and in the CNS [Sawynok, 1998; Sawynok and Liu, 2003; Inoue et al., 2005]. ATP has been reported to stimulate sensory nerve endings causing pain and a marked increase in the discharge from sensory neurons and is involved in the initiation of different types of nociception and pain [Burnstock, 2007]. Adenosine regulates pain transmission by actions at spinal, supraspinal and peripheral sites [Sawynok, 1998, 1999], and may play a particularly important role in inflammatory and neuropathic pain [Dickenson et al., 2000]. Endogenous adenosine can also be released from both the

spinal cord and from peripheral tissues, and the regulation of such release by various pharmacological agents can alter pain processing through activation of adenosine A_1 receptors on neurons, and perhaps other receptors on adjacent structures [Sawynok and Liu, 2003].

Cerebrospinal fluid (CSF) surrounds the brain and spinal cord and fills up the ventricles as well as the subarachnoid space. It is a secretion product of various CNS structures and obtains its ultimate composition through exchange with the blood and the adjacent brain tissue. Changes in composition of CSF may accurately reflect changes in the brain and spinal cord tissue [Castro-Gago et al., 1995; Rodriguez-Núñes et al., 2000; Palmer et al., 2004; Sethuraman et al., 2006]. There are several studies investigating the role of potential biochemical markers of pain transmission in the cerebrospinal fluid (CSF); these include excitatory amino acids, nitric oxide, substance P, and others [Olofsson et al., 1997; Hsu et al., 2001; Larson et al., 2000; Sethuraman et al., 2006; Alexander et al., 2007; Sarchielli et al., 2007]. The presence of purines and their metabolites in the human CSF has been extensively demonstrated elsewhere [Castro-Gago et al., 1995; Regner et al., 1997; Rodriguez-Nunes et al., 2000]. Previous studies have investigated the role of extracellular purines in the CSF in several conditions, such as traumatic brain injury and epilepsy [Castro-Gago et al., 1995; Rodriguez-Nunes et al., 2000]. Although extracellular purines play a pivotal role on pain transmission [Sawynok and Liu, 2003], to date, there are no studies correlating CSF purine levels and pain conditions.

Given the pivotal role of purines and their receptors in the mechanisms underlying pain transmission, this study was designed to evaluate the roles of CSF purines (adenine-, guanine-based purines and their metabolites) in chronic pain. Additionally, we also

investigated the physiological roles of purines in CSF of subjects who were experiencing acute pain conditions.

Subjects and methods:

Subjects: A prospective study of adult patients was performed in two tertiary care hospitals in Brazil. The protocol was evaluated and approved by our Institutional Research and Ethics Committee. Written informed consent was obtained from all patients. In this study, 104 patients, ASA I – III, were enrolled with ages ranging from 18 to 70 years old. This study involved four groups of patients: i. patients who were experiencing pain during the last 6 months (chronic pain group, n = 16); ii. patients without pain scheduled to elective or emergent surgery (control group, n = 52); iii. patients who were experiencing chronic and acute pain immediately before the procedure (chronic/acute pain group, n = 20); iv. patients who were experiencing acute pain immediately before the procedure and without history of chronic pain (acute pain group, n = 16). The pain etiology of all patients was exclusively vascular or orthopedic. Exclusion criteria were patients who presented cognitive impairment or difficulty in understanding verbal commands, or presented with unstable cardiovascular or pulmonary diseases, or received preanesthetic medications before evaluation. None of the patients enrolled in the study received oral premedication or analgesics immediately before evaluation and CSF collection. Demographic characteristics were also collected (gender, age, weight, comorbidities and preoperative medications).

All patients were asked to report any pain in two self-assessment instruments – a verbal scale (VPS) and a visual analogue scale (VAS). In the first one, the reported pain was graded from 1 to 4, according to intensity: (1) none, (2) slight, (3) moderate, or (4) severe [Schmidt et al., 2007b]. The VAS [Scott and Huskisson, 1976] is widely used as a

measure of self-reported pain assessment. The scale consists of a 100-mm line, that pictorially represents a continuum between two extremes: no pain (score of 0) and extreme pain (score of 100). The use of the VAS was explained to all patients before evaluation. In order to stratify the data of VAS, cutoff points were established from percentiles 25, 50 and 75 of the measures, corresponding to 0.1, 1.0 and 5.0 cm. Based on these cutoff points, the clinical significance of the values and the methodological strategy used by Collins *et al.* [1997], absence of pain corresponded to the range from zero to percentile 25 (0.1 cm); mild pain corresponded to the range from the first percentile to the median (0.2 to 1.0 cm); moderate pain corresponded to the range from the median to percentile 75 (1.0 to 5.0 cm), and intense pain corresponded to the scores above the second percentile (5.0 to 10 cm).

A power analysis was performed using CSF purine levels as the primary outcome measure. The sample size was calculated so that a mean difference of 10 % between the groups would permit a type 1 error probability of $\alpha = 0.05$ (two-tailed test) with altered CSF purine levels in the labor pain group, and the null hypothesis would be retained with $\beta = 0.10$. This indicated that 15 patients would have to be included in each group (total = 60 patients). A higher number of patients were included to allow more adequate control of the potential confounding effect of variables.

Collection of CSF samples: Patients in all groups received a combined spinal epidural (CSE) or a single spinal technique for pain relief and anesthesia. All the CSF was collected by experienced anesthesiologists. The first 0.5 ml of CSF aspirated was discarded to reduce contamination. The CSF samples were inspected visually and discarded if blood contamination was present. A total of 0.5 ml of CSF was collected from the patients after successful subarachnoid puncture before the intrathecal injection of anesthetics or

analgesics. All samples were centrifuged at 10,000 g in an Eppendorf centrifuge during 5 min to obtain cell-free supernatants, stored at -70 ^{o}C within 30 min of collection and not thawed until laboratory evaluations.

HPLC procedure: High-performance liquid chromatography (HPLC) was performed with CSF cell-free supernatants aliquots for determination of purines concentration, according to Domanski et al [2006]. CSF concentrations of the following purines were determined: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine, inosine monophosphate (IMP), inosine, hypoxanthine, xanthine, and uric acid. Analyses were performed with Shimadzu Class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto injector valve with 50 μL loop, and an UV detector. Separations were achieved on a Supelco C18 250 mm x 4.6 mm, 5 μm particle size column. The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24 ^{o}C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 10 μL were injected into the injection valve loop. Absorbance was read at 254 nm. CSF concentrations of purines are expressed as mean ± SEM in μM.

Statistical analysis: Data were stored in EPI-INFO software version 6.0 and analyzed by Statistical Package for the Social Sciences (SPSS) for Windows version 12.0

(registered trademark). Data are expressed as mean ± standard deviation (SD). Data were submitted to Kolmogorov-Smirnov, Levene and Bartlett tests for normality evaluation. Statistical analysis between groups was performed using student *t* test or one-way ANOVA followed by the *post-hoc* Tukey-Kramer multiple comparisons test when applicable for parametric data. Non-parametric data were analyzed by using Mann-Whitney *U* test or Kruskal-Wallis test followed by Dunn test as a *post hoc* when appropriate. Correlations are presented as linear Pearson's or nonparametric Spearman's coefficient. $P < 0.05$ was considered for statistically significant differences.

Results:

The average age of the patients assessed was 57.5 ± 1.5 years old for patients with and without pain and no difference was found between groups ($P = 0.61$). Eighty patients were male and 24 were female (77 % vs. 23 %). However, there was no other difference on demographic characteristics between groups (data not shown). The most prevalent preoperatively diseases included: systemic hypertension, diabetes mellitus, chronic pulmonary obstructive disease, obesity, or compensated heart failure. Types of surgery performed during this study included: abdominal or vaginal hysterectomy, inguinal herniorrhaphy, open or trans-urethral prostatectomy, arterial embolectomy, peripheral arterial bypass, saphenectomy, lower limb amputation, total hip or knee arthroplasty, and femoral osteosynthesis. All patients enrolled to pain groups were predominantly experiencing somatic pain.

As shown in Fig. 1, chronic pain patients presented increased CSF levels of IMP, inosine, guanosine and uric acid as compared to control ($P < 0.05$). Notably, chronic pain

did not affect significantly CSF levels of ATP, ADP, AMP, GTP, GDP, GMP, adenosine, xanthine, hypoxanthine, and uric acid (Fig. 1).

Fig. 2 shows a comparison between all groups regarding the CSF purines levels. Interestingly, patients presenting chronic and acute pain presented similar changes in the CSF purines concentration as compared to control group. Patients in the chronic and acute/chronic pain groups presented a significant increase in the CSF levels of IMP, guanosine, inosine and uric acid ($P < 0.05$). However, when comparing the acute pain group, this change was only significant in CSF inosine and uric acid levels ($P < 0.05$). Notably, chronic and/or acute pain did not affect the CSF levels of ATP, ADP, AMP, GTP, GDP, GMP, adenosine, xanthine, hypoxanthine, and uric acid (Fig. 2).

As shown in Fig. 3, the CSF levels of IMP, inosine, guanosine and uric acid were significantly correlated with chronic pain intensity when evaluated by the VAS (IMP – r = 0.60, $P = 0.013$; inosine – r = 0.78, $P = 0.0006$; guanosine – r = 0.62, $P = 0.010$; and uric acid – r = 0.74, $P = 0.002$). Fig. 4 depicts the correlation of CSF purines levels with pain intensity measured by the VPS. A significant correlation was found only for IMP and guanosine, but not inosine or uric acid (IMP – r = 0.68, $P = 0.007$; inosine – r = 0.42, $P = 0.120$; guanosine – r = 0.58, $P = 0.010$; and uric acid – r = 0.36, $P = 0.184$).

Discussion:

In this study, the CSF levels of IMP, inosine, guanosine and uric acid were significantly higher in chronic pain patients when compared to control patients and significantly correlated with pain intensity measured by a visual analogue scale. Patients presenting chronic and acute pain presented similar CSF purines levels. Patients displaying exclusively acute pain showed only significant changes in CSF inosine and uric acid levels.

Chronic and/or acute pain did not affect the CSF levels of ATP, ADP, AMP, GTP, GDP, GMP, adenosine, xanthine, hypoxanthine, and uric acid.

The population sample studied was homogenous since the preanesthetic characteristics (gender, age, weight, and preoperative comorbidities) of the patients were quite similar. Although patients presenting two pain etiologies (vascular or orthopedic) were included in this study, the predominant characteristic is somatic with some patients displaying neuropathic pain characteristics (mainly those presenting pain from vascular/ischemic origin). Importantly, several patients scheduled for orthopedic or vascular surgery did not refer acute or chronic pain symptoms at the evaluation and were enrolled to control groups. Although no sufficiently powerful, a subgroup analysis indicated that similar CSF purines changes were found between groups. Perhaps future studies may focus on the role of purines in other pain conditions, such as neuropathic pain.

There is general acceptance of widespread physiological and pathological roles of purines acting via extracellular receptors and a number of recent studies implicate the purinergic system as essential in nociceptive pathways [Sawynok and Liu, 2003; Liu and Salter, 2005; Boison, 2008]. Adenine-based purines (ABPs) have been considered important targets for the development of new drugs for treating pain, since the nucleoside adenosine and its analogs induce antinociceptive effects following both systemic and central administration [Sawynok and Liu, 2003]. Adenosine can alter pain transmission by actions on both nociceptive afferent and transmission neurons, and these actions are mediated primarily by adenosine A_1 receptors [Sawynok and Liu, 2003]. Additional actions on inflammatory cells at peripheral sites and on glia in the CNS [Gebicke-Haerter et al., 1996] mediated by adenosine A_{2A}, A_{2B} and A_3 receptors also occur, and these potentially can produce indirect effects on pain transmission.

Although ABPs play an important role on pain transmission, we did not find any correlation between CSF ABPs levels and chronic pain. In a recent study, we demonstrated that rats submitted to a partial sciatic nerve ligation which caused thermal hyperalgesia produced a significant increase in CSF adenosine levels as compared to control animals [personal communication]. However, in the present study, no significant modulation of CSF adenosine levels was produced in humans displaying chronic somatic/neuropathic pain syndromes. Although not statistically significant, chronic pain patients tended to present higher CSF levels of AMP and GMP and reduced levels of ATP. Additionally, we found a significant increase in CSF levels of IMP, inosine and uric acid in chronic pain patients. Altogether these alterations may be related to the breakdown of ATP to adenosine and consequently to inosine and its final product uric acid. Interestingly, both inosine and uric acid are also increased in patients displaying only acute pain.

The physiopathological implications of these differences are difficult to interpret, but may represent an alteration in the brain and spinal cord energy metabolism, as reflected by increased CSF levels of the purine metabolites inosine and uric acid [Castro-Gago et al., 1995; Rodriguez-Núñes et al., 2000]. However, these changes may be partially compensated by homeostasis, since no significant effects in CSF levels of ATP were produced by both acute and chronic pain. The accumulation of IMP in the CSF may be due to an enhanced breakdown of ABPs followed by increased levels of inosine. The accumulation of inosine and the final product uric acid may cause a reduced degradation of IMP or its synthesis *de novo* by the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) [Schmidt et al., 2007a]. This hypothesis may similarly explain the increased CSF levels of guanosine in chronic pain patients. Considering that there was no accumulation of IMP and guanosine in patients displaying only acute pain, we

may speculate that persistent or sustained noxious stimuli are needed to generate these alterations.

