Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Curso de Pós-Graduação em Ciências Biológicas: Bioquímica

NEUROTOXICIDADE GLUTAMATÉRGICA E VIAS DE NEUROPROTEÇÃO: PARTICIPAÇÃO DO SISTEMA ADENOSINÉRGICO

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Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica, da Universidade Federal do Rio Grande do Sul (RS) como requisito parcial para a obtenção do grau de DOUTOR em Ciências Biológicas: Bioquímica.

Porto Alegre, março de 2004

Para a minha Constelação preferida:

Riro, minha família e meus amigos.

AGRADECIMENTOS

O fundamental nutriente deste trabalho foi a vontade de aprender e de buscar respostas acompanhada de parcerias que ampararam o dia-a-dia.

Á Deusa um **muito obrigado** pela oportunidade de **crescer**!

Ao Diogo por ser como o Menino Maluquinho do Ziraldo: braços que abraçam o mundo e macaquinhos no sótão.

Aos meus queridos companheiros de bancada, o meu grande apoio: Mauro, Eduardo (Dudis), Flúvia, Marcelo (Ganzella) e Amilcar.

Ao Sarkis pela experiência.

Ao André (Schmidtinho) pelo grande auxílio no trabalho das convulsões em camundongos.

À UFRGS, ao CPG em Bioquímica e à CAPES pela oportunidade e pelo investimento.

Aos funcionários do ratário e em especial à Cecília (sala de Cultura de Células) e à Cléia pela dedicação exemplar.

Aos figurantes desta tese, com maior ou menor participação, mas todos importantes:

Lisi

Ricardo

Bibi

Rô

Mônica

Jean

Ju (agora Frizzo)

Francine

Carmem

Ale Bruno

Homens do lab 26

Pessoal do lab. 28

Mulheres dos labs. 24 e 22

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A neurotransmissão excitatória no Sistema Nervoso Central (SNC) é mediada por aminoácidos excitatórios (EAAs), tais como: L-aspartato, D-aspartato e por glutamato. O glutamato é o principal neurotransmissor excitatório e está envolvido tanto em sinalizações fisiológicas quanto patológicas na sinapse, através da ativação dos receptores ionotrópicos (iGluR) e metabotrópicos (mGluR). O acúmulo de glutamato na fenda sináptica leva a excitotoxicidade celular devido à excessiva estimulação de seus receptores, principalmente os receptores ionotrópicos NMDA. Os aminoácidos L-aspartato e D-aspartato mimetizam muitas ações do glutamato, pois são agonistas dos receptores NMDA e são captados pelos transportadores de aminoácido excitatório (EAATs). Acredita-se que a rápida remoção dos EAAs da fenda sináptica pelos EAATs seja o maior mecanismo de regulação e neuroproteção no sistema glutamatérgico. A ativação dos receptores NMDA leva ao aumento extracelular do nucleosídeo adenosina, considerado um importante neuromodulador. O aumento nos níveis de adenosina se deve à sua liberação como tal pelo transportador bi-direcional de nucleosídeo ou formada a partir da degradação de nucleotídeos liberados para o meio extracelular por ação da cascata das ecto-enzimas, ATP difosfoidrolase e 5'-nucleotidase. O pré-condicionamento é um

mecanismo de proteção celular devido à indução de tolerância à morte a partir de um estímulo subtóxico. O pré-condicionamento com NMDA protege neurônios em cultura contra a morte celular induzida por glutamato.

No presente estudo se observou que a neurotoxicidade induzida por altas doses dos EAAs glutamato, L-aspartato ou D-aspartato diminui a captação de glutamato, mas, por outro lado os agonistas de receptores glutamatérgicos NMDA e SR-ACPD aumentam esta captação. Estes resultados sugerem que o transportador EAATs pode ser regulado diferentemente, contribuindo para os processos de neurotoxicidade ou de neuroproteção celular. Doses neurotóxicas de glutamato ou NMDA estimulam a atividade da ecto-ATP difosfoidrolase e ecto-5'-nucleotidase de neurônios em cultura. Tem-se proposto que baixas concentrações extracelulares de adenosina são originadas da sua liberação como tal e assim ativa preferencialmente os receptores A_1. Enquanto altas concentrações são originadas da sua formação pela cascata das ecto-enzimas, favorecendo a ativação dos receptores A_{2A}. A excitotoxicidade desencadeada pelos EAAs observada aqui estimula a produção de adenosina no espaço extracelular pela ativação da ecto-5'-nucleotidase em neurônios. Adenosina formada pode contribuir para o mecanismo de neurotoxicidade ou de neuroproteção, dependendo se ativará receptores A_1 ou A_{2A}, respectivamente. Pois, tem-se descrito que a ativação dos receptores A_1 reduz a morte celular e a liberação de EAAs. Enquanto que a ativação dos receptores A_{2A} aumenta a liberação de glutamato sobre condições normais e durante insulto isquêmico, potencializando assim a neurotoxicidade glutamatérgica. Porém, os antagonistas dos receptores A_1 (CPT) ou A_{2A} (ZM 241385) de adenosina não afetaram essa neurotoxicidade. Um mecanismo de proteção bem estabelecido é o induzido pelo pré-condicionamento, onde doses subtóxicas de uma toxina protegem de um posterior insulto tóxico. O envolvimento dos receptores de adenosina neste processo tem sido sugerido. Nos resultados apresentados nesta tese, observou-se que o bloqueio dos receptores A_1 de adenosina, mas não dos receptores A_{2A}, durante o período de pré-condicionamento com NMDA previnem a neuroproteção contra a morte celular induzida por glutamato. Após o período do pré-condicionamento, verificou-se também uma inibição na atividade da ecto-5'-nucleotidase estimulada por glutamato e uma diminuição da funcionalidade dos receptores A_{2A}.

Estes mecanismos desfavorecem a ativação dos receptores A_{2A}, o que poderia potencializar a neurotoxicidade, mas cooperam para que os receptores A_1 sejam ativados quando ocorrer o insulto neurotóxico, contribuindo assim para a neuroproteção decorrente

do pré-condicionamento com NMDA. Em experimentos realizados *in vivo*, observou-se que o pré-condicionamento com NMDA previne convulsões e a conseqüente morte celular induzidas pela administração intra-cerebroventricular de ácido quinolínico em camundongos. Neste mecanismo de proteção, a participação dos receptores A_1 foi fundamental contra as convulsões, mas não contra a morte celular. Estes resultados sugerem que os mecanismos de proteção induzidos pelo pré-condicionamento com NMDA contra as convulsões e morte celular *in vivo* ocorreram por diferentes vias de proteção: uma dependente exclusivamente da ativação do receptor NMDA e outra dependente da cooperação dos receptores A_1 de adenosina.

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Tese de Doutorado em Ciências Biológicas: Bioquímica

Porto Alegre, março de 2004.

ABSTRACT

GLUTAMATERGIC NEUROTOXICITY AND NEUROPROTECTION PATHWAY: PARTICIPTION OF ADENOSINERGIC SYSTEM

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Excitatory neurotransmission in the mammalian CNS is mediated by excitatory amino acids (EAAs), such as L-aspartate, D-aspartate and glutamate. Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS) and it is involved in physiological pathways as well as contributes to neuronal damage. Glutamate acts through activation of ionotropic (iGluR) and metabotropic receptors (mGluR). Excessive accumulation of glutamate in the synaptic cleft may lead to an excitotoxic neuronal damage due to over activation of these receptors, mainly the ionotropic NMDA receptors. The EAAs L-aspartate and D-aspartate mimic most of the actions of glutamate, since they are putative NMDA receptors agonists and are uptake by excitatory amino acid transporters (EAATs). Rapid uptake of the amino acid excitatory through EAATs is believed to be the major regulatory and neuroprotective mechanism in the glutamatergic system. The activation of the NMDA receptors promotes the increase of extracellular adenosine levels, which is an important neuromodulator in the CNS. The extracellular adenosine can be released via a bi-directional transporter or formed from adenine nucleotides released that are degraded by an extracellular chain of ecto-nucleotidases,

ATP diphosphohydrolase and 5'-nucleotidase. The preconditioning is a mechanism of protection evoked by subtoxic stimulus. NMDA preconditioning leads to tolerance to the subsequent lethal stimulus, which protects cultured neurons against glutamate-induced cellular death.

In the present study, the neurotoxicity induced by high doses of glutamate, L-aspartate or D-aspartate inhibited glutamate uptake, but opposite, the glutamate receptors agonists, NMDA and SR-ACPD increase the glutamate uptake. These results suggest that the EAATs may be regulated by different mechanisms, contributing to neurotoxic or neuroprotective cellular process in the glutamatergic pathway. Cultured neurons in the presence of neurotoxic concentration of glutamate or NMDA show a stimulation of the ecto-ATP diphosphohydrolase and ecto-5'-nucleotidase activities. Extracellular adenosine at lower concentrations is released from bi-directional adenosine transporter and activates predominately adenosine A_1 receptors, whereas at higher concentrations it is formed from ecto-nucleotidases pathway and activates of adenosine A_{2A} receptors. The EAAs-induced neurotoxicity observed herein stimulates the production of adenosine by ecto-5'-nucleotidase activity in neurons. Adenosine can cooperate with neuroprotection or neurotoxicity depended whether it would act on adenosine A_1 or A_{2A} receptors, respectively. Adenosine has been shown to reduce the cellular damage by activating adenosine A_1 receptors inhibits EAAs release, otherwise by activating adenosine A_{2A} receptors stimulates glutamate release upon normoxic or ischemia conditions. But, the adenosine receptors A_1 (CPT) or A_{2A} (ZM 241385) antagonists did not affect the neurotoxicity induced by L- or D-aspartate. The preconditioning is a neuroprotective mechanism, where subtoxic doses of a toxin protect against a subsequently lethal dose. In the present study, the neuroprotection evoked by NMDA preconditioning against glutamate-induced cellular damage was prevented by the presence of adenosine A_1 receptor antagonist, CPT, but not by the adenosine A_{2A} receptors antagonist, ZM 241385. Moreover, NMDA preconditioning prevented the stimulatory effect of glutamate on AMP hydrolysis and it promoted a desensitization of the adenosine A_{2A} receptor. Taken together, the results described herein suggest that the neuroprotection evoked by NMDA preconditioning against cellular damage elicited by glutamate occurred through mechanisms involving adenosine A_{2A} receptors desensitization co-operating with adenosine A_1 receptors activation in cerebellar granule cells. We also observed *in vivo*

experiments that NMDA preconditioning led to protection against seizures and cellular death induced by quinolinic acid in mice. In this neuroprotective mechanism the participation of adenosine A_1 receptors was crucial against quinolinic acid-induced seizures but not cellular damage. These results suggest that the neuroprotective mechanisms evoked by NMDA preconditioning against quinolinic acid-induced seizures and cellular damage occurred by a different pathway: one dependent exclusively on the NMDA receptor activation and other dependent on the co-operation of adenosine A_1 receptors.