In the present study, we predominantly enrolled patients presenting with vascular pain, which includes ischemic muscle pain, lumbar and chronic pelvic pain in women [Burnstock, 1996]. In these patients, pain occurs during the reactive hyperemic phase that follows local vasospasm, and there is an overwhelming acceptance that ATP plays a role in this process [Burnstock, 1996]. During reactive hyperemia, large amounts of ATP are released from vascular endothelial cells, which act on its specific receptors on endothelial cells, resulting in glial activation and release of several substances, including reactive oxygen species, nitric oxide, arachidonic acid, and pro-inflammatory cytokines. [Liao and Chen, 2001; Watkins and Milligan, 2001; Watkins and Maier, 2003; Burnstock, 2007]. These processes are largely involved in the mechanisms underlying chronic pain generation [Millan et al., 1999] and our results may reflect a sustained release of ATP from CNS cells following nerve damage or sustained noxious stimulation.

In the present study, CSF levels of guanosine were significantly increased in chronic and acute pain patients, an effect that may represent an endogenous modulation of pain transmission. Since nucleosides guanosine and adenosine closely interact in modulating several functions of the CNS [Dobolyi et al., 2000], guanosine might well play a significant role on pain transmission and nociception. There is increasing evidence that guanosine acts as an intercellular signaling molecule. It is released from cells and has several diverse effects *in vivo* and *in vitro*, particularly trophic effects and glutamatergic modulation [Schmidt et al., 2007a]. *In vitro*, guanosine has been shown to be neuroprotective [Rathbone et al., 1999; 2008; Di Iorio et al., 2001], stimulates nerve regeneration [Jiang et al., 2003; Bau et al., 2005], and prevents ischemic injury (Frizzo et

al., 2002) and NMDA-induced excitotoxicity [Ciccarelli et al., 2001]. *In vivo*, acute or chronic administration of guanosine prevents seizures and toxicity induced by drugs that overstimulate the glutamatergic system [Baron et al., 1989; Malcon et al., 1997; Schmidt et al., 2000; 2005; Lara et al., 2001; Vinadé et al., 2003], is amnesic and anxiolytic in rodents [Vinadé et al., 2003; 2004; 2005], and is neuroprotective against stroke and spinal cord injury [Jiang et al., 2003; 2007; 2008; Chang et al., 2008]. Recently, we demonstrated that anticonvulsant guanosine produced consistent and dose-dependent antinociceptive effects in several pain models in mice [Schmidt et al., 2008]. Additionally, administration of guanosine in mice, in antinociceptive doses, produced a significant increase in CSF levels of guanosine and its metabolites (xanthine and uric acid) in mice [Schmidt et al., 2008]. Since there is data supporting the existence of specific receptor-like binding sites for guanosine on brain membrane preparations, it is tempting to propose that guanosine could act as an endogenous antinociceptive substance likewise the nucleoside adenosine. Although the overall effects of guanosine seem to be related to attenuating glutamatergic overstimulation, its precise mechanism of action remains unclear. Guanosine may exert antinociceptive effects through an increased production and release of trophic factors, activation of the adenosinergic system, and/or modulation of glutamatergic pathways [Schmidt et al., 2007a]. Additionally, receptor-independent mechanisms may mediate guanosine actions. It could also act as an alternative source of energy for neural cells after further metabolism, as previously demonstrated in spinal cord cultures [Jurkowitz et al., 1998; Litsky et al., 1999]. Thus, guanosine and its analogs may be of potential interest in the development of new drugs for managing chronic pain conditions.

In summary, this is the first study investigating the effects of pain on the CSF levels of purines in humans. This study provides additional evidence on the role of extracellular

purines in the CNS and suggests that the breakdown of ABPs and accumulation of the by-products inosine, guanosine and uric acid in the CSF may represent shifts in neuronal and glial energy metabolism. These findings suggest that purines, in special inosine and guanosine, are associated with the mechanisms underlying pain transmission.

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Legends:

Figure 1: CSF levels of purines in patients displaying chronic pain (chronic pain group) and patients without acute or chronic pain (control group). Panel A: ATP, ADP, AMP, adenosine (ADO); Panel B: GTP, GDP, GMP, guanosine (GUO); Panel C: IMP, inosine (INO), hypoxanthine (HX), xanthine (XA) and uric acid (UA). The columns represent mean (μM) and vertical bars represent SEM. N = 52 (control) and 16 (chronic pain); * = $P < 0.05$ and ** = $P < 0.01$, Student *t* test.

Figure 2: CSF levels of purines in patients displaying chronic pain (chronic pain group), patients displaying chronic associated to acute pain (chronic and acute pain group), patients displaying only acute pain (acute pain group) and patients without acute or chronic pain (control group). Panel A: ATP, ADP, AMP, adenosine (ADO); Panel B: GTP, GDP, GMP, guanosine (GUO); Panel C: IMP, inosine (INO), hypoxanthine (HX), xanthine (XA) and uric acid (UA). The columns represent mean (μM) and vertical bars represent SEM. N = 52 (control), 16 (chronic pain), 20 (chronic and acute pain), and 16 (acute pain); * = $P < 0.05$ and ** = $P < 0.01$ as compared to control, one-way ANOVA followed by Student-Newman-Keuls test.

Figure 3: Correlation between CSF levels of IMP (panel A), guanosine (panel B), inosine (panel C) and uric acid (panel D) and pain scores measured by the visual analogue scale (VAS). (A) IMP CSF levels (Pearson's coefficient $r = 0.60$; $P = 0.013$); (B) Guanosine CSF levels (Pearson's coefficient $r = 0.62$; $P = 0.010$); (C) Inosine CSF levels (Pearson's

coefficient $r = 0.78$; $P = 0.0006$); (D) Uric acid CSF levels (Pearson's coefficient $r = 0.74$; $P = 0.002$).

Figure 4: Correlation between CSF levels of IMP (panel A), guanosine (panel B), inosine (panel C) and uric acid (panel D) and pain scores measured by the verbal pain scale (VPS). (A) IMP CSF levels (Spearman's coefficient $r = 0.68$; $P = 0.007$); (B) Guanosine CSF levels (Spearman's coefficient $r = 0.58$; $P = 0.010$); (C) Inosine CSF levels (Spearman's coefficient $r = 0.42$; $P = 0.12$); (D) Uric acid CSF levels (Spearman's coefficient $r = 0.36$; $P = 0.184$).

Figure 1:

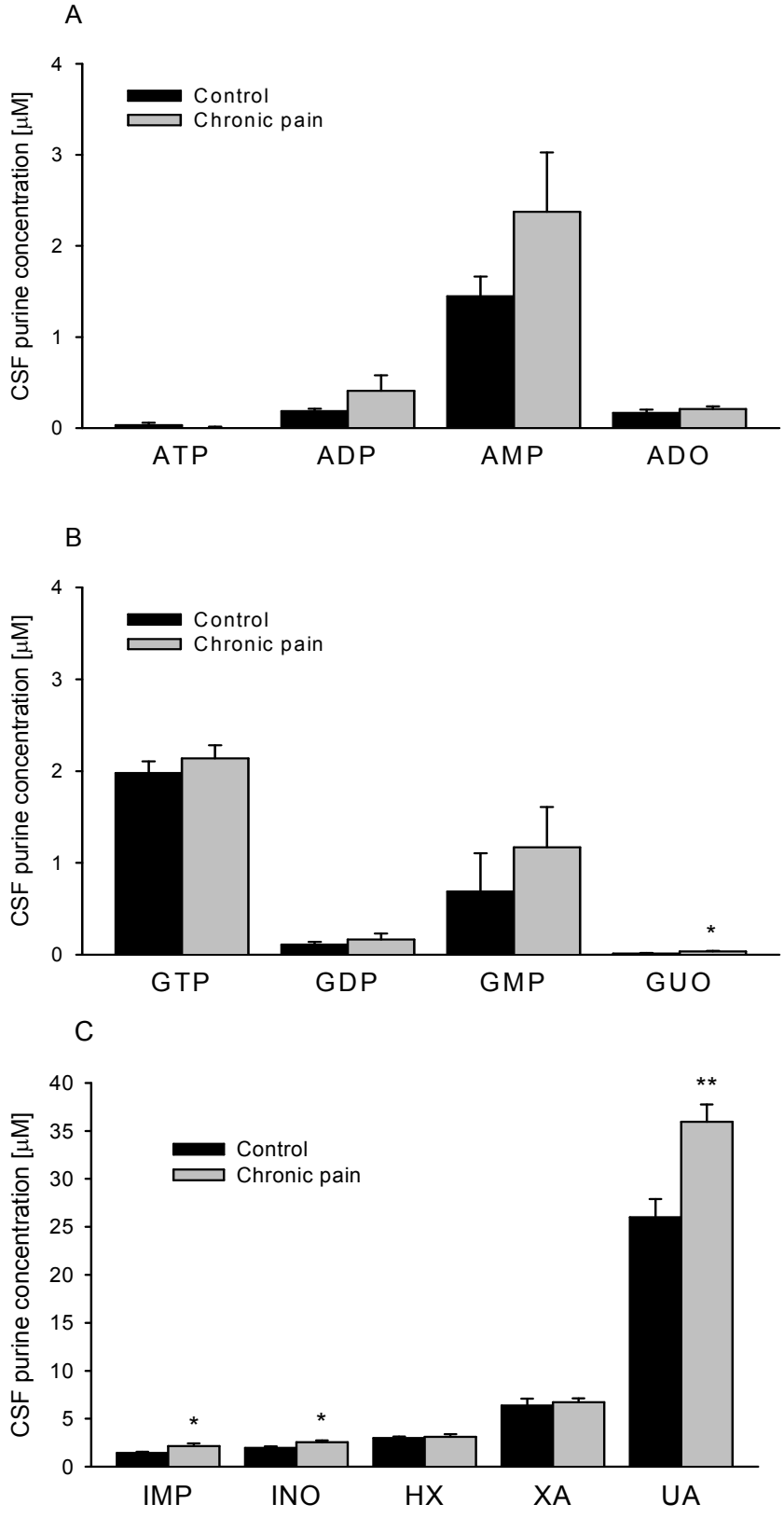

A
CSF purine concentration [µM]
Control
Chronic pain
ATP
ADP
AMP
ADO
B
GTP
GDP
GMP
GUO
*
C
IMP
INO
HX
XA
UA
**

Figure 2:

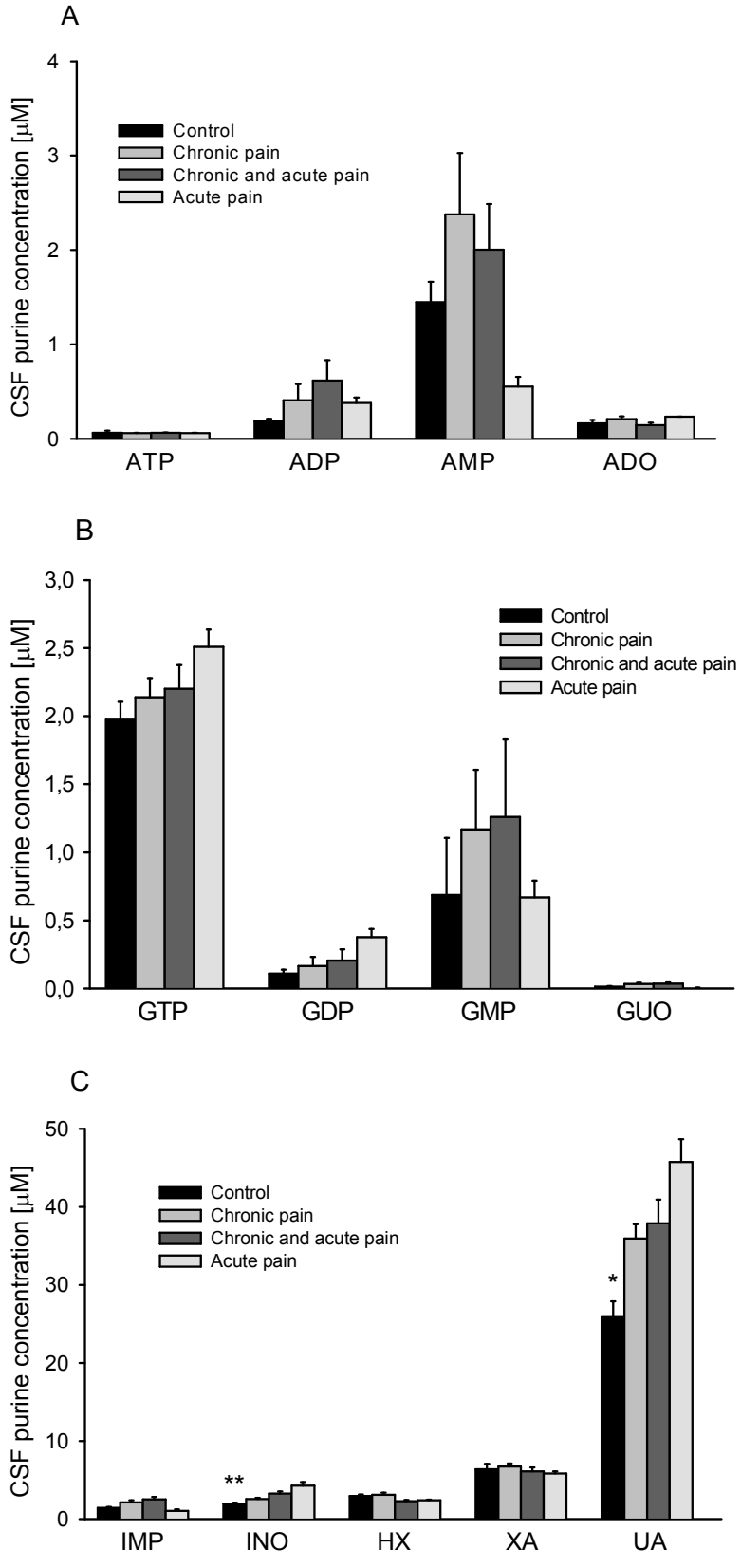

A
CSF purine concentration [µM]
Control
Chronic pain
Chronic and acute pain
Acute pain
ATP
ADP
AMP
ADO
B
CSF purine concentration [µM]
Control
Chronic pain
Chronic and acute pain
Acute pain
GTP
GDP
GMP
GUO
C
CSF purine concentration [µM]
Control
Chronic pain
Chronic and acute pain
Acute pain
**
*
IMP
INO
HX
XA
UA

Figure 3:

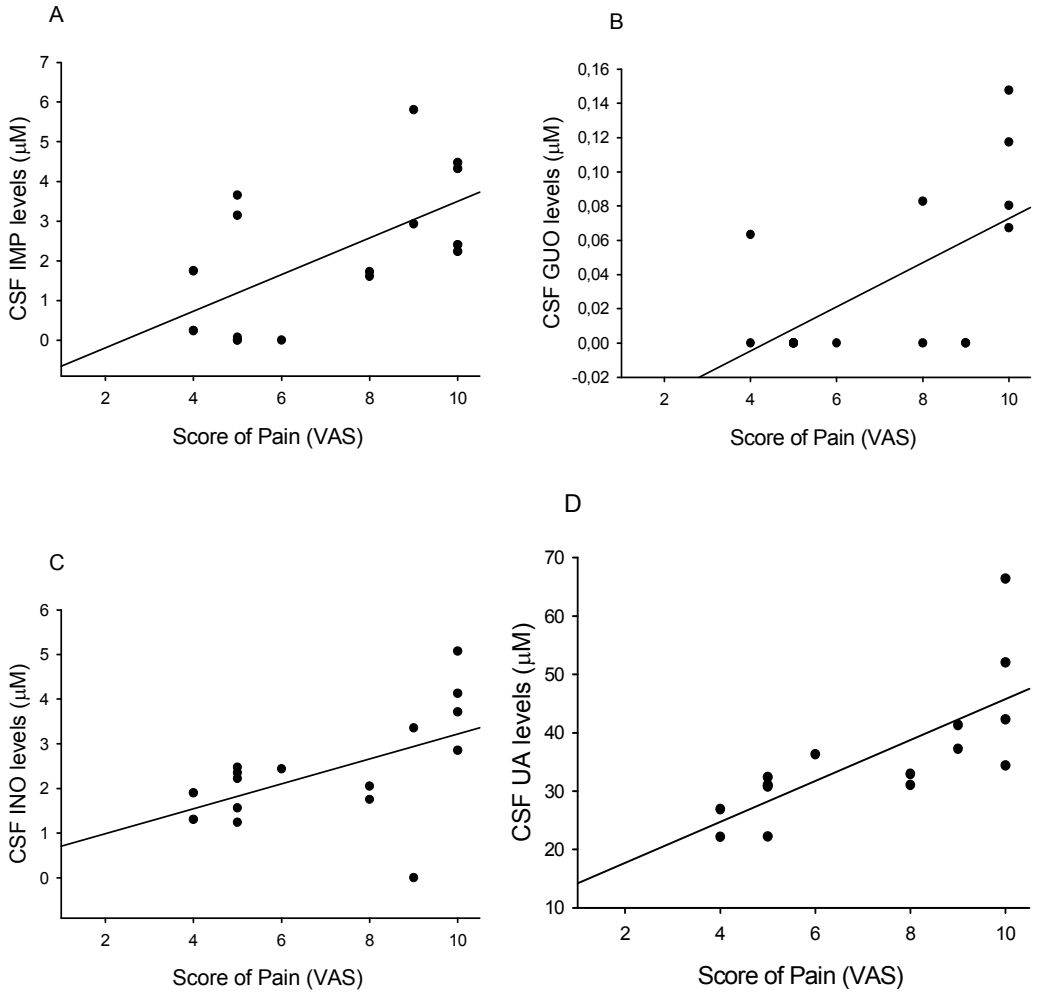
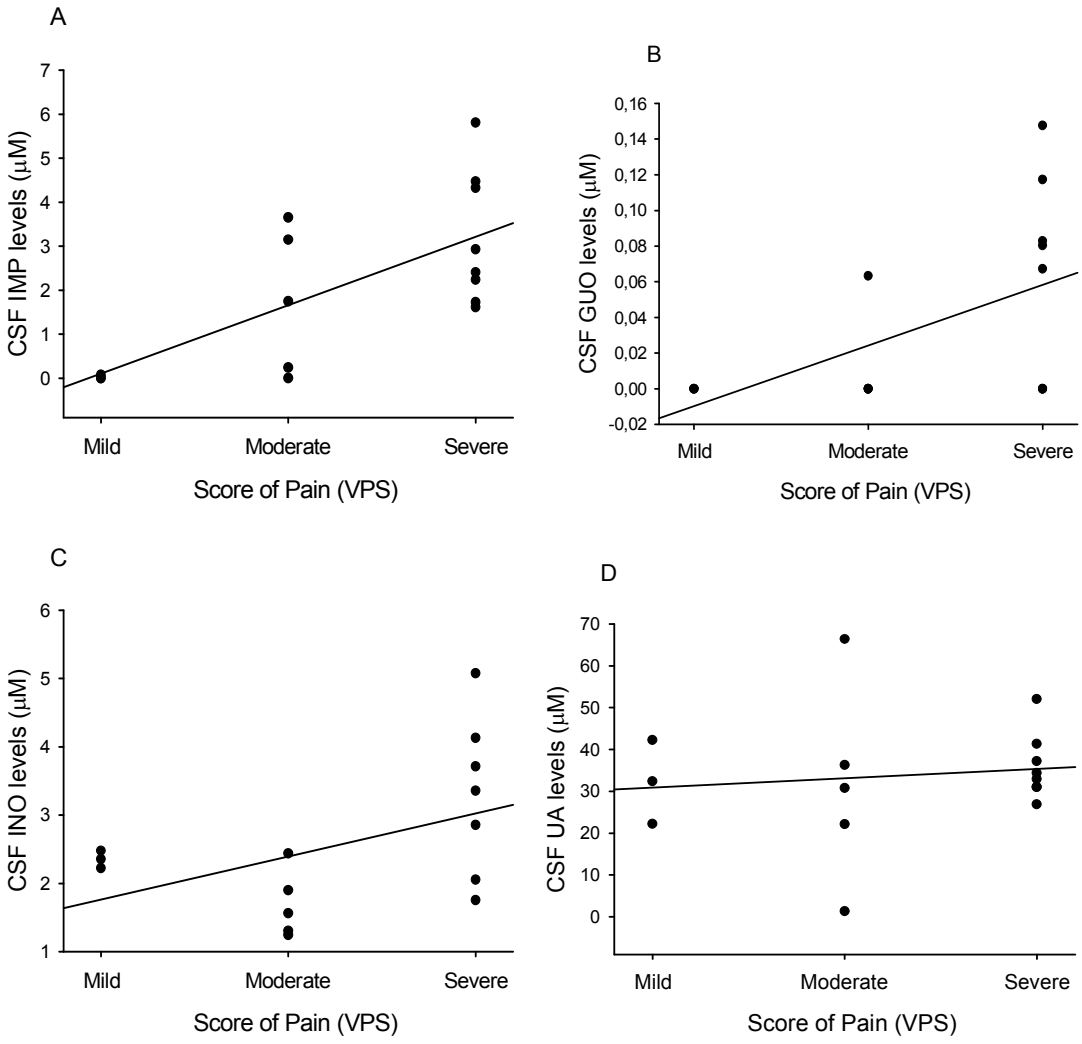

A
CSF IMP levels (μM)
Score of Pain (VAS)
B
CSF GUO levels (μM)
Score of Pain (VAS)
C
CSF INO levels (μM)
Score of Pain (VAS)
D
CSF UA levels (μM)
Score of Pain (VAS)

Figure 4:


A
CSF IMP levels (μM)
7
6
5
4
3
2
1
0
Mild
Moderate
Severe
Score of Pain (VPS)
B
CSF GUO levels (μM)
0,16
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
-0,02
Mild
Moderate
Severe
Score of Pain (VPS)
C
CSF INO levels (μM)
6
5
4
3
2
1
Mild
Moderate
Severe
Score of Pain (VPS)
D
CSF UA levels (μM)
70
60
50
40
30
20
10
0
Mild
Moderate
Severe
Score of Pain (VPS)

PARTE III

Onde os resultados são discutidos e as perspectivas são traçadas.

III.1. DISCUSSÃO DOS RESULTADOS

III.1.a. Revisão sobre o papel dos derivados da guanina no SNC

O primeiro capítulo desta tese teve como objetivo realizar uma breve revisão crítica dos efeitos biológicos já documentados dos derivados da guanina. Este estudo de revisão demonstrou que têm surgido informações cada vez mais consistentes sobre o papel dos derivados da guanina no espaço extracelular. Diversos grupos de trabalho além da nossa instituição têm demonstrado e replicado tais efeitos biológicos *in vitro* e *in vivo*.

Através deste estudo, podemos revisar de forma didática alguns dos aspectos históricos sobre o sistema purinérgico. É importante ressaltar que muita resistência do meio cientifico ocorreu na época em que o eminente pesquisador Geoffrey Burnstock propôs o ATP como neurotransmissor e, posteriormente, caracterizou o sistema purinérgico de neurotransmissão [Burnstock, 1972]. Isto também pode ser uma realidade em relação às purinas derivadas da guanina. Como nossa revisão procura demonstrar, os derivados da guanina apresentam cada vez mais embasamento para suas funções biológicas extracelulares, adicionalmente aos já bem documentados efeitos intracelulares. Estes efeitos podem ser resumidos em três grandes correntes: i. efeitos modulatórios sobre o sistema glutamatérgico; ii. efeitos comportamentais; iii. efeitos tróficos em células neurais.

Entre os principais efeitos biológicos dos derivados da guanina no extracelular podemos citar: *in vitro* – aumentam a captação de glutamato em culturas de astrócitos e fatias cerebrais [Frizzo et al., 2001, 2002, 2003, 2005], reduzem a toxicidade induzida por NMDA em neurônios [Caciagli et al., 2000], inibem a ligação de glutamato e seus agonistas aos seus receptores [Baron et al., 1989; Souza e Ramirez, 1991; Burgos et al.,

1998], apresentam efeitos neuroprotetores contra excitotoxicidade [Frizzo et al., 2002; Moretto et al., 2005], modulam a apoptose celular [Pettifer et al., 2004; Di Iorio et al., 2004] e possuem efeitos tróficos em células neurais [Rathbone et al., 1999]; *in vivo* – evitam convulsões induzidas por agentes glutamatérgicos [Schmidt et al., 2000; Lara et al., 2001], são amnésicos e ansiolíticos em ratos e camundongos [Roesler et al., 2000; Vinadé et al., 2003, 2004, 2005], e são neuroprotetores contra isquemia cerebral e lesões medulares [Malcon et al., 1997; Jiang et al., 2003; 2007; 2008; Chang et al., 2008].

Portanto, os efeitos neuromodulatórios dos derivados da guanina sobre o sistema glutamatérgico e seus efeitos neurotróficos sobre células neurais são potencialmente relevantes. Além disso, a capacidade e o perfil dos derivados da guanina em modular o sistema glutamatérgico parece muito interessante para a busca de novas alternativas terapêuticas em diversas doenças que cursam com hiperativação glutamatérgica. Dentre estas condições patológicas podemos citar a dor aguda e crônica, como veremos nas discussões subseqüentes a respeito dos demais estudos desta tese.

III.1.b. Efeitos anticonvulsivantes dos derivados da guanina

Apesar desta tese versar predominantemente sobre modelos de dor e os efeitos dos derivados da guanina, optamos por incluir este trabalho com o intuito de exemplificar o trabalho desenvolvido durante minha graduação sobre o papel dos derivados da guanina em modelos de convulsão e doenças neurodegenerativas em animais. Além disso, este estudo propõe novas informações sobre o potencial mecanismo de ação dos derivados da guanina e sobre qual componente deste sistema desempenha papel neuroprotetor *in vivo*.

Diversos estudos anteriores demonstraram que os derivados da guanina são anticonvulsivantes contra fármacos que estimulam o sistema glutamatérgico [Schmidt et al., 2000; Lara et al., 2001; Vinadé et al., 2003, 2004; Soares et al., 2004; Saute et al., 2006]. Neste estudo, a administração intracerebroventricular (i.c.v.) de GTP, GDP, GMP e guanosina foi capaz de inibir as convulsões induzidas por ácido quinolínico em camundongos. Entretanto, os resultados não foram replicados quando análogos pobremente hidrolisáveis do GTP e GDP foram administrados. Estes resultados, somados a dados de estudos anteriores [Soares et al., 2004; Saute et al., 2006], demonstram que os efeitos dos nucleotídeos da guanina GTP, GDP e GMP são condicionados a sua capacidade de conversão até o nucleosídeo guanosina.