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LISTA DE ABREVIATURAS

ADP: adenosine 5´-difosfato

AMP: adenosine 5´-monofosfato

AMPc: adenosine 5´-monofosfato cíclico

ATP: adenosine 5´-trifosfato

AP-5: [L(±)-2-Amino-3-phosphonopropionic acid] ácido L(±)-2-amino-3-fosfopropiônico

BDNF: (brain-derivated neurotrophic factor) fator neurotrófico derivado do cérebro

CGS 21680: 2-[4-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine

CPA: (N-cyclopentyladenosine) N-ciclopentiladenosina

CPT: (8-cyclopentyl-1,3-dimethylxanthine) 8-ciclopentil-1,3-dimetilxantina

EAAT: (excitatory amino acid transporter) transportador de aminoácido excitatório

EAAs: (excitatory amino acid) aminoácidos excitatórios

L-AP3: [(±)-2-amino-5-phosphonopentanoic acid] ácido (±)-2-amino-5-fosfopentanóico

MK-801: (dizocilpine) dizocilpina

NGF: (nerve growth factor) fator de crescimento neural

NMDA: (N-methyl-D-asparate) N-metil-D-aspartato

NT-4/5: (neurotrophin-4/5) neurotrofina-4/5

8-PT: (8-phenyltheophylline) 8-fenilteofilina

SR-ACPD: [(1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid] (1*S*,3*R*)-1-amino-ciclopentano-1,3-dicarboxílico

Trk: (receptor tyrosine kinase) receptor tirosina quinase

ZM 241385: 4-(2[7-amino-2-(2-furyl {1,2,4}-triazolo{2,3-a{1,3,5}triazian-5-yl-aminoethyl)phenol

APRESENTAÇÃO

Esta tese apresenta no item **I. INTRODUÇÃO** um embasamento teórico sobre o estudo realizado. No item **II. ARTIGOS CIENTÍFICOS** encontram-se os artigos que representam a íntegra deste estudo. Material e Métodos, Resultados e Discussão dos Resultados estão descritos nos artigos científicos.

O item **III. DISCUSSÃO**, apresentado no final da tese, contém interpretações e comentários gerais sobre o estudo da tese.

O item **V. REFERÊNCIAS BIBLIOGRÁFICAS** refere-se somente às citações que aparecem nos textos dos itens **I. INTRODUÇÃO** e **III. DISCUSSÃO**.

I. INTRODUÇÃO

I.1. Sistema Glutamatérgico

O glutamato é considerado o principal aminoácido excitatório no Sistema Nervoso Central (SNC) que atua como um transmissor sináptico, participando de muitas sinalizações celulares excitatórias e de fenômenos plásticos vinculados à aprendizagem e memória (FONNUM, 1984; IZQUIERDO, 1989), como a indução da potenciação de longa duração (LTP) e a depressão de longa duração (LTD) (ARTOLA & SINGER, 1987; ITO, 1989). Além destas funções, o glutamato também participa do desenvolvimento do SNC, incluindo a diferenciação e morte celular (McDONALD & JOHNSTON, 1990; MELDRUM, 2000).

O glutamato exerce suas funções sinalizadoras sinápticas agindo em proteínas específicas localizadas na membrana celular neural. Os receptores glutamatérgicos são classificados de acordo com as suas propriedades farmacológicas e funcionais, e são divididos em receptores ionotrópicos (iGluR) e metabotrópicos (mGluR). Os receptores ionotrópicos são canais iônicos que quando ativados tornam-se permeáveis a cátions e são divididos em: NMDA, AMPA e cainato (MORI & MISHIMA, 1995). A estimulação de qualquer destes receptores ionotrópicos leva ao influxo de cálcio (Ca^{2+}) e/ou sódio (Na^{+}) resultando na despolarização da membrana, ativando assim, indiretamente os canais de cálcio dependentes de voltagem. Os oito tipos de receptores

metabotrópicos são associados a sistemas transmembrana acoplados a proteínas-G divididos em três grandes famílias de receptores de acordo com as suas seqüências homólogas e com a modulação de efetores intracelulares. O Grupo I inclui os receptores mGluR 1 e 5, estão associados a ativação da fosfolipase C (PLC); o Grupo II (mGluR 2 e 3) e o Grupo III (mGluR 4, 6, 7 e 8) estão associados negativamente a adenilato ciclase (CONN & PIN, 1997).

A extensão da estimulação dos receptores glutamatérgicos é determinada pela concentração do glutamato no espaço extracelular. É de grande importância para as células que o glutamato seja mantido em baixas concentrações, pois a excessiva liberação e o conseqüente aumento nos seus níveis extracelulares o torna tóxico em decorrência da excessiva estimulação de seus receptores (LIPTON & ROSENBERG, 1994). Desde que não há evidência da presença de uma enzima que degrade o glutamato extracelular, o sistema de transporte de glutamato é a via mais rápida de remoção do glutamato extracelular, possibilitando que seja armazenado em vesículas citoplasmáticas no neurônio e, conseqüentemente, interrompendo a sua ação nos receptores (ROSENBERG et al., 1992). Os transportadores de glutamato estão presentes na membrana celular de neurônios e em astrócitos, sendo os transportadores na glia considerados mais importantes, pois além de manter baixa a concentração extracelular do glutamato, transforma o glutamato a glutamina no citoplasma (SCHOUSBOE, 1981).

Os aminoácidos L-aspartato e D-aspartato são considerados neurotransmissores excitatórios, pois mimetizam praticamente todas as ações

do glutamato nos receptores NMDA e nos transportadores. A afinidade do L-aspartato e do D-aspartato aos receptores NMDA é dez vezes menor que a do glutamato (OLVERMAN et al., 1988). Tem-se relacionado o D-aspartato à regulação da maturação e diferenciação de neurônios e células endócrinas (SCHELL et al., 1997; SAKAI et al., 1998; WOLOSKER et al., 2000). Ambos os aminoácidos são armazenados em vesículas (FLECK, et al., 2001), mas há controvérsia sobre a dependência de íons Ca^{2+} para a sua liberação (LEWIN et al., 1996; JENSEN et al., 2000; NAKATSUKA et al., 2001).

I.1.1. Excitotoxicidade Glutamatérgica

O conceito de excitotoxicidade foi introduzido por OLNEY (1970) para descrever a neurotoxicidade associada com a administração exógena de altas concentrações glutamato, ou de compostos que agem como agonistas em receptores glutamatérgicos. A excitotoxicidade glutamatérgica não é o único fator desencadeante de neuropatologias, mas tanto pode ser um evento primário, como uma conseqüência de um evento danoso já ocorrido. Com isto, tem-se sugerido que alterações na captação e/ou liberação do glutamato possa estar envolvida em várias condições neuropatológicas crônicas e agudas nas quais o glutamato participa, como por exemplo: a isquemia, a hipoglicemia, o edema cerebral, a lesão por trauma mecânico, a epilepsia, a demência de Alzheimer, a enfermidade de Huntington e em as doenças neurológicas relacionadas a AIDS (OBRENOVITCH et al., 2000).

I.1.2. Receptores NMDA

Os receptores NMDA são os mais estudados dos receptores glutamatérgicos. Na célula em repouso, o canal-receptor NMDA é bloqueado pelo íon magnésio (Mg^{2+}), que é liberado após a despolarização induzida pela ativação do receptor, facilitando assim o influxo de íons Na^+, K^+ e principalmente Ca^{2+} (MAcDERMOTT et al., 1986). O potencial excitatório mediado por receptores NMDA é mais prolongado do que o mediado pelos receptores AMPA ou cainato (DALE & ROBERTS, 1985). Fisiologicamente, a ativação de receptores NMDA está associada ao desenvolvimento do neurônio e à formação da sinapse (RABACCHI et al., 1992), e em processos importantes de plasticidade sináptica, tais como aprendizagem e memória (BLISS & COLLINGRIDGE, 1993). A excessiva ativação destes receptores está envolvida na epilepsia e em danos cerebrais associados à isquemia/hipoglicemia (OLNEY, 1994). Esta excessiva ativação dos canais-receptores NMDA desfaz a homeostase celular dos íons Ca^{2+}, levando a excitotoxicidade e ao dano no neurônio provocado pelo aumento do seu influxo. Há mecanismos de lesão celular e subseqüente morte por necrose e/ou apoptose que ocorrem como resultado deste acúmulo intracelular de Ca^{2+} (ARUNDINE & TYMIANSKI, 2003). Com isto, relaciona-se este aumento nos níveis intracelulares dos íons Ca^{2+} com a morte celular induzida pela excessiva ativação dos canais-receptores NMDA. Em muitos modelos de excitotoxicidade em que os níveis extracelulares

de glutamato estão elevados, antagonistas dos receptores glutamatérgicos são utilizados como neuroprotetores. Os antagonistas dos receptores NMDA, AP-5 (competitivo, bloqueia o sítio de ligação de NMDA ao receptor) e MK-801 (não competitivo, bloqueia o canal iônico no receptor) (MONAGHAN et al., 1989) fazem parte do grupo de antagonistas utilizados para bloquear respostas neurotóxicas desencadeadas pela ativação dos canais-receptores NMDA, devido a liberação de aminoácidos durante um insulto isquêmico ou convulsivo (OLNEY et al., 1987; NOVELLI et al., 1988; BREUKEL et al., 1998; SIERRA-PAREDES et al., 2000; MELONI et al., 2002).

I.1.2.1. Ácido Quinolínico

O ácido quinolínico é um metabólico endógeno do triptofano e uma potente neurotoxina que normalmente está presente em concentração nanomolar no cérebro e no fluído cérebro-espinhal (LAPIN, 1978). Contudo, um acúmulo do ácido quinolínico é observado no cérebro e fluído cérebro-espinhal em pacientes com doenças infecciosas e inflamatórias, na doença de Huntington, em doenças neurológicas relacionadas a AIDS e na etiologia da epilepsia (HEYES et al., 1996). O exato envolvimento do ácido quinolínico nestas patologias ainda não está definido, mas provavelmente parte da sua toxicidade seja mediada pela ativação dos canais-receptores NMDA (STONE, 2001). A neurotoxicidade do ácido quinolínico é semelhante àquela observada com NMDA, contudo o ácido quinolínico apresenta menor excitabilidade e

potência em deslocar ligantes unidos aos receptores (SCHWARCZ et al., 1983; STONE & PERKINS, 1981). A toxicidade do ácido quinolínico está relacionada com: a ausência de um mecanismo de remoção do ácido do meio extracelular (FOSTER et al., 1984), com a sua habilidade em causar estresse oxidativo (RÍOS & SANTAMARÍA, 1991), em promover liberação de glutamato e aspartato (CONNICK & STONE, 1988) e inibir a captação de glutamato em astrócitos (TAVARES et al., 2002). A maior evidência das ações tóxicas do ácido quinolínico nos receptores NMDA é devido a inibição de muitas destas respostas pelo antagonista dos receptores NMDA, o MK-801 (FOSTER et a., 1988; SHAN et al., 1997; SANTAMARÍA & RÍOS, 1993; TAVARES et al., 2002). A administração tópica do ácido quinolínico tem sido usada como um modelo de convulsão e uma estratégia de avaliação da excitotoxicidade induzida pelos receptores NMDA *in vivo*. A convulsão induzida pelo ácido quinolínico certamente envolve receptores NMDA, pois o MK-801 também é capaz de evitar que este evento acorra (FEDELE & FOSTER, 1993; SCHMIDT et al., 2000).

I.1.3. Sistema de Transporte de Glutamato

Os Transportadores de Aminoácidos Excitatórios (EAATs) são proteínas que realizam o transporte de glutamato e aspartato do meio extracelular para o meio intracelular com alta afinidade e dependência de íons Na^+, finalizando a ação excitória destes aminoácidos no terminal nervoso (MANGANO &

SCHWARCZ, 1981; ROSENBERG et al., 1992; ROBINSON et al., 1993). Assim, para remover o glutamato do espaço extracelular a captação é o mecanismo elementar, pois contribui para a proteção contra a excitotoxicidade neural provocada pelo excesso deste neurotransmissor (ROBINSON & DOWD,1997). Cinco tipos de transportadores para glutamato já foram identificados e clonados a partir de isoformas de roedores; dois deles são expressos principalmente em células da glia: GLT-1 (EAAT1) e GLAST (EAAT2) (STORCK et al., 1992; PINES te al., 1992), embora há evidências de sua expressão em neurônios em desenvolvimento (ROTHESTEIN et al., 1994; PLACHEZ et al., 2000). Em neurônios os transportadores predominantes são: EAAC1 (EAAT3) (KANAI & HEDIGER, 1992) e EAAT4 (FAIRMAN et al., 1995), além do EAAT5 que é restritamente expresso nas células da retina (ARRIZA et al., 1997). O transporte de glutamato é termodinamicamente acoplado ao co-transporte de dois a três íons Na^+, um próton (H^+) e o contra-transporte de um íon potássio (K^+) (ZERANGUE & KAVANAUGH, 1996). A carga movida durante a atividade dos transportadores é maior que a esperada pelo fluxo iônico acoplado ao transporte de aminoácidos. Isto se deve a permeabilidade aos íons cloro (Cl^-) através dos EAATs, principalmente por EAAT4 e EAAT5, ou talvez, através de um canal de Cl^- acoplado ao transportador (FAIRMAN et al., 1995). Isto sugere outra função aos transportadores que não apenas neutralizar a neurotransmissão glutamatérgica, mas também regular a excitabilidade e sinalização em neurônios (AMARA & FONTANA, 2002). O estudo das funções dos EAATs no sistema glutamatérgico é realizada pela utilização de diferentes

tipos de ligantes/inibidores. Os inibidores são classificados em substratos e não-substratos (KOCH et al., 1999). Os inibidores substratos, por serem captados, podem tanto ligar ao sítio extracelular quanto ao sítio intracelular do carreador. O mecanismo de inibição devido à união ao sítio intracelular é conhecido como trans-inibição (DEBERNARDI et al., 1999). Os inibidores não-substratos não são transportados pelos carreadores e bloqueiam a captação, provavelmente devido à ocupação do sítio de ligação extracelular no transportador. A captação reversa é um mecanismo patofisiológico de liberação citoplasmática do aminoácido (não-vesicular) e independente dos íons Ca^{2+}. A reversão nos EAATs ocorre em situações em que há falha energética, como por exemplo durante um insulto isquêmico (LONGUEMARE & SWANSON, 1995; ROSSI et al., 2000; BONDE et al., 2003). Com isto, os inibidores substrato são usados para inibir a captação reversa e conseqüentemente auxiliar na proteção contra insultos em que há liberação de glutamato. Os aminoácidos L-aspartato e D-aspartato são utilizados como inibidores da captação do glutamato, pois são captados pelos transportadores com capacidade igual ao do glutamato (KOCH et al., 1999). DHK (di-hidrocainato) também é um bloqueador da captação, mas não é substrato para o transportador. O efeito de inibidores também depende do tipo de célula que se está utilizando, pois há inibidores específicos para certos tipos de EAATs. Tem-se relacionado a inibição do bloqueador DHK com o transportador GLT1 (EAAT1), contudo em altas concentrações ele é capaz de inibir o transportador EAAC1 em neurônios (VELAZ-FAIRCLOTH, 1996). Esta hipótese é confirmada quando se observa

um aumento na morte celular pela presença de DHK em neurônios (WANG et al., 1998).

I.2. Mecanismos de neuroproteção

A neuroproteção é um processo que interrompe e/ou previne uma cascata patológica que ocorre durante um processo de insulto tóxico. Com isto, contra uma excitotoxicidade glutamatérgica, tem-se utilizado como neuroprotetores antagonistas dos receptores NMDA, quelantes de íons Ca^{2+}, bloqueadores de canais de Ca^{2+} e Na^{+}, administração em altas concentrações de íons Mg^{2+} (McINTOSH, 1993; GAGLIARDI, 2000). Muitas vezes, a efetividade destas drogas depende que outros processos neuroprotetores sejam ativados para que ocorra o mecanismo de proteção (OBRENOVITCH et al., 2000).

I.2.1. Sistema Adenosinérgico

O nucleosídeo purinérgico adenosina desempenha uma função fundamental como neuromodulador no SNC (BRUNDEGE & DUNWIDDIE, 1997). Normalmente, adenosina está presente em baixas concentrações no fluído extracelular, porém altas concentrações são observadas durante um aumento da atividade nervosa, durante a hipóxia ou isquemia. Este aumento nos níveis de adenosina indica a sua participação nestes processos, contudo o

seu envolvimento no mecanismo neuroprotetor ou neurotóxico dependerá da atividade do nucleosídeo nos seus diferentes subtipos de receptores.

I.2.1.1. Origem de Adenosina Extracelular

O nucleotídeo ATP é fundamental no meio intracelular como fonte energética e no meio extracelular um neurotransmissor de ação excitatória rápida e neuromodulador no SNC (NEARY et al., 1996; VIZI et al., 1997). O ATP exerce suas funções agindo em receptores do tipo P_2, que são subdivididos em: receptores ionotrópicos P_{2x} (ligados a canais iônicos) e metabotrópicos P_{2y} (ligados a proteínas-G). O ATP liberado para o espaço extracelular despolariza a membrana devido à ativação dos receptores P_{2X} na pós-sinapse, promovendo a entrada dos íons Na^+ e Ca^{2+} e saída do íon K^+ por canais iônicos (BEAN & FRIEL, 1990). A excessiva ativação dos receptores P_{2X} leva a excitotoxicidade. A participação dos receptores P_2 na morte celular foi observada em neurônios e em astrócitos após dano induzido por glutamato ou hipóxia/hipoglicemia (VOLONTÉ et al., 1999; CAVALIERE et al., 2001). A ação do ATP é finalizada pela sua conversão a adenosina por ação de uma cascata enzimática de ecto-nucleotidases (DUNWIDDIE, 1985). A hidrólise extracelular do ATP pode ocorrer tanto pela ação de uma ecto-ATPase quanto por uma ecto-ATP difosfoidrolase (ecto-apirase, EC 3.6.1.5), pois ambas as enzimas são expressas no cérebro (KEGEL et al., 1997). A ecto-ATP difosfoidrolase é a enzima responsável pela defosforilação do ADP a AMP, que será degradado a

adenosina pela ação da ecto-5´-nucleotidase (EC 3.1.3.5). A ecto-5'-nucleotidase é considerada a enzima marca-passo na formação de adenosina no meio extracelular pela degradação de nucleotídeos liberados. Em um insulto isquêmico foi detectado o aumento na expressão da ecto-ATP difosfoidrolase e da ecto-5'-nucleotidase no hipocampo de ratos, resultando numa maior eficiência no catabolismo dos nucleotídeos liberados durante o processo isquêmico (BRAUN et al., 1997; BRAUN et al., 1998). Com isto, o aumento na expressão destas enzimas além de contribuir para a eliminação dos efeitos citotóxicos causados pelo excesso de nucleotídeos, contribui para a formação de adenosina e a sua subseqüente ação nos seus receptores (DUNWIDDIE, 1985). Além da via das ecto-enzimas, a adenosina extracelular pode ser originada da sua liberação como tal pela atividade do transportador de nucleosídeo, que regula tanto as concentrações intracelulares e extracelulares de adenosina, dependendo do seu gradiente de concentração (SWEENEY et al., 1996). Além do transportador, a ecto-adenosina deaminase que degrada adenosina a inosina, ajuda a finalizar as ações de adenosina nos seus receptores.