Através destes resultados com a administração i.c.v. de derivados da guanina e utilizando as mesmas doses anticonvulsivantes de GMP e guanosina, foram iniciados os trabalhos que avaliam os efeitos dessas substâncias sobre a transmissão dolorosa.

III.1.c. Efeitos antinociceptivos dos derivados da guanina

Nos capítulos II.2.b., II.2.f., II.2.g. e II.2.h., podemos observar grande quantidade de resultados da investigação dos derivados da guanina, mais precisamente a guanosina, em diversos modelos de dor em ratos e camundongos. Entretanto, nos estudos iniciais incluindo o capítulo II.2.c. desta tese, a guanosina e o GMP não apresentaram efeitos antinociceptivos significativos no modelo de "tail-flick" em ratos, quando utilizadas doses até 7,5 $mg.kg^{-1}$, por via intraperitoneal (i.p.), semelhante a um estudo anterior [Saute et al., 2006].

Devido aos resultados iniciais desanimadores com a administração sistêmica de guanosina e GMP, optamos por utilizar uma abordagem semelhante à do capítulo II.2.a. em termos de via de administração e doses dos derivados da guanina para investigá-los como potenciais substâncias antinociceptivas. Podemos observar neste capítulo que doses anticonvulsivantes de guanosina e GMP, administrados por via i.c.v., foram antinociceptivos em quatro modelos de dor aguda em camundongos. Os derivados da guanina foram avaliados em dois modelos térmicos ("tail-flick" e "hot-plate" ou placa quente) e dois modelos químicos, com injeção de substâncias irritantes na pata do animal. A administração de AOPCP, um inibidor da enzima 5'-nucleotidase e conseqüentemente da conversão de GMP em guanosina, reverteu os efeitos antinociceptivos do GMP no modelo da capsaicina, sugerindo, de forma semelhante a outros estudos [Frizzo et al., 2003; Soares et al., 2004; Saute et al., 2006] e ao estudo já exposto na seção anterior, que os efeitos do GMP sobre a dor estão condicionados a sua conversão até guanosina. Portanto, nos demais estudos desta tese não utilizamos o nucleotídeo GMP com objetivo de limitar o uso desnecessário de animais, utilizando apenas a guanosina como principal representante e efetor do sistema purinérgico derivado da guanina.

Como observado nos capítulos subseqüentes (II.2.f. e II.2.g.) a guanosina foi amplamente investigada em diversos modelos de dor e através de diferentes vias de administração. No capítulo II.2.f. utilizamos a via intratecal (i.t.) e no capítulo II.2.g. as vias i.p. e oral (p.o.). Novamente, os efeitos antinociceptivos da guanosina, por diferentes vias de administração, foram replicados na maior parte dos modelos testados. A administração de guanosina pelas vias i.c.v. e i.t. apresentou eficácia antinociceptiva em todos os modelos testados. Em relação à administração sistêmica da guanosina, apenas no modelo de "tail-flick" os efeitos não foram estatisticamente significativos, apesar de uma

haver uma tendência também neste modelo. Através da administração sistêmica de guanosina, também podemos observar que esta substância foi antinociceptiva no modelo de ácido acético para simular dor visceral e modelo da formalina, com eficácia significativa no componente antiinflamatório do modelo. A guanosina administrada por via sistêmica também inibiu o edema da pata induzida pela administração i.pl. de glutamato.

Os modelos incluídos nesta tese para investigação dos derivados da guanina *in vivo* são modelos tradicionais com embasamento relativamente bem estabelecido [Le Bars et al., 2001]. Os modelos apresentados (excluindo o modelo de dor neuropática do capítulo II.2.h.) são baseados em estímulos térmicos e químicos que induzem dor aguda em animais. Apesar desses modelos estarem relacionados à transmissão de dor aguda de curta duração, algumas diferenças importantes são encontradas. Os modelos térmicos "tail-flick" e da placa quente são semelhantes, mas o primeiro está mais relacionado a um reflexo espinhal, praticamente sem controle de estruturas superiores, enquanto que a placa quente trata-se de modelo mais robusto e complexo, com ativação de estruturas supra-espinhais, produzindo dois componentes comportamentais (lambida da pata e pulos), considerados como respostas de estruturas mais centrais [Le Bars et al., 2001]. Essas diferenças podem ter contribuído de forma significativa para as diferenças de eficácia da guanosina em ambos os modelos.

A injeção intraplantar (i.pl.) de substâncias algogênicas (capsaicina, glutamato e formalina) produz geralmente respostas comportamentais semelhantes e representa um estímulo de maior duração do que os estímulos térmicos (dor tônica versus dor fásica nos estímulos térmicos) [Le Bars et al., 2001]. Entretanto, a injeção i.pl. de glutamato produz resposta nociceptiva e edema fortemente mediados por receptores não-NMDA [Beirith et al., 2002], enquanto que o teste da capsaicina envolve um mecanismo mais complexo, predominantemente mediado pela liberação de aminoácidos excitatórios na medula,

ativação de receptores para taquicininas e receptores NMDA [Sakurada et al., 1993]. A formalina é a substância mais utilizada para injeções intradérmicas em modelos de dor e sua injeção acarreta uma resposta comportamental bifásica [Le Bars et al., 2001]. A primeira fase resulta essencialmente da estimulação direta dos nociceptores, semelhante ao modelo de glutamato i.pl. A segunda fase do teste da formalina envolve um período de sensibilização durante o qual o fenômeno inflamatório ocorre. Por fim, a injeção i.p. de ácido acético causa uma resposta comportamental caracterizada por constrições repetidas do abdômen após a injeção da substância algogênica. Esta resposta é dor de origem peritoneo-visceral e, apesar de apresentar baixa especificidade, é um modelo muito sensível e preditivo para testar novas moléculas ou analgésicos cujas propriedades farmacodinâmicas ainda são pouco conhecidas como a guanosina [Le Bars et al., 2001].

No capítulo II.2.h., a guanosina foi testada em um modelo de dor crônica neuropática em ratos. Os resultados deste estudo demonstraram que a guanosina, administrada de forma aguda ou crônica, foi capaz de inibir a hiperalgesia térmica induzida por um modelo de constrição crônica do nervo ciático. A guanosina também reverteu a perda de peso e redução da atividade locomotora de animais submetidos a este modelo.

Considerando os resultados expostos acima, podemos inferir que a guanosina é uma molécula endógena com propriedades antinociceptivas e, levando em consideração seus efeitos sobre o edema na pata induzido pelo glutamato e sobre a segunda fase do teste da formalina, também apresenta potenciais efeitos antiinflamatórios.

Com os efeitos comportamentais documentados, partimos agora para potenciais explicações para estes resultados. A guanosina pode exercer seus efeitos através de diversos mecanismos. As principais hipóteses incluem: estímulo à produção e liberação de fatores

tróficos neurais, ativação do sistema adenosinérgico endógeno, e modulação da atividade glutamatérgica [Schmidt et al., 2007; Rathbone et al., 2008].

As purinas apresentam importantes propriedades tróficas sobre células do SNC e SNP [Rathbone et al., 2008]. Em especial, guanosina estimula a síntese e liberação de diversos fatores tróficos de astrócitos, tais como fator de crescimento neural (NGF), fator neurotrófico ciliar, neurotrofinas, fator de crescimento de fibroblastos, proteína S100β, entre outros [Rathbone et al., 1999]. Normalmente, os fatores tróficos desempenham função estimuladora das vias de dor em nível medular, estando envolvidos em diversos mecanismos responsáveis pela manutenção do quadro doloroso [Jongen et al., 2005]. Entretanto, alguns resultados são contraditórios e estudo recente demonstrou que administração intratecal de fatores neurotróficos é capaz de tratar de forma eficiente quadros de dor neuropática [Pezet et al., 2008]. Em estudos recentes em nossa instituição, o modelo de dor neuropática foi capaz de induzir alterações significativas nos níveis liquóricos de diversos fatores tróficos [dados não publicados]. Caso a guanosina apresente algum efeito antinociceptivo por promover a liberação de fatores tróficos, estes devem estar predominantemente envolvidos nos modelos de dor crônica e não explicariam os efeitos da guanosina sobre a transmissão da dor aguda. Entretanto, estudos avaliando uma potencial correlação entre guanosina, fatores tróficos e transmissão da dor ainda necessitam ser realizados.

Quanto ao envolvimento dos derivados da adenina nos efeitos antinociceptivos da guanosina, os resultados desta tese indicam que são improváveis de contribuir de forma significativa. Estudos anteriores foram contraditórios, pois alguns demonstraram que guanosina induz efeitos biológicos através da liberação de adenosina [Ciccarelli et al., 2001]

e que seus efeitos tróficos são parcialmente inibidos pela adenosina deaminase e antagonistas de receptores adenosinérgicos [Ciccarelli et al., 2000]. Em contrapartida, a maioria dos resultados disponíveis na literatura exclui o envolvimento do sistema adenosinérgico sobre os efeitos da guanosina, como nos efeitos tróficos [Gysbers e Rathbone, 1996], na captação de glutamato [Frizzo et al., 2001], nos efeitos anticonvulsivantes [Lara et al., 2001] e sobre a memória [Vinadé et al., 2004].

Considerando os dados desta tese, a influência do sistema adenosinérgico nos efeitos da guanosina é improvável pelas seguintes observações: i. a antinocicepção induzida por guanosina i.c.v. não foi inibida pela administração de antagonistas dos receptores de adenosina; ii. através de dosagem por cromatografia de alta performance (HPLC), demonstramos que a guanosina, administrada de forma aguda ou crônica e por diferentes vias de administração, não foi capaz de elevar os níveis dos derivados da adenina em nenhum dos protocolos estudados. Ainda dentro deste contexto, mesmo a administração de guanosina e GMP em doses elevadas direto no SNC, acarretando um aumento de até 1800x na concentração de guanosina no líquido cefalorraquidiano (LCR), não obteve efeitos significativos sobre a concentração de adenosina neste ambiente. Entretanto, limitações nesta abordagem devem ser consideradas. Adenosina é uma molécula sujeita à rápida metabolização [Burnstock, 2007], o que pode explicar a ausência de um aumento liquórico deste composto. Além disso, as concentrações de purinas diretamente no tecido cerebral e medular não foram avaliadas e é possível que níveis alterados de adenosina possam ocorrer nestas circunstâncias.

Nesta tese demonstramos que a administração de guanosina promove um aumento significativo nos níveis liquóricos de oxipurinas como xantina e ácido úrico. Este efeito provavelmente se deve a degradação enzimática de guanosina *in vivo*, mas, apesar de

improvável, não podemos descartá-la como causa dos efeitos antinociceptivos da guanosina. O mesmo ocorre em relação ao papel da guanina nos efeitos da guanosina. Estudos recentes têm demonstrado que a administração crônica de guanosina causa aumento significativo dos níveis teciduais de guanina na medula espinhal e este efeito pode estar relacionado com sua capacidade neuroprotetora e trófica [Jiang et al., 2003;2007;2008]. Entretanto, nossa metodologia de dosagem de purinas através de HPLC ainda não está suficientemente acurada para dosagem de guanina, o que limita nossa investigação e permite apenas especulações sobre os potenciais efeitos da guanina na dor. Uma potencial perspectiva seria avaliar os efeitos da guanina em modelos de dor, mas sua baixa solubilidade em água é um fator limitante para esta investigação.

A principal hipótese para o mecanismo de ação antinociceptiva da guanosina e dos demais derivados da guanina é a capacidade destas substâncias em modular a atividade glutamatérgica. Nesta tese, podemos observar vários resultados que levam neste sentido. No capítulo II.2.f. e II.2.g. os resultados indicam que a administração de guanosina atenuou a resposta dolorosa provada por agonistas glutamatérgicos administrados diretamente no SNC de camundongos. A guanosina, tanto por via intratecal como sistêmica, atenuou os efeitos nociceptivos da administração intratecal de glutamato, AMPA, cainato e trans-ACPD, mas não de NMDA. Apesar de estudos prévios não demonstrarem guanosina como um potencial antagonista de receptores glutamatérgicos [Souza e Ramirez, 1991], os presentes dados permitem sugerir que a guanosina promove seus efeitos antinociceptivos, pelo menos em parte, por interagir com receptores glutamatérgicos do tipo não-NMDA (AMPA, cainato e metabotrópicos) ou então com seus mecanismos de transdução de sinal. Além disso, esses dados indicam que, apesar de guanosina também ser antinociceptiva por

via i.c.v., seus efeitos antinociceptivos provavelmente ocorrem predominantemente em nível medular.

Há crescentes evidências de que o sistema glutamatérgico interage amplamente com outros neuromoduladores e seus receptores como a substância P e os receptores vanilóides [Siebel et al., 2004]. A administração intratecal de guanosina atenuou de forma significativa a resposta nociceptiva à injeção intratecal de substância P, mas não à injeção de capsaicina (capítulo II.2.f.). Este resultado, apesar de não replicado após administração sistêmica de guanosina (capítulo II.2.g.), indica que receptores para taquicininas, em especial os receptores NK_1, podem estar envolvidos nos efeitos antinociceptivos da guanosina, assim como os receptores glutamatérgicos. Como demonstramos que a guanosina não é capaz de reverter os efeitos da administração intratecal de NMDA e que não interage com a via L-arginina-NO-GMPc (capítulo II.2.g.) após administração sistêmica, podemos sugerir que a guanosina não é um fármaco antagonista do receptor NMDA.