I.2.1.2. Receptores Adenosinérgicos

As ações fisiológicas da adenosina são mediadas por receptores específicos acoplados a proteínas-G que são divididos em A_1, A_{2A}, A_{2B} e A_3 (FREDHOLM et al., 2001). Os receptores A_1 e A_3 são acoplados a G_i, e os

receptores A_2 são acoplados a G_s, causando a inibição e a ativação da adenilato ciclase, respectivamente (DALY, 1985; PALMER & STILES, 1995).

Os efeitos inibitórios dos receptores A_1 resultam na neuroproteção tanto em modelos experimentais *in vitro* quanto *in vivo*, principalmente em modelos de hipóxia/isquemia e convulsões (RUDOLPHI et al., 1992; von LUBITZ, 1999). Estes efeitos são atribuídos à ativação da condutância de K^+ na pós-sinapse (hiperpolarizando a membrana celular) (DUNWIDDIE & DIAO, 1994) e à redução do influxo dos íons Ca^{2+} na pré-sinapse (WU et al., 1982; VACAS et al., 2003), o que por si só é protetor, mas que também promove uma redução na liberação de neurotransmissores, entre eles o glutamato e o aspartato (CORRADETTI et al., 1984; POLI et al, 1991). A ativação de receptores A_{2A} exerce uma ação tônica estimulatória na liberação de aminoácido excitatórios, contribuindo para a neurotoxicidade induzida pela isquemia (O'REGAN et al., 1992; CHEN et al., 1999). Com isto, tem-se atribuído aos antagonistas dos receptores A_{2A} o papel neuroprotetor contra a morte celular, provavelmente pela sua habilidade de reduzir a liberação de aminoácidos excitatórios (SIMPSON et al., 1992). A ativação dos receptores NMDA ou o processo isquêmico levam ao aumento extracelular de adenosina (HOEHN & WHITE, 1990; CRAIG & WHITE, 1993; MELANI et al., 1999). Como citado acima, a origem da adenosina no meio extracelular pode ser através da sua liberação pelo transportador equilibrativo de nucleosídeo (SWEENEY et al., 1996) ou produzida pela degradação dos nucleotídeos liberados por ação da cascata de ecto-enzimas (CUNHA et al., 1998). Em cultura de neurônios a excitotoxicidade

glutamatérgica induzida por glutamato, NMDA ou cainato estimula a degradação de nucleotídeos extracelulares por ação das ecto-enzimas ATP difosfoidrolase e 5'-nucleotidase, resultando na elevação dos níveis extracelulares de adenosina (BOECK et al., 1999; BOECK et al., 2000). Tem sido sugerido que a adenosina liberada age preferencialmente nos receptores A_1, enquanto que a adenosina formada pela via das ecto-nucleotidases favorece a ativação dos receptores A_{2A} (CUNHA et al., 1996). Como há uma intercomunicação entre os receptores A_1 e A_{2A} (LOPES et al., 1999), é provável que a regulação dos níveis extracelulares de adenosina através de uma ou de outra via favoreça as suas ações neuromodulatórias em situações de toxicidade celular.

I.2.2. Neuroproteção pelo Pré-condicionamento

O pré-condicionamento é um estímulo subtóxico ou sublimiar insuficiente para induzir algum dano, mas de alguma maneira, aumenta a capacidade das células de sobreviverem após um subseqüente estímulo danoso. As primeiras descrições de pré-condicionamento foram introduzidas por MURRY e colaboradores (1986) através de estudos em células do miocárdio após uma isquemia; e com KITAGAWA e colaboradores (1990) através da observação de que um breve período de isquemia global protegia neurônios cerebrais da morte necrótica induzida por uma posterior isquemia global severa. Com isto, estratégias de pré-condicionamento tais como: isquemia (CHEN & SIMON,

1997), hipóxia (GIDDAY et al., 1994) e hipotermia (NISHO et al., 2000) são protetoras contra o dano isquêmico. Em muitos modelos de excitotoxicidade os níveis extracelulares de glutamato estão elevados (como por exemplo, durante um insulto isquêmico ou convulsivo), o que ocasiona a excessiva ativação dos receptores glutamatérgicos, principalmente os canais-receptores NMDA. Assim sendo, em estudos de neuroproteção contra a excitotoxicidade glutamatérgica, tem-se utilizado o chamado pré-condicionamento químico (REJDAK et al., 2001) com doses subtóxicas de glutamato (JONAS et al., 2001), NMDA (CHUANG et al., 1992), ou ácido quinolínico (SEI et al., 1998). Observou-se em neurônios granulares do cerebelo em cultura que, a neuroproteção pelo pré-condicionamento com NMDA contra a neurotoxicidade induzida por glutamato é um processo mediado pela ativação dos receptores NMDA, pois este evento é bloqueado pelo antagonista MK-801. Esta neuroproteção não está associada à variação da expressão dos receptores NMDA, pois o influxo dos íons Ca^{2+} desencadeado pela ativação destes receptores é mantido (DAMSCHTODER-WILLIAMS et al., 1995). Para que a neuroproteção ocorra, é fundamental a participação de outras proteínas, além do receptor em questão, pois este evento é bloqueado pelo inibidor da síntese protéica (ciclohexamida) e pelo inibidor da síntese de RNA (actinomicina D) (MARINI & PAUL, 1992; DAMSCHTODER-WILLIAMS et al., 1995). A neuroproteção pelo pré-condicionamento com NMDA também tem sido observada contra a morte celular decorrentes da isquemia (GRABB & CHOI, 1999). Neste processo de neuroproteção há um aumento na expressão da proteína HSP70 (VALENTIM et

al., 2003). Estudos mostram que é fundamental a participação dos receptores A_1 de adenosina durante o pré-condicionamento isquêmico, pois esta neuroproteção é diminuída por antagonistas de receptores A_1 (HIRAIDE et al., 2001), e mimetizada por adenosina ou pelos agonistas destes receptores (EVANS et al., 1987; WARDAS, 2002). No pré-condicionamento com NMDA, o antagonista CPT também reverte a neuroproteção contra a morte celular induzida por cainato em experimentos realizados *in vivo* (OGITA et al., 2003).

Os fatores neurotróficos BDNF, NGF e NT-4/5 também participam do pré-condicionamento com NMDA em células em cultura (MARINI et al., 1998; ROCHA et al., 1999). O BDNF liberado para o meio extracelular durante o período do pré-condicionamento age nos receptores tirosina quinase tipo TrkB (MARINI et al., 1998). A ativação dos receptores TrkB e dos canais-receptores NMDA estimula a atividade dos fatores de transcrição nuclear, NF-kB *in* vitro (LIPSKY et al., 2001) e AP-1 *in vivo* (OGITA et al., 2003), que são responsáveis pela regulação dos fatores neurotróficos. Juntos todos estes mecanismos contribuem para que o processo de tolerância celular derivado do pré-condicionamento ocorra e seja mantido por um determinado período para proteger as células de um posterior insulto danoso.

OBJETIVOS

Devido à participação de glutamato em várias neuropatologias, é de grande importância se estudar mecanismos de regulação da sua excitotoxicidade. O objetivo principal deste trabalho é identificar vias de neuroproteção contra danos celulares causados por: altas concentrações de aminoácidos excitatórios em cultura de neurônios granulares do cerebelo; convulsões e excitotoxicidade induzidas pelo agonista glutamatérgico, o ácido quinolínico, em camundongos.

OBJETIVOS ESPECÍFICOS

I) Os transportadores de aminoácido excitatório têm uma função importante na manutenção da homeostase de glutamato, regulando a neurotransmissão e também a excitotoxicidade glutamatérgica.

No presente estudo, investiga-se a captação de glutamato em cultura de neurônios para determinar se ela é afetada por altas concentrações de aminoácidos excitatórios e/ou por agonistas de receptores glutamatérgicos.

I) Os aminoácidos excitatórios L-aspartato e D-aspartato são agonistas dos canais-receptores NMDA e substratos para os transportadores de aminoácido excitatório. A ativação dos receptores NMDA aumenta a degradação extracelular dos nucleotídeos, acarretando um aumento dos níveis extracelulares do nucleosídeo adenosina, o qual é considerado um

neuromodulador. Adenosina agindo em receptores A_1 desempenha funções neuroprotetoras e em receptores A_{2A} pode levar a uma neurotoxicidade.

No presente estudo, investiga-se a possibilidade dos aminoácidos mimetizarem a estimulação na atividade da ecto-5'-nucleotidase e se esse efeito é mediado pela ativação dos receptores NMDA. Além disso, verificar se antagonistas dos receptores de adenosina poderiam afetar a neurotoxicidade induzida por L-aspartato ou D-aspartato em cultura de neurônios.

II) O pré-condicionamento é um mecanismo de proteção celular devido à indução de tolerância ao dano, a partir de um estímulo subtóxico e por isto tem sido utilizado como mecanismo de neuroproteção contra insultos induzidos por isquemia ou glutamato. NMDA em doses subtóxicas protege neurônios contra a morte celular desencadeada por altos níveis extracelulares de glutamato. Além disso, o papel da adenosina neste mecanismo de neuroproteção tem sido investigado.

No presente estudo, investiga-se a estimulação da atividade da ecto-ATP difosfoidrolase e da ecto-5'-nucleotidase por glutamato, e a participação dos receptores adenosinérgicos, no mecanismo de neuroproteção pelo pré-condicionamento com NMDA em cultura de neurônios granulares do cerebelo.

III) A administração intra-cerebroventricular de ácido quinolínico induz convulsões e conseqüente morte celular em camundongos e ratos. O modelo de convulsões por ácido quinolínico é utilizado para medir a neurotoxicidade desta substância, mediada por receptores NMDA.

No presente estudo, investiga-se a efetividade do uso de doses subconvulsivas de NMDA, como um mecanismo de neuroproteção pelo pré-condicionamento, contra as convulsões e conseqüente morte celular induzidas pelo ácido quinolínico em camundongos. Além disso, verificar o envolvimento dos receptores de adenosina no pré-condicionamento induzido por NMDA.

II. ARTIGOS CIENTÍFICOS

II.1. CAPÍTULO 1 - Carina R. Boeck, Eduardo H. Kroth, Mauro J. Bronzatto, Fluvia Jardim, Diogo O. Souza and *Deusa Vendite. **Effects of glutamate transporter and receptor ligands on neuronal glutamate uptake.** Submetido ao periódico **Brain Research**.

Effects of glutamate transporter and receptor ligands on neuronal glutamate uptake

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Number of text pages of the whole manuscript: 30

Number of Table: 1

Number of Figures: 6

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Contract grant sponsor: FAPERGS, FINEP/CNPq and PRONEX

ABSTRACT

Previous studies have shown that Glu transporters and Glu receptors can modulate [^{3}H]-glutamate (Glu) uptake in cultured neural cells. The Glu transporters regulate the balance between physiological and pathological signaling over stimulation of the glutamatergic system pathway. The effect of Glu ligands, at neurotoxic concentrations, on Glu uptake in cerebellar granule cells was assessed. Pre-incubation of cells with Glu, L- or D-aspartate (Asp) inhibited [^{3}H]-Glu uptake and this inhibition was reversed when L-Asp was co-incubated with dihydrokainate (DHK). L- and D-Asp are agonists of the *N*-methyl-D-aspartate (NMDA) receptors, however, their effects upon Glu uptake were not altered in the presence of NMDA receptor antagonists. A robust Glu efflux was observed following the pre-incubation of cells with neurotoxic concentration of excitatory amino acids (EAAs). Thus, EAAs-evoked inhibition of [^{3}H]-Glu uptake may be due EAAs binding to extracellular and/or intracellular sites on the EAA transporters. In contrast, the Glu receptors agonists, NMDA and (1*S*,3*R*)-1-Aminocyclopentane-1,3-dicarboxylic acid (SR-ACPD), increased Glu uptake, whilst kainic acid (KA) had no effect. The NMDA effect was not altered by its antagonists (±)-2-amino-5-phosphonopentanoic acid (AP-5) or dizocilpine (MK-801), however MK-801 increased Glu uptake *per se*. The SR-ACPD effect was abolished by its antagonist, L(±)-2-Amino-3-phosphonopropionic acid (L-AP3), providing evidence that the SR-ACPD-induced increase was due to the activation of metabotropic Glu receptor. The present study indicates that the Glu ligands, at neurotoxic concentrations, modify Glu uptake via different mechanisms in cultured neurons. Thus, these events may contribute to neurotoxic or neuroprotective cellular process in the glutamatergic pathway.

Theme D: Neurotransmitters, modulators, transporters, and receptors

Topic: Excitatory amino acids: physiology, pharmacology and modulation

Keywords: cerebellar granule cells; glutamate uptake; glutamate transporters substrates; glutamate receptors agonists; glutamate receptors antagonists.

1. Introduction

Excitatory neurotransmission in the mammalian CNS is mediated by excitatory amino acids (EAAs), principally glutamate (Glu) and aspartate (Asp). Glu action is mediated via activation of ionotropic (iGluR) and metabotropic (mGluR) receptors, which are present throughout the nervous system of most, if not all, species. Accumulation of Glu in the synaptic cleft may lead to excitotoxic neuronal damage due to over activation of glutamatergic receptors [25]. Rapid uptake of these EAAs by the transporters localized on the cellular membrane is believed to be the major regulatory and neuroprotective mechanism. To date, five types of Na^+-dependent EAA transporters (EAATs) have been cloned. Two of them, GLT-1 and GLAST, are expressed primarily in glial cells, although there is evidence to demonstrate their neuronal expression during development and/or in cultured neurons [33,29,31,18]. EAAC1 and EAAT4 are neuronal transporters and EAAT5 expression is thought to be restricted to the retina [5,4]. Astroglial Glu/Asp uptake is considered most important for keeping the extracellular levels of Glu low and, therefore, essential for both normal synaptic transmission and protection of neurons against Glu excitotoxicity. Neuronal transporters are thought to occur in the postsynaptic region [2]. The extra-synaptic localization of the neuronal transporters and their expression in non-glutamatergic neurons suggest that they may also have other functions in addition to their participation in the extracellular clearance of glutamate [36].

Cultured cerebellar granule cells express Glu transporters and exhibited high-affinity Glu uptake [14]. Accordingly, in our laboratory, we have demonstrated that the activation of ecto-nucleotidases by Glu in cerebellar granule cells is dependent upon Glu uptake [6].

Glu/Asp transporters can be regulated at several levels. Acute regulation of uptake has been reported by: i) EAAT substrates [15,28], ii) redox sensitivity [14,1], iii) phosphorylation mediated by protein kinase A (PKA) and protein kinase C (PKC) [27,12] and iv) purine nucleoside guanosine, as recently demonstrated in our laboratory [17]. In addition, EAATs expression is developmentally regulated in neuronal cells [18,20]. Some of these regulations promote a change in catalytic efficiency of the transporters, which could be linked to the shift in the electrochemical gradients, or a change in the number of these transporters at the cell membrane.

Since Glu transporters play a key role in regulating extracellular Glu concentration in the synapse and in spillover from one synapse to another, the Glu uptake is crucial for the maintenance of Glu homeostasis and, thus, effective neurotransmission.

In the present study, we examined whether Glu transporter substrates and Glu receptor agonists have, at neurotoxic concentrations, any effect on Glu uptake in cultured cerebellar granule cells. Our results demonstrated a long lasting inhibition of Glu uptake evoked by substrates of transporters (Glu, L- or D-Asp) and a stimulation evoked by NMDA receptor ligands (NMDA and MK-801) and by Group I mGluR (activated by SR-ACPD).

2. Materials and methods

2.1. Materials

Unless otherwise stated, chemicals and reagents were obtained from Merck, Sigma or Life Technologies. L-[^{3}H]-Glu (specific activity, 33 Ci/mmol) was from Amersham Pharmacia Biotech.

2.2. Cerebellar Granule Cell Culture

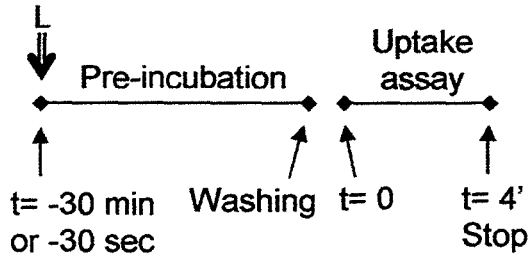
Primary cultures of cerebellar granule cells were prepared from 8-day-old Wistar rats as previously described [6]. Briefly, freshly dissected cerebella were dissociated by mild trypsinization (0.025% trypsin (w/v), 37 °C, 15 min) and disrupted mechanically with a fire-polished Pasteur pipette in the presence of DNase (0.08 mg/ml) and trypsin inhibitor (0.05% (w/v). Cells were seeded at a density of 3.2 x 10^5/cm^2 in a 24-well multiwell dish (Nunc) coated with 10 µg/ml poly-D-lysine in Eagle's basal medium (BME) supplemented with 10% fetal bovine serum, 25 mM KCl and 50 µg/ml gentamicin. The growth of non-neuronal cells was inhibited by addition of 20 µM cytosine arabinofuranoside 18-20 h after seeding and the medium was maintained without changing during the culture period. Due to medium evaporation, glucose (0.1% in water (w/v)) was added to a final volume of 100 µL at 7 days in vitro (DIV) [37]. These cultures contain > 90% granule cells, 4-6% GABAergic neurons, and a small number of glial (2-3%) and endothelial cells (< 1%) [22]. Neuronal survival was also assessed using morphological criteria by inverted phase-contrast microscopy at the end of experiments.

2.3. Determination of Glutamate Uptake

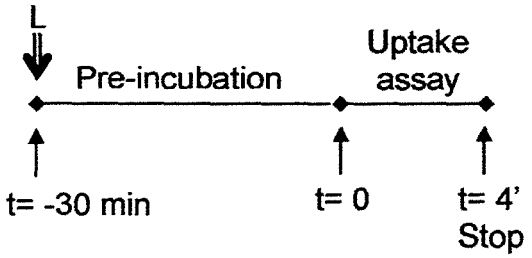
At 9 DIV, nutrient medium was carefully removed from dishes, and the cell monolayer was gently washed once with pre-warmed sodium-containing HEPES-buffered saline (in mM: 120 NaCl, 5 KCl, 2 $CaCl_2$ and 10 glucose), pH 7.4 or choline-containing assay medium (sodium free: NaCl replaced with choline chloride), as appropriate [6]. The experimental designs are illustrated below. Cells were pre-incubated in the absence or presence of Glu transporter substrates (Glu, L- or D-ASP) or of glutamatergic receptor agonists (NMDA, KA or SR-ACPD). As specified, dihydrokainate (DHK) (Glu transporter blocker), dizocilpine (MK-801) or (±)-2-amino-5-phosphonopentanoic acid (AP-5) (NMDA receptor antagonists), or L(±)-2-Amino-3-phosphonopropionic acid (L-AP3) (Group I mGluR receptor antagonist) were present. When mentioned, after the pre-incubation period, the compounds were removed by aspiration of medium and the cells were washed twice with sodium-containing assay medium before the [^{3}H]-Glu uptake assay period. Glu uptake was started by the addition of 100 µM [^{3}H]-Glu (final concentration) and incubated for 4 min at 37 °C in sodium-containing assay medium. Uptake was stopped with two rinses of ice-cold choline-containing assay medium. Cells were then lysed with 0.1 N NaOH/0.01% SDS and the suspension colleted for the measurement of the [^{3}H]-Glu taken up. Control cells were assayed with the treatment correspondent to that of the experimental group, but without addition of ligands. Non-specific uptake was performed in choline-containing (instead of sodium) medium. Glu uptake was defined as the difference between the uptake performed with sodium-containing medium and those with choline-containing medium. Protein assays were performed on sister wells using bovine serum albumin as standard [7].