Considerando que a manutenção de níveis fisiológicos de glutamato através de sua captação por transportadores específicos é crítica para a transmissão sensorial normal da medula espinhal [Liaw et al., 2005] e que a guanosina tem demonstrado diversos efeitos modulatórios sobre a captação de glutamato [Frizzo et al. 2001;2002;2003;2005; Vinadé et al., 2005], esta tese objetivou investigar se os efeitos antinociceptivos da guanosina se devem a este mecanismo. O modelo utilizado foi o de captação de glutamato em fatias de medula, que será discutido em seção subseqüente. Nos resultados *in vitro* demonstrados no capítulo II.2.e., podemos observar que a guanosina estimulou a captação de glutamato de forma significativa apenas em doses elevadas, em torno de 1 mM. Essas doses foram superiores as doses utilizadas em estudos anteriores que demonstraram efeitos

estimuladores sobre a captação de glutamato em fatias cerebrais [Frizzo et al., 2002]. Várias abordagens também foram realizadas nos estudos *in vivo*, com o intuito de investigar a captação global de glutamato em fatias de medula e córtex e resultados contraditórios foram demonstrados. Inicialmente, no capítulo II.2.b. demonstramos que a administração i.pl. de capsaicina causou queda significativa da captação de glutamato no córtex de camundongos, efeito revertido pela guanosina. Entretanto, nos experimentos apresentados no capítulo II.2.g., onde investigamos os efeitos da administração sistêmica de guanosina, não houve alteração na captação cortical de glutamato, mesmo após a administração de capsaicina. Esta diferença pode estar relacionada à presença de lesão induzida pela neurocirurgia no protocolo i.c.v. Quanto à captação de glutamato na medula espinhal, a injeção de capsaicina causou um aumento significativo da captação de glutamato na medula espinhal, efeito novamente revertido pela guanosina (capítulos II.2.f. e II.2.g.). No entanto, é importante ressaltar que em nenhum experimento a guanosina foi capaz de modular positivamente a captação de glutamato em fatias de córtex ou medula espinhal em condições basais.

No modelo de dor neuropática descrito detalhadamente no capítulo II.2.h., podemos observar que ocorreu uma redução significativa da captação de glutamato na medula espinhal e um aumento na captação cortical de glutamato nos animais portadores de dor crônica neuropática, 14 dias após a lesão nervosa. A guanosina foi capaz de reverter o efeito sobre a captação cortical de glutamato e as alterações comportamentais (resposta locomotora e dolorosa), mas não as alterações sobre a captação medular de glutamato.

Avaliando os dados em conjunto e considerando as limitações da metodologia adotada, não podemos concluir com precisão se a guanosina promove seus efeitos antinociceptivos através da modulação da captação de glutamato. Especulamos, no entanto,

que as alterações observadas na captação sejam conseqüência direta da modulação da resposta dolorosa pela guanosina e não como causa de sua antinocicepção. Novos estudos, com metodologias mais refinadas, devem determinar qual o real papel da captação de glutamato glial ou neuronal nos efeitos antinociceptivos da guanosina.

III.1.d. Mecanismos celulares da ação da guanosina sobre o SNC (receptores e mensageiros secundários): Hipóteses para os efeitos antinociceptivos da guanosina

Considerando os dados e argumentos discutidos na seção anterior, é muito provável que a guanosina exerça seus efeitos antinociceptivos por modular a atividade glutamatérgica. Entretanto, é difícil determinar e explicar como isto ocorre de fato. Nesta seção, procuraremos abordar, com base na literatura disponível, potenciais mecanismos de ação em nível celular pelos quais a guanosina poderia exercer seus efeitos sobre a nocicepção.

Há crescente evidência que os derivados da guanina, mais especificamente a guanosina, agem como moléculas sinalizadoras intercelulares. As células da glia, principalmente os astrócitos, são as principais fontes de purinas como os derivados da guanina [Rathbone et al., 1999]. Estas células expressam receptores purinérgicos envolvidos em processos tróficos e neuroprotetores [Neary et al., 1996; Ciccarelli et al., 2001]. Entretanto, é bem estabelecido que os derivados da guanina (GTP e guanosina) não são ligantes de receptores adenosinérgicos [Muller e Scior, 1993], sugerindo que estas substâncias podem possuir receptores específicos na membrana celular. O mesmo ocorre

em relação à liberação de adenosina que ocorre após a administração de guanosina [Rathbone et al., 1999]. No entanto, a adenosina não parece estar envolvida na maioria dos efeitos biológicos dos derivados da guanina, pois antagonistas dos receptores purinérgicos capazes de reverter efeitos adenosinérgicos não reverteram os efeitos guanosinérgicos [Gysbers e Rathbone, 1992; Frizzo et al., 2001; Tasca e Souza, 2000].

Uma outra hipótese para os efeitos da guanosina seria decorrente de sua intracelular após a sua captação para o interior da célula. Entretanto, muitos efeitos tróficos desencadeados pela guanosina não foram afetados pela administração de inibidores da recaptação de nucleosídeos como dipiridamol, indicando que sua ação se dá no meio extracelular [Gysbers e Rathbone, 1992].

Até o momento, apesar dos efeitos biológicos intensos demonstrados pelos derivados da guanina, as evidências de receptores específicos para guanosina ou os demais derivados da guanina têm sido desapontadoras, mas alguns estudos indicam potenciais sítios de ligação na membrana celular de células neurais [Vuorinen et al., 1992; Ciccarelli et al., 2001; Traversa et al., 2002; 2003]. Traversa et al. [2002; 2003] demonstrou que há um sítio específico para guanosina em preparação de membranas de cérebro de ratos, provavelmente ligado à proteína G. Inosina e guanina foram capazes de deslocar guanosina, mas não os derivados da adenina e os nucleotídeos da guanina. A ordem de potência foi guanosina = 6-tio-guanosina > inosina > 6-tio-guanina > guanina. Apesar destes estudos sugerirem fortemente que os efeitos guanosinérgicos são independentes do sistema adenosinérgico, os dados são bastante limitados. Os estudos disponíveis que abordam esta hipótese apresentam metodologias diversas e bastante limitadas, não permitindo até o momento conclusões definitivas a respeito do tema. Em nossa instituição, estamos

desenvolvendo protocolos que objetivam esclarecer a existência real de algum sítio de ligação para guanosina na membrana celular.

Até o momento, a guanosina já demonstrou interagir com diferentes cascatas intracelulares. Vários dos efeitos induzidos pela guanosina têm sido relacionados à ativação intracelular de vias ligadas a proteínas cinases ativadas por mitógenos (MAPK), via PI3K/Akt/PKB (fosfatidil-inositol-3-cinase (PI-3-K)/Akt/proteina cinase B) e aumento da disponibilidade de AMPc no interior da célula [Gysbers e Rathbone, 1996; Caciagli et al., 2000; Di Iorio et al., 2004; Pettifer et al., 2004; Tomaselli et al., 2005].

Alguns estudos sugerem que a guanosina promove seus efeitos neuroprotetores através de mecanismos receptor-independente. Considerando que poucos estudos têm encontrado evidências de sítios específicos na membrana celular para os derivados da guanina, esta hipótese deve ser fortemente considerada quando discutimos os potenciais efeitos antinociceptivos desta substância. Os estudos de Jurkowitz et al. [1998] e Litsky et al. [1999] demonstraram que os efeitos protetores de inosina e guanosina em culturas de células da medula espinhal eram revertidos pela administração de um inibidor de PNP, enzima responsável pela degradação de guanosina até guanina e de inosina até hipoxantina [Schmidt et al., 2007]. A hipótese principal concedida por estes estudos é a de que guanosina e inosina, ao serem metabolizadas, liberam um grupo ribose de sua composição. Esta ribose é convertida em intermediários da via glicolítica e culmina com a liberação de ATP necessário para a manutenção da integridade celular. Portanto, guanosina poderia servir como uma fonte de energia adicional para células neurais que apresentam algum grau de sofrimento. Estudos mais recentes demonstraram que a guanosina e outros nucleosídeos são capazes de estimular a bomba Na/K ATPase e aumentam a sobrevida de ratos submetidos a modelo de choque hipovolêmico [Darlington e Gann, 2005]. Estes efeitos não

foram revertidos por antagonistas adenosinérgicos, mas sim por inibidores da captação de nucleosídeos, sugerindo que este efeito se deve à ação intracelular da guanosina. Novamente, dados sugerem que a guanosina pode exercer efeitos citoprotetores por participar ativamente do metabolismo energético celular quando em concentrações suprafisiológicas. Apesar deste mecanismo dificilmente ser capaz de mediar a capacidade da guanosina em modular a dor aguda, sua influência sobre a dor crônica é perfeitamente plausível.

Considerando os dados expostos acima, podemos concluir que o nosso conhecimento sobre o mecanismo de ação da guanosina e dos demais derivados da guanina ainda é muito incipiente e contraditório. Talvez a melhor opção no momento seja pensar na guanosina como uma molécula multifacetada capaz de interagir com múltiplos mecanismos celulares dependentes ou não de receptores específicos. Novos estudos devem surgir em breve caracterizando, de forma mais fidedigna e acurada, possíveis receptores para guanosina e os mensageiros secundários envolvidos na transdução de sinal intracelular.

III.1.e. Investigação de outros efeitos comportamentais e da toxicidade mediada pelos derivados da guanina

A adenosina e seus análogos têm sido relacionados a efeitos adversos como sedação, disfunção motora, entre outros [Sawynok e Liu, 2003]. Portanto, caso a guanosina e os demais derivados da guanina apresentem efeitos semelhantes isto poderia contribuir para mascarar os efeitos antinociceptivos apresentados. Considerando estes fatores, praticamente todos os protocolos experimentais incluíram testes comportamentais que avaliaram a função motora dos animais durante o tratamento com derivados da guanina. Alguns testes

iniciais de toxicidade hepática e renal e de farmacocinética da guanosina também foram realizados.

Considerando os dados provenientes do modelo de placa perfurada ("hole board"), do rotarod e das caixas de locomoção, não observamos efeitos significativos da guanosina por nenhuma via de administração. O baixo potencial tóxico da guanosina também foi demonstrado pelo cálculo de índice de letalidade, onde não foi observada qualquer morte em doses de até 960 $mg.kg^{-1}$ ($LD_{50} > 960\ mg.kg^{-1}$). A guanosina não causou redução de peso ou alterações na temperatura corporal, mesmo nos animais submetidos a doses elevadas do composto. Nos animais submetidos a injeções diárias de guanosina, também não observamos morte ou sinais grosseiros de toxicidade. Notadamente, os animais submetidos ao modelo de dor neuropática apresentaram até um ganho de peso ao longo do tratamento, o que pode estar relacionado com a melhora do quadro doloroso. Este perfil baixo de toxicidade, como boa tolerabilidade à administração central ou sistêmica da guanosina corrobora diversos estudos anteriores [Lara et al., 2001; Vinadé et al., 2003;2005]. Apenas o GMP em doses acima de 1 μmoles, quando administrado por via i.c.v. demonstraram sinais de toxicidade ao SNC com a ocorrência de crises convulsivas [capítulo II.2.a.].

Com intuito de verificar se a guanosina possui propriedades sedativas, investigamos o composto por via sistêmica e em doses analgésicas no modelo de sono induzido por fenobarbital em camundongos. De forma surpreendente, a guanosina produziu um efeito excitante ao SNC e reduziu significativamente o tempo de sono induzido. Este efeito excitante assemelha-se ao ocasionado por alguns antagonistas gabaérgicos e adenosinérgicos [El Yacoubi et al., 2003] e merece ser mais estudado em estudos futuros.

A administração de guanosina, independente da dose, não alterou a função renal dos animais. Entretanto, quando doses maiores do que 240 $mg.kg^{-1}$ foram administradas por via sistêmica, sinais de toxicidade hepática foram observados, com aumento significativo das enzimas hepáticas ALT e AST. Estudo recente demonstrou que a concentração de guanosina aumenta em até 2,8 x no fígado, atingindo o pico 15 minutos após sua administração (guanosina 8 $mg.kg^{-1}$) [Jiang et al., 2008]. Entretanto, aumento significativo da concentração de guanosina também ocorreu nos rins, pulmões e SNC. Ainda não há disponível conhecimento adequado a respeito da farmacocinética da guanosina, mas estudos recentes começaram a investigar estes aspectos. Na presente tese, podemos observar que a guanosina apresenta significativa penetração no SNC de ratos e camundongos quando administrada por via sistêmica. Após uma administração intraperitoneal, ocorreu um aumento significativo da concentração de guanosina no LCR com pico em 30 min e manutenção dos níveis aumentados até 360 min após, retornando ao basal 24 horas após como demonstrado no capítulo II.2.h. em ratos.

A guanosina apresenta diversos efeitos potencialmente benéficos (como neuroproteção, antinocicepção e efeitos tróficos). No entanto, apesar de um bom perfil de segurança sem efeitos tóxicos graves demonstrados nesta tese, a guanosina e outros derivados da guanina (GMP) apresentam um importante efeito adverso já bem estabelecido: déficits cognitivos – amnésia [Roesler et al., 2000; Vinadé et al., 2003; 2004; 2005; Saute et al., 2006]. Estes déficits são comuns a drogas que interagem com o sistema glutamatérgico, pois, uma vez que o glutamato constitui o principal neurotransmissor excitatório do SNC, torna-se muito difícil não interferir nos diversos circuitos que ele participa ao se empregar medicações não seletivas a determinados tipos de receptores. Esta pode ser uma grande limitação para sua aplicação na prática clínica.