Outline of the experimental protocols (L, ligands added to Glu transporters or receptors):

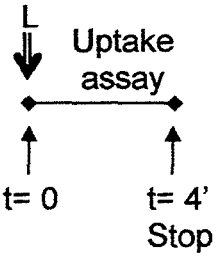
Protocol 1: with wash step before Glu uptake assay



Protocol 2: without wash step before Glu uptake assay



Protocol 3: ligands added just during Glu uptake assay



2.4. Determination of Glutamate Release

In order to investigate whether EAAs efflux occurred during the Glu uptake period, we mimicked the same condition of the pre-incubation with EAAs, using

[^{3}H]-Glu to load cells. The Glu release was assayed in the presence or absence of EAAs during the correspondent uptake assay period (release assay period). According to Protocol 1, the cells were pre-incubated in the presence of 500 µM [^{3}H]-Glu for 30 min, after that the medium was aspired and the cells were washed twice with sodium-containing assay medium before the release assay period. [^{3}H]-Glu release was started by the addition of 100 µM Glu, L-, D-Asp for 4 min at 37 °C in sodium-containing assay medium. After that, the medium was collected for scintillation counting to evaluate extracellular [^{3}H]-Glu and the cells were washed twice with ice-cold choline-containing assay medium. Cells were then lysed with 0.1 N NaOH/0.01% SDS and collected for the measurement of the intracellular [^{3}H]-Glu content. Control cell groups were assayed with the treatment correspondent to that of the experimental group, but without addition of ligands during the release assay period. Release was quantified as the percentage of total loaded [^{3}H]-Glu (counts in medium and lysates combined) released into the medium (counts in the medium only) [3].

3. Statistical Analysis

All assays were carried out in triplicate or quadruplicate and each value is the mean ± SD of independent experiments that correspond to each culture. The significance of the difference among groups was evaluated by a one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test. Differences were considered significant at < 95% confidence.

3. Results

Considering recent evidence that Glu/Asp transporters can be rapidly regulated by a variety of signaling pathways, cultured cerebellar granule cells were exposed to EAAT or to Glu receptors ligands to evaluate their effect on Glu uptake activity.

For a preliminary characterization of Glu uptake, cultured cerebellar granule cells were incubated with 100 μM [^{3}H]-Glu for different times and uptake was linear with time up to 4 min, reaching a plateau at 8 min (data not shown). Thus, further experiments on Glu uptake kinetics studies were performed for 4 min. The Michaelis-Menten constant (K_m, approximately) and a maximal velocity (V_{max}, approximately) were calculated from the Eadie-Hofstee plot obtained with [^{3}H]-Glu in the final concentration range of 1-100 μM (Fig. 1). The apparent K_m and V_{max} values were 16.5 ± 4 μM and 4.5 ± 2 nmol Glu/mg protein/min, respectively.

To evaluate the effect of Glu transporters substrates, cells were pre-incubated with Glu, L-, D-Asp, DHK or L-Asp plus DHK for 30 min, and washed before [^{3}H]-Glu uptake assay (Protocol 1). Glu, L- or D-Asp decreased the uptake from 3.4 ± 0.8 to 2.4 ± 0.6, to 2.2 ± 0.5 or to 1.7 ± 0.5 nmol Glu/mg protein/min, respectively ($P < 0.01$) (Fig. 2). DHK had no effect, but DHK blocked the inhibitory effect of L-Asp when co-applied with L-Asp during the pre-incubation period (Fig. 2). When added just during the [^{3}H]-Glu uptake assay period, DHK alone blocked [^{3}H]-Glu uptake (2.1 ± 0.4 to 1.3 ± 0.4 nmol Glu/mg protein/min, $P < 0.01$).

Since the EAAs are NMDA receptor agonists, the cells were pre-incubated with L- or D-Asp in the presence of NMDA receptor antagonists to investigate the possible involvement of NMDA receptors on the EAAs inhibitory effect. As

show in Fig. 3, cells were pre-incubated for 30 min with L- or D-Asp in the presence or absence of MK-801 or AP-5 and washed before [^{3}H]-Glu uptake assay. In this experimental protocol, each antagonist alone had no effect on Glu uptake and neither was able to modify the inhibitory effect of L- or D-Asp (Fig. 3A and 3B).

In order to further characterize putative mechanisms of uptake inhibition following pre-incubation with EAAs, the released Glu was determined. We considered the possibility that Glu uptake inhibition, induced by pre-incubation with EAAs, could be due to the release of these ligands during the uptake assay period. Thus, we pre-loaded the cells with 500 µM [^{3}H]-Glu for 30 min and the effect of EAAs on Glu release for 4 min was determined. The basal [^{3}H]-Glu release from the cells corresponded to 26.3 ± 5 µM (27.7 ± 7 % of the total loaded) and this release did not change when the cells were incubated with 100 µM EAAs (Table 1). Considering the possibility that the inhibitory effect of EAAs was due to their release during the Glu uptake assay, their ability to affect Glu uptake, at their correspondent concentrations released, was tested. The EAAs, at this concentration, inhibited Glu uptake (Fig. 4A). In addition, the inhibitory effect of EAAs was further investigated by measuring Glu uptake after a brief pre-incubation (30 sec) with 500 µM EAAs, considering that this time may be enough for the EAAs to bind to the transporters. Under these conditions, we observed that EAAs also inhibited the Glu uptake (Fig. 4B).

In further experiments, neuronal cells were treated with NMDA, SR-ACPD or KA during pre-incubation and/or [^{3}H]-Glu uptake assay periods (Fig. 5). When these agonists were washed before the Glu uptake assay, they had no effect (see Materials and methods Section: Protocol 1, Fig. 5A). On the other hand,

when they were present during pre-incubation and the [^{3}H]-Glu uptake assay periods, NMDA and SR-ACPD increased the [^{3}H]-Glu uptake from 2.9 ± 0.2 to 4.0 ± 0.9 or to 4.5 ± 1.5 nmol Glu/mg protein/min, respectively ($P < 0.05$), and KA had no effect (Protocol 2, Fig. 5B). When they were present just during the [^{3}H]-Glu uptake assay period, only NMDA increased the uptake, whilst SR-ACPD and KA have no effect (Protocol 3, Fig. 5C).

The Glu uptake stimulation by SR-ACPD or NMDA occurred via different mechanisms (Fig. 6). As shown in Fig. 6B, the SR-ACPD effect was due to receptor activation, since co-application of L-AP3 (mGluR receptor antagonist) abolished the SR-ACPD-evoked Glu uptake increase using Protocol 2. In contrast, both AP-5 and MK-801 (NMDA receptor antagonists) were ineffective in blocking the stimulatory effect of NMDA (Fig. 6A). Neither AP-5 nor L-AP3 had any effect on Glu uptake when present alone during pre-incubation and the [^{3}H]-Glu uptake assay period, whilst MK-801 was able to increase the Glu uptake *per se*. This effect was also observed when MK-801 was present just during the [^{3}H]-Glu uptake assay period (from 1.8 ± 0.1 to 2.6 ± 0.3 nmol Glu/mg protein/min, $P < 0.01$).

4. Discussion

The present report demonstrates that ligands to EAATs and Glu receptors are able to modify, in a different manner, the Glu uptake by cerebellar granule cells. The inhibitory effect of EAAs (Glu, L-, and D-Asp) on [^{3}H]-Glu uptake remained even after their washing before the uptake assay, indicating a long lasting effect. However, the stimulatory effect of NMDA and SR-ACPD on Glu uptake was

dependent upon their presence during pre-incubation and [^{3}H]-Glu uptake assay periods (or just during the [^{3}H]-Glu uptake assay period for NMDA).

Transportability displayed by L- and D-Asp on EAATs in cerebellar granule cells is known to competitively inhibit Glu uptake [14,19]. In our experimental protocol conditions, cells were washed prior to uptake measurements (Protocol 1), therefore, the EAAs were transported and accumulated inside the cells during the pre-incubation period and, consequently, released during the Glu uptake assay, inhibiting [^{3}H]-Glu uptake. When we measured the EAAs release using [^{3}H]-Glu, during the pre-incubation period, and EAAs during the uptake assay period, we observed that EAAs did not change the baseline of the [^{3}H]-Glu released. In our study, no EAAs-evoked Glu release was observed, probably due to the use of higher concentration of EAAs than other studies describing Glu efflux induced by the reversal of uptake or heteroexchange [3,26,23,16]. In subsequent experiments, the EAAs, at concentration corresponding to their released amounts, were enough to inhibit Glu uptake in cerebellar granule cells. In another experiment, when 500 μM EAAs were pre-incubated briefly for 30 seconds, the same inhibitory effect was observed. These findings could indicate that the EAAs-evoked Glu uptake inhibition was due to the occupancy of the extracellular sites on the transporters by EAAs. This suggestion is supported the fact that the L-Asp effect was blocked by the presence of the non-transportable inhibitor, DHK, during the pre-incubation period. The inhibition observed suggests that DHK could be blocking L-Asp binding on the transporter and, subsequently, pre-loading (Protocol 1), whilst the remaining Glu uptake processes are unaffected. In the experiments described herein, DHK, when pre-incubated alone and washed before the [^{3}H]-Glu uptake

assay period, had no effect on Glu uptake, however it blocked the Glu uptake when added just during the [^{3}H]-Glu uptake assay period (Protocol 3). Cerebellar granule cells display a high density of EAAC1 and DHK is considered selective blocker of the GLT-1 transporter, although it blocks EAAC1-mediated transport at milimolar concentrations [20,35]. Nevertheless, the presence of a GLT-1 transporter cannot be ruled out, since its expression has been observed not to be restricted to glial cells, demonstrated by the expression in cultured neurons [31,20,9,11]. In addition, the lack of effect of the NMDA receptor antagonists, pre-incubated with EAAs, ruled out the contribution of NMDA receptors on the EAAs-evoked inhibitory effect, since L- and D-Asp can also mimic most of the actions of Glu by binding to NMDA receptor [24,21 for Review]. The inhibitory effect of Glu, L-, or D-Asp contrasted with the reported stimulatory effect of EAAs on Glu uptake in astrocytes, which is due to an up-regulation of the GLAST transporter [15]. In neuronal cultures contaminated with astrocytes, EAAs also increase Glu uptake activity. In contrast, however, they promote a decrease in the amount of EAAC1 on the cell surface [28]. Another likely explanation for this EAAs-evoked inhibitory effect could be due to their binding to an intracellular site on the transporter having as a consequence the phenomenon described as *trans*-inhibition [13]. Taken together, the results described herein suggest that the long lasting EAAs-evoked effect represents a competitive inhibition by binding to an extracellular and/or intracellular site on the EAATs. The EAAs-evoked inhibitory effect on Glu uptake in cerebellar granule cells may reflect a competitive inhibition of the transport in whichever direction that it is taking place.

Additionally, Glu receptors agonists were also tested for their ability to modify Glu uptake using neurotoxic doses. NMDA and SR-ACPD increased [^{3}H]-Glu uptake in neuronal cells when present during pre-incubation and [^{3}H]-Glu uptake assay periods (Protocol 2), whereas KA had no effect. L-AP3 (mGluR antagonist) blocked this stimulatory effect of SR-ACPD on Glu uptake (see Fig. 5B and 6A). SR-ACPD has been widely used as a non-specific mGluR agonist. The increase in astroglial Glu uptake by SR-ACPD pre-incubation has been previously attributed to the fact that the RS-ACPD enantiomer, but not SR-ACPD, may itself serve as a substrate for Na^+-dependent EAATs [15,38]. Moreover, the lack of [^{3}H]-*t*-ACPD uptake in our preparations (unpublished observations) and the fact that the effect of SR-ACPD was blocked by L-AP3 suggest that the SR-ACPD-evoked increase of uptake was due to the activation of metabotropic receptors. In addition, the direct effect of L-AP3 on Glu uptake was ruled out, since it has no effect on Glu uptake assayed in the control groups. It is known that SR-ACPD stimulates PKC in cerebellar granule cells [30] and that EAAC1 transporter activity is increased by activation of PKC [12]. Indeed, a modulation of postsynaptic mGluRs has been described in parallel fiber synapses on Purkinje cell dendrites in response to neuronal Glu uptake. Probably, a functional interplay between neuronal mGluRs and Glu transporters could regulate Glu clearance in addition to neurotransmission [8]. The SR-ACPD effect clearly depends on the activation of mGluR in neurons, which increased intracellular signaling response. In contrast to the SR-ACPD effect, NMDA stimulation was the same when it was present either during the pre-incubation assay and the Glu uptake assay periods (Protocol 2, Fig. 5B) or only during the Glu uptake assay period (Protocol 3, Fig. 5C). Interestingly, the NMDA

antagonists, AP-5 and MK-801, did not modify the NMDA-evoked activation of Glu uptake, even though MK-801 had a stimulatory effect *per se*. An increase in Glu uptake following Glu pre-incubation has been demonstrated in 'astrocyte-poor' neuronal cultures, but this effect was not blocked by the presence of MK-801, although NMDA pre-incubation also increased the Glu transporter activity [28]. Other studies have found that MK-801 may affect noradrenaline, serotonin, dopamine release and uptake via membrane depolarization, and that these effects are not mediated by its blockade of the NMDA receptors [26,32,10]. In fact, our observations, with regard to the NMDA and MK-801 effects, could indicate a shift in the uptake driving force induced by raising extracellular K^+ concentrations. In contrast to the EAAs effects, cells were not pre-loaded with these ligands, therefore, the mechanism by which NMDA or MK-801 evoke an increase in Glu uptake is unknown in neuronal cells. We are currently endeavoring to clarify this interesting matter.

In conclusion, our data show that the Glu transporter substrates, Glu, L- and D-Asp, have direct inhibitory effects on Glu uptake activity. In contrast, Glu receptor agonists increase Glu uptake activity in cerebellar granule cells in different manners. Taken together, these results suggest that the Glu removed from the synaptic cleft by the neuronal transporter may be modified by different mechanisms in response to cellular glutamatergic excitotoxicity, as a consequence of over-stimulated glutamatergic neurotransmission. Since disturbances of glutamatergic neurotransmission lead to neuronal damage, the characterization of the mechanisms that can modify Glu uptake is important for understanding the physiological and pathological signaling pathways activated by the glutamatergic system.

Acknowledgements

This research was supported by grants from FAPERGS, FINEP/CNPq and PRONEX. Carina R. Boeck was the recipient of a CAPES fellowship.

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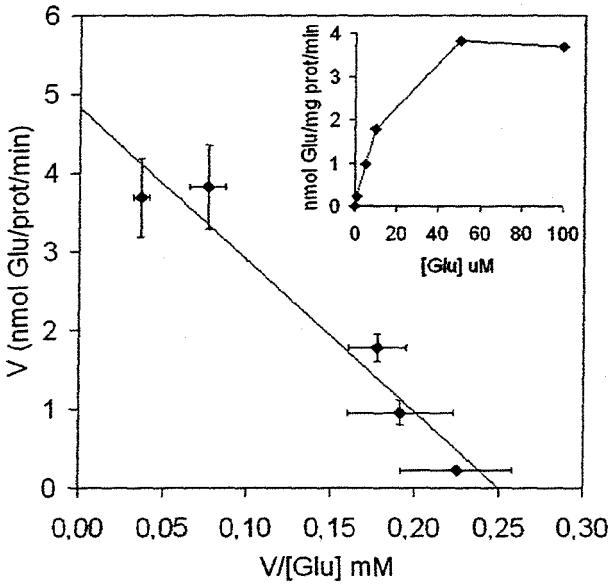

Fig. 1. Eadie-Hofstee plots of the saturation kinetics of [^{3}H]-Glu uptake in cerebellar granule cells. The inset shows activation of the transporter by Glu in the range of 1-100 μM for 4 min. Values are mean ± S.E.M. of independent cultures (n = 8-10).

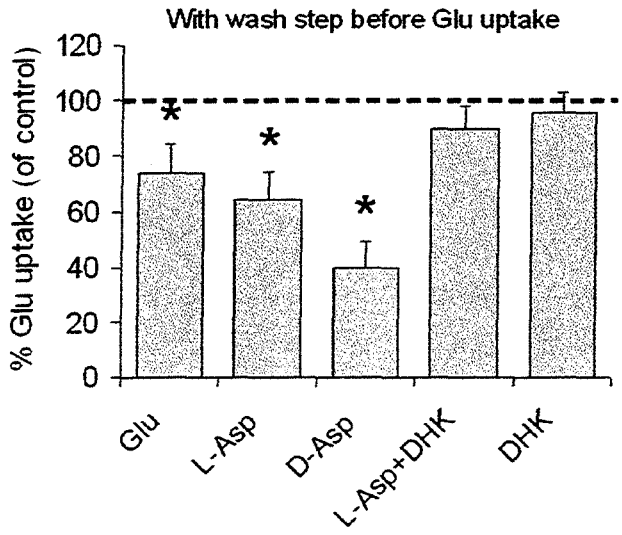

Fig. 2. Effect of Glu, L- or D-Asp on [^{3}H]-Glu uptake in cerebellar granule cells. According to Protocol 1, the cells were pre-incubated for 30 min in the presence of 500 μM EAAs with or without 500 μM DHK and washed prior to the [^{3}H]-Glu uptake assay period. Values are mean ± SD of independent cultures (n = 3-6). *Significantly different from control and other groups ($P < 0.01$).

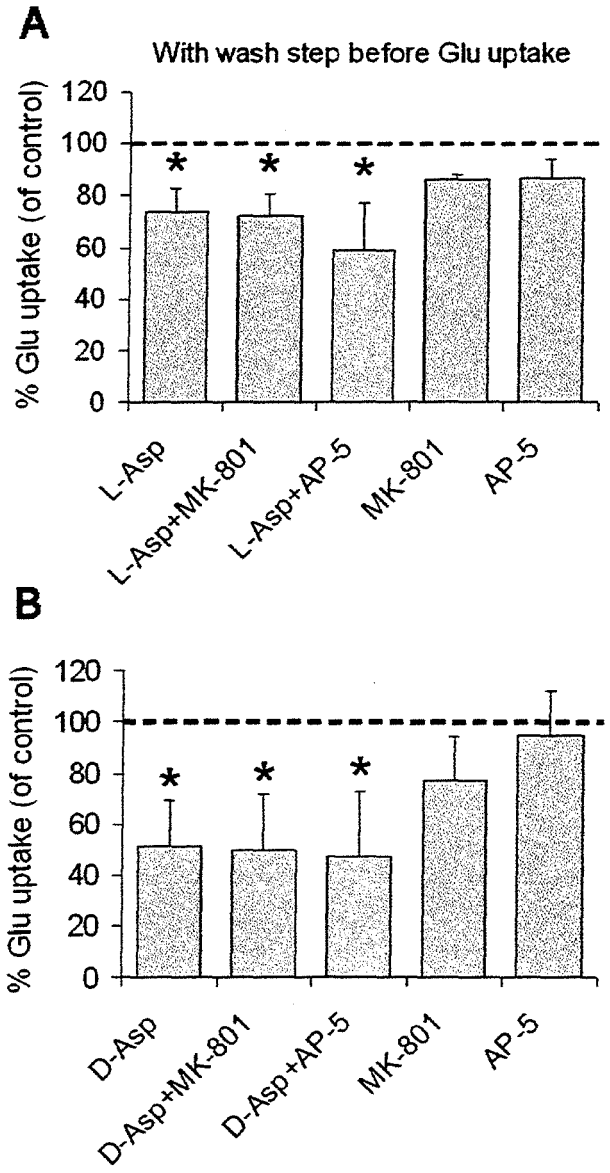

Fig. 3. Effect of NMDA receptor antagonists on [^{3}H]-Glu uptake inhibited by L- and D-Asp. According to Protocol 1, the cells were pre-incubated for 30 min in the presence of 500 μM (A) L-Asp or (B) D-Asp with or without 1 μM MK-801 or 100 μM AP-5 and washed prior to the [^{3}H]-Glu uptake assay period. Values are mean ± SD of independent cultures (n = 3-9). *Significantly different from control and other groups ($P < 0.01$).