III.1.f. Caracterização de um modelo para investigar a captação global de glutamato na medula espinhal de camundongos

Com o objetivo de avaliar o papel dos derivados da guanina na nocicepção e propor um mecanismo de ação que envolva a captação de glutamato, objetivamos padronizar um método para avaliação da captação global de glutamato em medula espinhal de camundongos. Este estudo proposto no capítulo II.2.e. desta tese procurou utilizar como base modelos já bem estabelecidos em nossa instituição e onde importantes avanços no entendimento do mecanismo de ação da guanosina foram obtidos [Frizzo et al., 2002; 2005; de Oliveira et al., 2004; Moretto et al., 2005; 2008; Leke et al., 2006; Thomazi et al. 2008].

Há crescente evidência na literatura de que o glutamato é liberado no SNC após estímulos dolorosos periféricos e que alterações no metabolismo e vias glutamatérgicas em nível medular contribuem para a gênese de hiperalgesia e sensibilização central [Willis, 2001]. Considerando que a captação de glutamato através de transportadores específicos é a principal forma de manter a concentração deste aminoácido na sinapse dentro de níveis fisiológicos [Danbolt, 2001; Liaw et al., 2005] e que alterações na expressão e eficácia destes transportadores está relacionada à disfunção na transmissão sensorial [Liaw et al., 2005; Weng et al., 2006; 2007], o estudo da captação medular de glutamato é extremamente importante. Estudo anterior demonstrou que a constrição crônica do nervo ciático causa um padrão bifásico na expressão de transportadores de glutamato na medula [Sung et al., 2003]. Nos primeiros 4 dias após a lesão ocorre um aumento da expressão dos transportadores glutamatérgicos no corno dorsal da medula espinhal, seguida de uma diminuição progressiva. Neste mesmo estudo, a captação de glutamato diminuiu de forma

significativa após 5 dias da lesão. Portanto, a diminuição da captação de glutamato devido a redução da expressão de transportadores parece contribuir para os mecanismos de sensibilização central após lesões nervosas como a constrição crônica do nervo ciático [Willis, 2001]. Nesta tese, observamos que o modelo de captação global de glutamato em fatias de medula espinhal foi capaz de detectar diversas alterações induzidas por modelos de dor. Em modelos de dor aguda observamos uma resposta com aumento da captação global de glutamato após a administração de agentes algogênicos como a capsaicina. Em contrapartida, no modelo de contrição crônica do nervo ciático, observamos uma redução significativa na captação de glutamato em fatias de medula 14 dias após a lesão inicial. Estes dados corroboram os expostos acima, e sugerem que há uma retro-alimentação positiva aumentando a quantidade de transportadores após um estímulo agudo, ainda dentro de parâmetros fisiológicos. Entretanto, caso este estímulo persista (modelo de constrição nervosa crônica), alterações neuroplásticas e retro-alimentação negativa de transportadores ocorrem, causando alterações fenotípicas características em animais (hiperalgesia e alodinia). Portanto, estes resultados apóiam a proposta de que o modelo de captação de glutamato em fatias de medula espinhal é método sensível e reprodutível na investigação da capacidade funcional dos transportadores de glutamato de células neurais em nível medular, a exemplo do que já é tradicionalmente aceito para fatias cerebrais [Frizzo et al., 2002].

Os dados expostos neste capítulo permitem inferir que glutamato é efetivamente captado por fatias de medula espinhal de ratos e camundongos. Algumas evidências sugerem que este efeito se dá pela ação de transportadores, pois o acúmulo de glutamato no interior da fatia apresentou um padrão ao longo do tempo e correlacionou-se de forma significativa com a quantidade de proteína e com a concentração de glutamato,

apresentando um padrão de saturação semelhante ao demonstrado anteriormente em fatias de córtex e hipocampo [Frizzo et al., 2002].

Considerando que a captação de glutamato em nível medular é crítica para a manutenção da transmissão sensorial normal [Liaw et al., 2005; D'Mello e Dickenson, 2008] e que inibição da captação de glutamato na medula espinhal causa hiperalgesia e amplifica as respostas neuronais à estimulação das vias aferentes primárias [Weng et al., 2006], a padronização de um modelo simples e consistente que avalie a captação de glutamato em medula é método interessante para investigação dos efeitos de novos fármacos como a guanosina.

Os estudos recentes que avaliam a captação de glutamato em nível medular geralmente utilizam modelos eletrofisiológicos, abordagens guiadas por microdiálise e preparação de sinaptossomas de medula espinhal [Weng et al., 2006;2007]. Apesar destas abordagens apresentarem metodologia mais refinada e mais específica, também apresentam maior complexidade, custo e exigem maior treinamento. O método proposto nesta tese apresenta diversas limitações: altas concentrações de glutamato na preparação das fatias (100 μM); contribuição diferencial de células gliais e neurônios para a captação não foi avaliada; contribuição diferencial do corno dorsal e ventral da medula não foi avaliada; morte celular significativa (~30%) após o período de pré-incubação. Apesar dessas limitações, argumentamos que o modelo é simples e de baixo custo, além de permitir uma forma de triagem da influência de modelos animais e fármacos sobre o estado funcional dos transportadores neurais de glutamato na medula espinhal.

III.1.g. Efeitos antiglutamatérgicos da guanosina e interação com o antagonista do receptor NMDA MK-801

Nos capítulos II.2.c. e II.2.d., foram demonstrados os resultados de que a guanosina é capaz de contrabalançar efeitos comportamentais paradoxais induzidos pelo antagonista do receptor NMDA MK-801. A hipótese que se levantou para explicar tal resultado foi embasada nos trabalhos da nossa instituição que demonstram que a guanosina estimula a captação de glutamato pelos astrócitos [Frizzo et al, 2001, 2002, 2003], principal mecanismo de remoção do glutamato da fenda sináptica [Danbolt, 2001], associado com a potencial dependência glutamatérgica da ação de antagonistas NMDA [Moghaddam et al, 1997].

Embora pareça um pouco paradoxal que antagonistas glutamatérgicos exerçam seus efeitos por um aumento de atividade glutamatérgica, tem sido demonstrado que, de fato, muitas das ações dos antagonistas de receptores glutamatérgicos do tipo NMDA parecem ser devidas a um aumento da liberação de glutamato em algumas regiões cerebrais, o que resultaria numa maior ativação de receptores não-NMDA [Moghaddam et al., 1997].

Uma explicação possível de como isso ocorreria é imaginar que neurônios GABAérgicos não estão sendo mais estimulados por aferências glutamatérgicas via receptor NMDA. Assim, estes neurônios exerceriam uma menor inibição sobre outros neurônios. Em particular, a desinibição de neurônios glutamatérgicos permitiria uma maior liberação de glutamato e subseqüente aumento de sua ação em receptores não-NMDA do tipo AMPA e cainato. Contudo, embora este esquema envolvendo sinapses GABAérgicas fornece uma boa explicação, esta hipótese é puramente especulativa e o verdadeiro mecanismo responsável pelo aumento de glutamato extracelular em algumas regiões

cerebrais proporcionado por antagonistas NMDA permanece em estudo. Entretanto, é altamente provável que muitos dos efeitos adversos psicomiméticos desencadeados por altas doses de antagonistas do receptor NMDA como cetamina e MK-801 podem se dever a uma hiperativação glutamérgica, desencadeada pela liberação maciça de aminoácidos excitatórios no espaço extracelular.

Esta hipótese é amplamente descrita e fundamentada nos dois capítulos supracitados. O capítulo II.2.c. descreve e fundamenta as bases para esta teoria. Neste trabalho, investigamos os efeitos do antagonista NMDA MK-801 em diferentes doses sobre a transmissão de dor através de um modelo de dor aguda. Além disso, investigamos a interação entre o MK-801 e outras substâncias antiglutamatérgicas como os derivados da guanina e o riluzole. De forma interessante, demonstramos que o MK-801 promove um aumento significativo nos níveis liquóricos de aspartato e glutamato, evento que se correlaciona significativamente com os níveis de dor medidos de forma indireta pela retirada da cauda do animal a um estimulo doloroso artificial. Como guanosina, GMP, riluzole e o controle positivo morfina foram capazes de reverter os efeitos comportamentais e neuroquímicos, podemos inferir que o MK-801, em doses elevadas, causa hiperalgesia por promover um aumento significativo nos níveis extracelulares de aminoácidos excitatórios. Entretanto, através apenas da metodologia abordada neste estudo, não podemos determinar por que mecanismo este evento ocorreu. Visto que diferentes fármacos com mecanismos de ação diversos causaram o mesmo resultado, é possível que diferentes mecanismos estejam envolvidos.

O capítulo II.2.d. procura discutir de forma crítica o trabalho realizado por outra instituição [Deutsch et al., 2008]. Neste estudo, efeitos comportamentais induzidos pelo MK-801 foram novamente revertidos por guanosina. Os autores interpretam a reversão dos

efeitos comportamentais do MK-801 pela guanosina, como conseqüente a um déficit de inibição do receptor NMDA desencadeado pela menor quantidade de glutamato disponível na fenda sináptica [Deutsch et al., 2001, 2008]. Entretanto, utilizamos esta metodologia e resultados como nova oportunidade para discutir as hipóteses supracitadas e que podem explicar nossos resultados sobre a dor e os resultados prévios sobre atividade locomotora [Tort et al., 2004].

Uma vez que o uso de antagonistas NMDA tem sido apontado como um modelo farmacológico para a esquizofrenia [O'Neill e Shaw, 1999], um futuro papel para a guanosina como medicação antipsicótica é possível e já foi proposto anteriormente [Tort et al., 2004]. Ainda, como a guanosina é capaz de reverter efeitos adversos do MK-801, é possível que sua administração conjunta com MK-801 ou outros antagonistas NMDA, em doses adequadas, aumente a efetividade destes fármacos no tratamento de eventos neurotóxicos ou mesmo em quadros dolorosos crônicos.

III.1.h. Efeitos antinociceptivos do alopurinol: o papel do sistema purinérgico

Como a utilização de agonistas do sistema purinérgico como adenosina e guanosina ainda é possibilidade relativamente distante, uma abordagem que tem sido utilizada para investigar o potencial deste sistema na prática clínica é a administração de derivados do sistema purinérgico como o alopurinol [Schmidt et al., 2007]. Fármacos como o alopurinol apresentam grande experiência de uso e perfil de segurança no tratamento de outras doenças como gota e apresentam documentada capacidade em modular de forma indireta

(através da regulação dos níveis de purinas) a atividade do sistema purinérgico [Marro et al., 2006].

No capítulo II.2.i. apresentamos uma investigação ampla do alopurinol em modelos de dor aguda em camundongos. Os resultados demonstram efeitos significativos em quatro modelos clássicos de dor aguda e sugerem que estes efeitos estejam relacionados à capacidade do alopurinol em aumentar os níveis de purinas como guanosina e principalmente a adenosina. Os antagonistas adenosinérgicos cafeína e DPCPX (antagonista seletivo A_1) reverteram os efeitos antinociceptivos do alopurinol, um efeito não replicado pelo antagonista de receptores opióides naloxona e pelo antagonista de receptor A_{2a} SCH58261. Estes dados corroboram a existência de efeitos antinociceptivos agudos do alopurinol, já demonstrados em parte anteriormente, mas nunca efetivamente estudados [Pinelli et al., 1991; Daskalopoulou et al., 2005; Inkster et al., 2007; Hacimuftuoglu et al., 2006].

O mecanismo de ação básico do alopurinol é o de inibir a enzima xantina oxidase ligando-se de forma intensa à forma reduzida desta enzima. Este evento leva a uma redução da formação de ácido úrico a partir de seus precursores xantina e hipoxantina [Day et al., 2007]. Conseqüentemente, acumulo de adenosina, guanosina e inosina podem ser observados [Marro et al., 2006]. Neste estudo, observamos o aumento significativo de xantina, hipoxantina, guanosina, adenosina, inosina e AMP. Os níveis de adenosina e guanosina aumentaram em 7 e 14 vezes, respectivamente, após a administração de alopurinol (200 $mg.kg^{-1}$). Este estudo também demonstrou que o alopurinol atinge níveis significativos no SNC de camundongos, e a concentração estimada 30 min após a sua administração na dose de 200 $mg.kg^{-1}$ foi de aproximadamente 58 μM.

Apesar de ser ainda precoce a utilização de derivados da guanina e da adenina em estudos clínicos no tratamento da dor ou de doenças do SNC, uma abordagem interessante e viável é a utilização de derivados de purinas como o alopurinol. O alopurinol já possui diversas evidências que demonstram sua eficácia no tratamento de esquizofrenia, comportamento agressivo, quadros psicóticos e epilepsia [Tada et al., 1991; Wada et al., 1992; Zagnoni et al., 1994; Lara et al., 2000; 2003; Machado-Vieira et al., 2001; Akhondzadeh et al., 2005; Brunstein et al., 2005]. Nossa hipótese principal se baseia no aumento indireto do tônus purinérgico representado pelos nucleosídeos adenosina e guanosina, como demonstrado nesta tese [capítulo II.2.i.]. Considerando a capacidade das purinas derivadas da guanina e da adenina em modular a dor e os efeitos significativos do alopurinol sobre níveis de purinas no SNC, é relevante propor esta estratégia de tratamento em diversos quadros clínicos, tais como: dor aguda e/ou crônica, doenças psiquiátricas e doenças neurodegenerativas. Esta perspectiva parece bastante promissora e está atualmente em desenvolvimento em nossa instituição.

III.1.i. Modulação *in vivo* do sistema purinérgico pelo exercício físico: efeitos sobre a transmissão da dor

Esta tese objetiva demonstrar os efeitos do sistema purinérgico sobre a dor e demonstrar funções biológicas importantes deste sistema em nossa homeostase. No capítulo II.2.j. desta tese, objetivamos utilizar um modelo de exercício físico voluntário tradicionalmente utilizado em nossa instituição [Dietrich et al., 2005;2008] com intuito de demonstrar uma forma de modular, em um contexto fisiológico, o sistema purinérgico *in vivo*.

Muitos estudos têm demonstrado que a atividade física promove diversas alterações neuroplásticas no SNC de mamíferos [Cotman e Berchtold, 2002; Larsen et al., 2000; Tillerson et al., 2003]. Neste protocolo ainda em andamento, demonstramos sucintamente que 45 dias de exercício voluntário apresenta efeito antinociceptivo em três modelos clássicos de dor aguda. Estes efeitos parecem ser parcialmente modulados pelo sistema opióide e pelo sistema purinérgico. Os efeitos antinociceptivos apresentam caráter dinâmico e evoluem ao longo do tempo de acordo com a exposição ou não ao exercício. Alterações neuroplásticas devem ocorrer ao longo da exposição ao exercício, pois mesmo após a suspensão da atividade física por um período de 30 dias, os animais apresentaram uma capacidade de modular, de forma significativamente mais rápida, o limiar de dor no modelo de capsaicina. Entretanto, este efeito não foi replicado nos modelos térmicos de dor, o que pode estar relacionado a alterações intrínsecas de cada modelo [Le Bars et al., 2001].

Em relação ao sistema purinérgico, este estudo demonstra evidências, através de técnicas bioquímicas e de biologia molecular, que receptores adenosinérgicos A_1, localizados em nível medular, parecem estar envolvidos a neuroplasticidade mediada pelo exercício físico. Além disso, liberação endógena de derivados da adenina e da guanina parece contribuir com este processo. Um importante substrato para a liberação de adenosina, poderia ser o plexo coróide. Neste estudo, observamos a capacidade desta estrutura vascular em secretar ativamente todos os integrantes do sistema purinérgico. Esta abordagem encontra-se em fase bastante inicial, mas após a exposição desta estrutura ao ATP, a avaliação da hidrólise de nucleotídeos demonstra que há acúmulo de ADP e inosina e queda na formação de xantina. A liberação de purinas pelo plexo coróide pode explicar também as alterações significativas observadas nos níveis de purinas no LCR dos animais submetidos ao exercício físico voluntário. Observamos nestes animais um aumento significativo de

GTP, GDP, guanosina e adenosina no LCR e uma queda nos níveis liquóricos de hipoxantina, xantina e ácido úrico.

Os efeitos induzidos pelo exercício físico voluntário sobre o metabolismo de purinas no LCR e no plexo coróide *ex vivo*, parecem simular um efeito inibitório sobre a enzima PNP, responsável pela transformação de guanosina em guanina e de inosina em hipoxantina [Schmidt et al., 2007]. Para testar esta hipótese, administramos um inibidor específico desta enzima (imucilina H), por via i.c.v., em camundongos. Notadamente, a administração de imucilina H foi capaz de replicar os efeitos antinociceptivos do exercício físico sobre o modelo da capsaicina.

Apesar de incipientes e ainda passíveis de replicação, estes resultados apresentam-se promissores, sugerindo que o exercício físico voluntário é capaz de modular de forma fisiológica o sistema purinérgico e o limiar de dor em camundongos. Este efeito pode estar relacionado a mecanismos multifatoriais, mas são mediados, pelo menos parcialmente, por receptores adenosinérgicos do tipo A_1 em nível medular.

III.1.j. Efeitos da dor sobre os níveis liquóricos de purinas em humanos

A dor em humanos é um evento complexo e multifatorial, sendo, portanto, muito difícil de ser avaliada adequadamente [Millan, 1999]. Vários modelos humanos de dor têm sido propostos, mas apresentam limitações inequívocas quanto a questões metodológicas e aplicabilidade dos resultados [Le Bars et al. 2001]. Nesta tese, procuramos avaliar pacientes portadores de quadros dolorosos agudos e/ou crônicos de diferentes etiologias e detectar alterações nos níveis liquóricos de componentes do sistema purinérgico destes pacientes. Através desta abordagem simples e de baixo custo, sem causar sofrimento adicional aos

pacientes, procuramos correlacionar as alterações nas concentrações de purinas no SNC com a intensidade, duração e tipo de dor em pacientes submetidos a cirurgias sob anestesia espinhal. Os modelos de dor escolhidos para estes estudos detalhados nos capítulos II.3.a e II.3.b. foram: dor aguda: dor no trabalho de parto (dor predominantemente visceral) e dor proveniente de fratura ortopédica ou isquemia aguda de membros (dor predominantemente somática); dor crônica: ortopédica e vascular (dor predominantemente somática com componente neuropático em determinados casos).

Através da quantificação de purinas no LCR de gestantes com ou sem dor, e pacientes portadores ou não de quadros dolorosos crônicos podemos investigar o papel dessas substâncias no mecanismo de transmissão dolorosa. Entretanto, simplesmente quantificar os níveis liquóricos de purinas em pacientes com dor não estabelece uma relação causal entre a dor e as alterações purinérgicas encontradas. Correlacionar os níveis de purinas com a intensidade e o tempo de exposição à dor e propor hipóteses para eventuais alterações significativas foram os objetivos principais destes estudos. É impossível determinar, através desta metodologia, se as alterações observadas sobre os níveis extracelulares de integrantes do sistema purinérgico representam causa ou conseqüência das alterações álgicas estudadas.

A gestação cursa freqüentemente e de forma aguda, durante o período do trabalho de parto, com dor de forte intensidade. Portanto, podemos considerar o trabalho de parto como um modelo fidedigno de dor aguda em humanos, principalmente de caráter visceral [Melzack, 1993]. Considerando o papel fundamental das purinas na transmissão dolorosa [Sawynok e Liu, 2003], o objetivo principal do estudo exposto no capítulo II.3.a. foi o de comparar os níveis de purinas no LCR em quatro grupos de pacientes: i. gestantes como dor; ii. gestantes sem dor; iii. mulheres sem dor; iv. homens sem dor. Os dois últimos

grupos seriam controles absolutos. Este estudo foi composto apenas por pacientes sem comorbidades significativas. Estes pacientes foram submetidos a procedimentos eletivos ou de urgência, mas não faziam uso de fármacos específicos e eram classificados como pacientes classe I pela Associação Americana de Anestesiologistas (ASA), ou seja, pacientes completamente hígidos. É importante ressaltar que cálculo amostral foi realizado e erro tipo II é improvável, pois 168 pacientes, com características homogenias, foram incluídos neste estudo. Resumidamente, os resultados encontrados demonstraram que os níveis de adenosina estavam significativamente reduzidos e correlacionados negativamente como a intensidade da dor relacionada ao trabalho de parto. Também observamos que os níveis liquóricos de algumas purinas como adenosina e guanosina estavam significativamente aumentados em pacientes gestantes quando comparadas a não gestantes.

Como o sistema purinérgico parece desempenhar papel relevante principalmente nos mecanismos de manutenção de quadros dolorosos [Sawynok e Liu, 2003; Sperlágh e Illes, 2007], no capítulo II.3.b., apresentamos um estudo em pacientes portadores de dor aguda e/ou crônica. Neste estudo, objetivamos avaliar a correlação entre alterações nas concentrações liquóricas de purinas e quadros álgicos crônicos. Sucintamente, os resultados demonstraram que pacientes portadores de quadros dolorosos crônicos de origem predominantemente somática apresentaram níveis elevados de IMP, inosina, guanosina e ácido úrico. Surpreendentemente, os níveis destas purinas estavam significativamente correlacionados com a intensidade da dor, mas não com a duração da dor.

As implicações fisiopatológicas dos achados apresentados nesta tese são difíceis de interpretar devido à metodologia empregada. Entretanto, os dados apresentados acima apontam para um papel relevante do sistema purinérgico, incluindo os derivados da guanina, nos mecanismos de transmissão e manutenção da dor em humanos. Estas

alterações talvez representem mudanças no metabolismo energético cerebral e medular que pode ocorrer durante diversos insultos ao SNC [Castro-Gago et al., 1995; Rodriguez-Núñes et al., 2000]. Considerando o papel essencial do ATP extracelular como agente pró-nociceptivo no SNC [Liao e Chen, 2001; Burnstock, 2007], nossos resultados podem perfeitamente refletir liberação celular de ATP e sua conseqüente metabolização em nível extracelular. Esta liberação de ATP pode ser conseqüência de um estimulo doloroso inicial, mas pode participar também dos mecanismos de manutenção e persistência da dor [Burnstock, 2007]. ATP age em receptores específicos localizados na glia e está intimamente relacionado à ativação glial que ocorre após estímulos dolorosos sustentados como os expostos acima [Liao e Chen, 2001; Watkins e Milligan, 2001; Watkins e Maier, 2003; Burnstock, 2007]. Portanto, podemos especular, de forma ainda incipiente, que as alterações encontradas nos níveis de purinas no LCR de pacientes com dor representam metabolização do ATP e reiteram o papel deste nucleotídeo nas vias de transmissão dolorosa.

III.1.l. Considerações finais

A presente tese apresentou alguns resultados experimentais obtidos das investigações do potencial efeito antinociceptivo dos derivados da guanina, em especial da guanosina, em diversos modelos animais de dor, bem como apresentou também algumas abordagens farmacológicas e neuroquímicas para auxiliar no entendimento do mecanismo de ação responsável pelos efeitos destas substâncias no SNC de mamíferos.

Durante a formulação desta tese e através da seqüência apresentada dos trabalhos, podemos observar que houve uma certa evolução no conhecimento sobre potenciais

benefícios e funções para os derivados da guanina. Uma nova utilização e um novo papel biológico para essas substâncias foi proposto e isto certamente foi a maior contribuição da presente tese.

Os resultados farmacológicos indicam inequivocamente e de forma razoavelmente reprodutível que os derivados da guanina, em especial a guanosina, são antinociceptivos em roedores. A investigação farmacológica também demonstrou que este sistema provavelmente desempenha este "novo" papel independentemente dos derivados da adenina e do clássico sistema opióide. Através ainda dos estudos farmacológicos com agonistas do sistema glutamatérgico, demonstramos que a guanosina provavelmente exerce suas funções antinociceptivas por modular vias glutamatérgicas. Entretanto, a principal hipótese para seus efeitos sobre o sistema glutamatérgico (efeitos sobre a captação de glutamato) não foi efetivamente demonstrada ao longo dos trabalhos apresentados nesta tese. *In vitro*, demonstramos estes efeitos apenas em doses elevadas de guanosina. *In vivo*, estes efeitos, de fato, nem ocorreram. A guanosina foi capaz, no máximo, de modular a captação de glutamato em fatias como provável conseqüência de seus efeitos antinociceptivos e não como causa ou mecanismo responsável por estes efeitos. Além disso, esta tese também apresentou alguns resultados contraditórios, tanto em paradigmas comportamentais como na abordagem neuroquímica do mecanismo de ação da guanosina.

Estes efeitos contraditórios podem se dever a múltiplos fatores, tais como: i. a limitação do modelo de captação em fatias de medula; ii. a falta de análogos e de antagonistas específico de guanosina ou de derivados da guanina; iii. ao total desconhecimento de seus receptores ou da real existência dos mesmos; iv. a falta de conhecimento adequado sobre a farmacocinética da guanosina e dos demais derivados da guanina e de sua real potência farmacológica *in vitro* e *in vivo*; entre outros diversos fatores.

Estes aspectos ainda necessitam ser investigados com mais profundidade em pesquisas futuras.

Por fim, observo que esta tese demonstrou resultados de significado clínico incontestável. Estes efeitos foram obtidos através da investigação de um sistema relativamente novo de neurotransmissão: o sistema purinérgico. Especificamente em relação ao sistema purinérgico dos derivados da guanina, demonstramos pela primeira vez a existência de efeitos antinociceptivos *in vivo* e a futura utilização deste sistema como alvo para desenvolvimentos de novos fármacos analgésicos deve ser fortemente considerada.

III.2. CONCLUSÕES

A partir dos resultados expostos nesta tese, as seguintes conclusões podem ser formuladas:

III.2.a. Estudo de revisão:

- Através dos dados disponíveis na literatura, um novo sistema de neurotransmissão foi proposto: o sistema purinérgico dos derivados da guanina. Neste contexto, os derivados da guanina desempenham diversos efeitos extracelulares como modulação da atividade glutamatérgica, efeitos tróficos em células neurais, efeitos neuroprotetores contra estímulos nocivos ao SNC e efeitos comportamentais sobre memória e aprendizado. Esses achados demonstram que este sistema pode ser um novo alvo para o desenvolvimento de novos fármacos neuroprotetores.

III.2.b. Resultados experimentais com animais:

- Novos dados ratificam os derivados da guanina como anticonvulsivantes em modelos de convulsão induzida por agonistas glutamatérgicos (ácido quinolínico).

- O nucleotídeo GMP e o nucleosídeo guanosina possuem efeitos antinociceptivos em modelos animais de dor aguda.

- A guanosina, quando administrado por via intracerebroventricular, intratecal e sistêmica, demonstra efeitos antinociceptivos em diversos modelos animais de dor.

- A guanosina é capaz de reduzir os níveis de dor e melhorar a capacidade funcional dos animais submetidos a modelo de dor crônica neuropática.

- Os efeitos comportamentais dos derivados da guanina parecem ser mediados pela sua conversão até guanosina.

- MK-801 induz hiperalgesia em modelo animal de dor aguda por aumentar os níveis de glutamato e aspartato no líquido cefalorraquiano: guanosina reverte estes efeitos por modular a atividade glutamatérgica.

- O modelo de captação de glutamato em fatias de medula é método simples e consistente para investigar a modulação do sistema glutamatérgico na medula espinhal.

- A guanosina é capaz de modular a captação de glutamato em fatias de medula espinhal apenas em altas doses *in vitro*.

- Os efeitos antinociceptivos da guanosina parecem estar relacionados a sua capacidade de modular vias glutamatérgicas.

- O alopurinol apresenta efeitos antinociceptivos em diversos modelos de dor aguda, efeito que parece dever-se ao acumulo de adenosina e de guanosina.

- O exercício físico voluntário é capaz de modular o sistema purinérgico *in vivo* e apresenta efeitos antinociceptivos relacionados ao aumento do tônus do sistema purinérgico endógeno.

III.2.c. Resultados experimentais com humanos:

- Os níveis liquóricos das purinas são modulados pela dor aguda e/ou crônica em humanos.

- ATP e adenosina estão associados com os mecanismos de dor visceral no trabalho de parto em humanos.

- As purinas, em especial a inosina e a guanosina, estão relacionadas aos quadros dolorosos somáticos crônicos e agudos em humanos.

III.3. PERSPECTIVAS

Em relação ao modelo de captação de glutamato em fatias de medula espinhal proposto nesta tese, novas investigações ainda devem ser realizadas com intuito aprimorar este modelo. O objetivo principal é o de utilizar este modelo na investigação do sistema glutamatérgico nos mais diversos modelos de dor e sua modulação por fármacos como a guanosina. O modelo parece interessante também para a investigação do potencial efeito neuroprotetor da guanosina sobre as células neurais na medula espinhal, proposto por outros trabalhos. Além disso, outros fármacos e sistemas de neurotransmissão podem ser avaliados e investigados utilizando o modelo proposto que, apesar de limitado, apresenta-se viável, consistente e simples.

Durante a realização desta tese, tivemos a oportunidade de padronizar diversos modelos de dor aguda e crônica nesta instituição, o que permitirá importantes interações com outros pesquisadores no sentido de investigar novas propostas terapêuticas como as apresentadas nesta tese. Especial atenção deve ser concedida ao modelo de dor neuropática apresentado nesta tese, que já foi replicado em camundongos dentro de nossa instituição e apresenta-se como ferramenta robusta na investigação da fisiopatologia da dor. Nossa instituição apresenta infra-estrutura diferenciada na forma de metodologias para avaliação dos sistemas glutamatérgico e purinérgico que podem ser amplamente investigados através da utilização deste modelo animal.

Com relação aos resultados experimentais obtidos com a utilização de guanosina, adenosina e alopurinol, as perspectivas que naturalmente surgem são as de testar estes compostos na prática clínica, a fim de verificar suas potenciais ações antinociceptivas em humanos. É possível que a guanosina venha a apresentar efeitos clínicos semelhantes aos

encontrados com o uso de outros fármacos antiglutamatérgicos como a cetamina, o riluzole, e a lamotrigina. De fato, os análogos de adenosina já apresentam algum resultado clínico, mas ainda poucos estudos foram realizados e mais evidência é preciso surgir. Contudo, a ausência de estudos prévios empregando a guanosina em humanos torna necessária primeiramente a realização de uma série de testes para verificar sua segurança. Dados iniciais desta tese demonstram um bom perfil de segurança em animais, mas ainda são bastante incipientes. Ou seja, neste momento, o uso da guanosina na prática clínica constitui uma perspectiva ainda bastante distante.

Por outro lado, substâncias análogas do sistema purinérgico, como por exemplo o alopurinol, já vêm sendo utilizadas clinicamente há anos, e são consideradas drogas seguras e bem toleradas pela maioria dos pacientes, de maneira que a perspectiva de testá-las na prática clínica é bem mais próxima. De fato, a avaliação de um destes compostos como potencial medicação antinociceptiva já está em andamento. Atualmente estou coordenando dois ensaios clínicos randomizados no Hospital de Clínicas de Porto Alegre, de caráter duplo-cego e controlados por placebo, que visam inicialmente estudar o efeito do alopurinol como fármaco antinociceptivo contra dores aguda e crônica. Estes projetos possuem perspectivas de resultados para o final do próximo ano e dados preliminares apresentam resultados altamente promissores no tratamento de pacientes portadores de fibromialgia, síndrome dolorosa refratária aos tratamentos analgésicos convencionais.

Também como perspectiva de utilizar metodologias não-farmacológicas no tratamento da dor, emerge o exercício físico como uma possibilidade de modular o sistema purinérgico e a transmissão dolorosa de forma significativa. Este estudo apresentado nesta tese ainda está em desenvolvimento e uma ampla investigação neuroquímica ainda está em andamento. O objetivo e perspectivas são de entender um pouco mais sobre o como o

sistema purinérgico pode ser modulado *in vivo* e de como este sistema é capaz de modular as vias de transmissão nervosa.

Esta tese apresenta alguns dados experimentais em humanos demonstrando que somos capazes de interagir ciência de área básica e clínica de forma efetiva. A idéia de investigar os sistema purinérgico *in vivo* em humanos não é inovadora nem complexa, mas permitiu documentar que a presença de dor, seja ela aguda ou crônica, é capaz de promover alterações altamente significativas nos níveis de purinas no espaço extracelular do SNC, representado pelo líquido celaforraquidiano. Ainda através destes estudos, demonstramos que a investigação do líquido celaforraquidiano humano ainda é capaz de nos fornecer ferramentas fundamentais para o entendimento da homeostase. Também é importante ressaltar a utilização de modelos de dor aguda e crônica em humanos de forma bastante simples e eficaz, sem acarretar sofrimento adicional ao já existente ou a utilização de modelos humanos de dor em voluntários saudáveis.

É importante ressaltar que os projetos iniciais com humanos ainda contemplavam a utilização deste material biológico escasso e nobre (líquido celaforraquidiano) na realização de estudos adicionais, investigando o papel dos aminoácidos e de fatores tróficos e a potencial correlação destas substâncias com quadros dolorosos agudos ou crônicos. Os dados provenientes destes estudos não foram incluídos nesta tese por questões didáticas, de espaço e enfoque, mas permitem inferir resultados altamente promissores sobre o papel destas substâncias na transmissão dolorosa. Além disso, esta abordagem nos permite correlacionar os mais diversos sistemas de neurotransmissão e neuromodulação *in vivo* com a presença de características clínicas específicas como a dor.

Por fim, como uma perspectiva mais ampla e de longo prazo, fica o plano de se implantar uma linha consistente de pesquisa dentro de nossa universidade que vise fazer

novos estudos avaliando o sistema purinérgico e seus derivados na transmissão da dor, na anestesiologia e na medicina intensiva. Em 2009 tenho previsto a realização de projetos associados a um grupo de pesquisa em anestesiologia clínica e básica, onde espero adquirir conhecimentos e novas metodologias que ajudem a implementar tal campo de pesquisa dentro de nossa instituição.

III.4. REFERÊNCIAS

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ANEXOS

A.1. Formação acadêmica e produção científica durante o doutoramento (2004-2008)

Formação Acadêmica:

- Especialização em Anestesiologia pela Disciplina de Anestesiologia da Faculdade de Medicina da Universidade de São Paulo (2003-2005).

- Preceptoria em anestesia e certificação em anestesia para transplantes de órgãos pela Disciplina de Anestesiologia da Faculdade de Medicina da Universidade de São Paulo (2006).

- Título Superior em Anestesiologia pela Sociedade Brasileira de Anestesiologia (2006).

- Certificação na área de atuação em dor pela Associação Médica Brasileira e Sociedade Brasileira de Anestesiologia (2008).

Lista de artigos científicos publicados em periódicos internacionais indexados com índice de impacto conhecido durante a realização desta tese:

1. **Schmidt AP**, Böhmer AE, Schallenberger C, Antunes C, Pereira ML, Leke R, Wofchuk ST, Elisabetsky E, Souza DO. Spinal mechanisms of antinociceptive action caused by guanosine in mice. Eur J Pharmacol 2009, no prelo.

2. **Schmidt AP**, Böhmer AE, Antunes C, Schallenberger C, Porciúncula LO, Elisabetsky E, Lara DR, Souza DO. Antinociceptive properties of the xanthine oxidase inhibitor allopurinol in mice: role of A_1 adenosine receptors. Br J Pharmacol 2009; 156:163-172.

3. **Schmidt AP**, Tort AB, Silveira PP, Böhmer AE, Hansel G, Knorr L, Schallenberger C, Dalmaz C, Elisabetsky E, Crestana RH, Lara DR, Souza DO. The NMDA antagonist MK-

801 induces hyperalgesia and increases CSF excitatory amino acids in rats: Reversal by guanosine. Pharmacol Biochem Behav 2009; 91:549-553.

4. Tango HK, **Schmidt AP**, Mizumoto N, Lacava M, Auler JO. Low hematocrit levels increase intracranial pressure in an animal model of cryogenic brain injury. Journal of Trauma 2009; 66:720-726.

5. **Schmidt AP**, Böhmer AE, Leke R, Schallenberger C, Antunes C, Pereira MS, Wofchuk ST, Elisabetsky E, Souza DO. Antinociceptive effects of intracerebroventricular administration of guanine-based purines in mice: evidences for the mechanism of action. Brain Res 2008; 1234:50-58.

6. **Schmidt AP,** Tort ABL, Lara DR, Souza DO**.** Guanosine and its modulatory effects on the glutamatergic system. Eur Neuropsychopharmacol 2008; 18:620-622.

7. Tavares RG, **Schmidt AP**, Tasca CI, Souza DO. Quinolinic acid-induced seizures stimulate glutamate uptake into synaptic vesicles from rat brain: effects prevented by guanine-based purines. Neurochem Res. 2008; 33(1): 97-102.

8. **Schmidt AP**, Lara DR, Souza DO. Proposal of a guanine-based purinergic system in the mammalian central nervous system. Pharmacol Ther 2007; 116(3):401-416.

9. **Schmidt AP**, Valinetti EA, Bertachi MF, Simões CM, Bandeira D, Auler JO. Effects of midazolam, clonidine or dexmedetomidine on postoperative pain and anxiety in children. Ped Anesth 2007; 17:667-74.

10. Leke R, Oliveira DL, **Schmidt AP**, Avila TT, Jorge RS, Fisher A, Wofchuck S, Souza DO, Portela LV. Methotrexate induces seizure and decreases glutamate uptake in brain slices: prevention by ionotropic glutamate receptors antagonists and adenosine. Life Sci 2006; 80(1):1-8.

11. Lelis RG, Krieger JE, Pereira AC, **Schmidt AP**, Carmona MJ, Oliveira SA, Auler JO. Apolipoprotein E4 genotype increases the risk of postoperative cognitive dysfunction in patients undergoing coronary artery bypass graft surgery. J Cardiovasc Surg 2006;47(4):451-456.

12. Otsuki D, Cardieri FA, **Schmidt AP**, Brudniewski M, Fantoni D, Auler JO. Comparison between intermittent and continuous measurement of cardiac output after acute normovolemic hemodilution in pigs. Artif Organs. 2006; 30(6):458-466.

13. Tavares RG, **Schmidt AP**, Abud J, Tasca CI, Souza DO. In vivo quinolinic acid increases synaptosomal glutamate release in rats: reversal by guanosine. Neurochem Res 2005; 30(4):1-6.

14. **Schmidt AP**, Takahashi ME, Posso IP. Phantom limb pain induced by spinal anesthesia. Clinics 2005; 60(3):263-264.

15. **Schmidt AP**, Ávila TT, Souza DO. Intracerebroventricular guanine-based purines protect against seizures induced by quinolinic acid in mice. Neurochem Res 2005; 30(1):69-73.

16. Vinadé ER, **Schmidt AP**. Effects of chronic administered guanosine on behavioral parameters and b glutamate uptake in rats. J Neurosci Res 2005; 79:248-253.

17. Machado-Vieira R, **Schmidt AP**. Increased cerebrospinal fluid levels of S100B protein in rat model of mania induced by ouabain. Life Sciences 2004; 76:805-811.

18. **Schmidt AP**, Schmidt SR. Treatment of refractory neuropathic pain related to a brachial plexus injury. Injury 2004; 35(5):528-530.

19. Soares FA, **Schmidt AP**, Farina M, Frizzo ME, Tavares RG, Portela LV, Lara DR, Souza DO. Anticonvulsant effect of GMP depends on its conversion to guanosine. Brain Res 2004; 1005(1-2):182-186.

20. Vinade ER, Izquierdo I, Lara DR, **Schmidt AP**, Souza DO. Oral administration of guanosine impairs inhibitory avoidance performance in rats and mice. Neurobiol Learn Mem 2004; 81(2):137-143.

21. Schmidt AP, Tort AB, Amaral OB, **Schmidt AP**, Walz R, Vettorazzi-Stuckzynski J, Martins-Costa SH, Ramos JG, Souza DO, Portela LV. Serum S100B in pregnancy-related hypertensive disorders: a case-control study. Clin Chem 2004; 50(2):435-438.