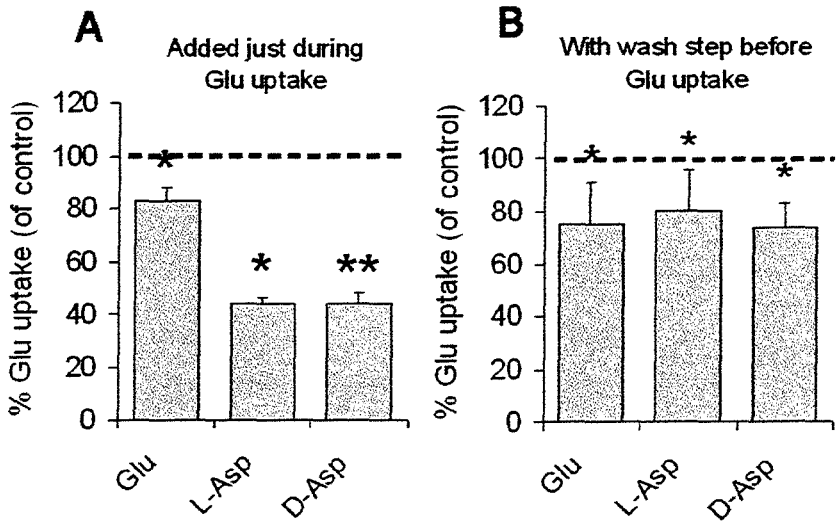

Fig. 4. Characterization of the long lasting effect of EAAs on [^{3}H]-Glu uptake. Cells were incubated in the presence of (A) 20 μM EAAs during the [^{3}H]-Glu uptake assay (Protocol 3) or (B) pre-incubated in the presence of 500 μM EAAs for 30 sec and washed prior to the [^{3}H]-Glu uptake assay (Protocol 1). Values are mean ± SD of independent cultures (n = 3-4). *Significantly different from control and Glu; **significantly different from other groups ($P < 0.01$).

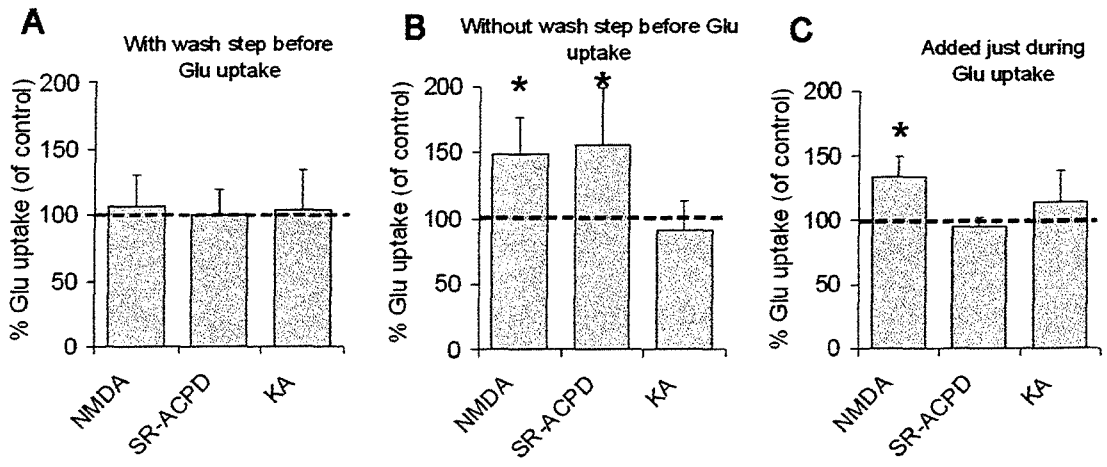

Fig. 5. Effect of Glu receptor agonists on [^{3}H]-Glu uptake. Cells were pre-incubated in the presence of 100 μM NMDA, SR-ACPD or KA for 30 min and (A) washed prior to the [^{3}H]-Glu uptake assay (Protocol 1) or (B) maintained during the [^{3}H]-Glu uptake assay (Protocol 2) or (C) agonists were added just during the [^{3}H]-Glu uptake assay period (Protocol 3). Values are mean ± SD of independent cultures (n = 5). *Significantly different from control and other groups ($P < 0.01$).

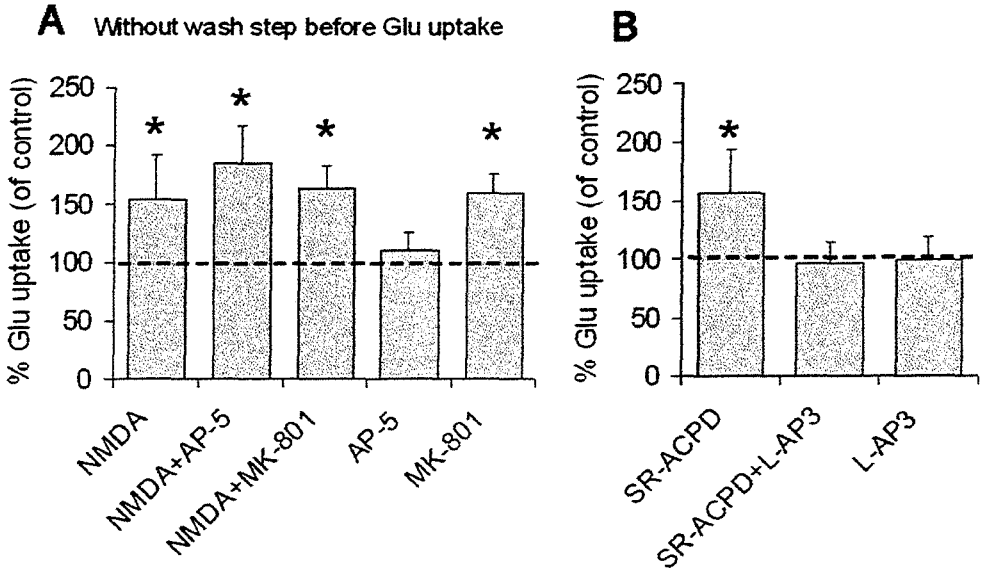

Fig. 6. Effect of receptor antagonists on [^{3}H]-Glu uptake activated by NMDA and SR-ACPD. According to Protocol 2, the cells were pre-incubated for 30 min in the presence of (A) 100 µM NMDA with or without 1 µM MK-801 or 100 µM AP-5 or (B) 100 µM SR-ACPD with or without 100 µM L-AP3, without wash step and maintained during the [^{3}H]-Glu uptake assay period. Values are mean ± SD of independent cultures (n = 4). *Significantly different from controls ($P < 0.01$).

Table 1. Effect of EAAs on [^{3}H]-Glu efflux.

Glu released	Control	D-Asp	L-Asp	Glu
μM	26.3 ± 5	20.5 ± 1	21.3 ± 3	26.1 ± 4
% total loaded	27.7 ± 7	27.1 ± 4	30.6 ± 5	31.3 ± 6

According to Protocol 1, the cells were pre-incubated for 30 min in the presence of 500 μM [^{3}H]-Glu and washed prior to incubation with 100 μM EAAs. Values are mean ± SD of independent cultures (n = 3).

II.2. CAPÍTULO 2 - Carina R. Boeck, Mauro J. Bronzatto, Eduardo H. Kroth, and *Deusa Vendite. **Neurotoxicity induced by L- or D-aspartate stimulates ecto-5'nucleotidase activity in cerebellar granule cells.** Submetido ao periódico **Neuroscience Letters**.

Neurotoxicity induced by L- or D-aspartate stimulates ecto-5'nucleotidase activity in cerebellar granule cells

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Number of text pages of the whole manuscript: 18

Number of Figures: 3

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Contract grant sponsor: FAPERGS, FINEP/CNPq and PRONEX.

Theme D: Neurotransmitters, Modulators, Transporters, and Receptors

Topic: Excitatory amino acids: excitotoxicity

Key words: L- and D-aspartate neurotoxicity; ecto-5'-nucleotidase; adenosine.

ABSTRACT

High concentrations of excitatory amino acids (EAAs) lead to cellular damage by excitotoxicity. Glutamate is an excitatory neurotransmitter and increases the extracellular adenosine levels, which is considered an important endogenous neuromodulator. The neurotoxicity induced by glutamate increase the ecto-5'-nucleotidase activity in neurons. Ecto-5'-nucleotidase produces adenosine from AMP. The EAAs L-aspartate and D-aspartate mimic most of the actions of glutamate in the NMDA receptors and on the EAAs transporters (EAATs). In the present study, both EAAs stimulated the ecto-5'-nucleotidase activity cerebellar granule cells. The antagonists of NMDA receptors, MK-801 and AP-5 prevented the stimulatory effect evoked by L-aspartate, but they did not affect the stimulation evoked by D-aspartate. Dihydrokainate, a blocker of excitatory amino acid transporters, prevented the L-aspartate and D-aspartate-evoked activation of ecto-5'-nucleotidase. Regarding the cellular damage, both NMDA receptors antagonists prevented completely the neuronal death induced by L-aspartate, but partially the D-aspartate-induced cellular death. Moreover, antagonist of adenosine A_1 or A_{2A} receptors did not prevent the cellular death induced by L-aspartate or D-aspartate. The present study indicated a different involvement of NMDA receptors on the L-Asp or D-Asp actions in cerebellar granule cells.

INTRODUCTION

Excitatory neurotransmission in the mammalian CNS is mediated by excitatory amino acids (EAAs), such as L-, D-aspartate (L-, D-Asp) and principally glutamate (Glu). Rapid uptake of these EAAs by the Na^+-dependent EAA transporters (EAATs) localized on the cellular membrane is believed to be the major regulatory and neuroprotective mechanism. Excitotoxicity mediated by Glu and L-Asp has been described in many instances to excessive activation of the N-methyl-D-aspartate (NMDA) receptors-channel complex [12]. The EAAs, L- and D-Asp, are putative NMDA receptors agonists mimicking most of the actions of Glu on NMDA receptors by sharing the same binding site [9]. The activation of NMDA receptors promotes the increase of extracellular adenosine levels [5], an important endogenous neuromodulator. The extracellular adenosine can be released via a bi-directional transporter or formed from adenine nucleotides released that are degraded by an extracellular chain of ecto-nucleotidases. The nucleotides ATP and ADP are hydrolyzed through ecto-ATP diphosphohydrolase activity resulting in the formation of AMP, which products adenosine by ecto-5'-nucleotidase activity. Ecto-5'-nucleotidase activity is a pivotal step of the extracellular adenosine production from enzymatic chain [15]. The activation of adenosine A_1 receptors inhibit neuronal firing, decrease calcium uptake [10] and inhibit release of excitatory neurotransmitters such as Glu and Asp [11], contributing to neuroprotection. The activation of adenosine A_{2A} receptors enhances synaptic transmission and the glutamate release [8], which could contribute to the neurotoxicity. In cultured neurons as well as hippocampal slices have been demonstrated that Glu stimulates

extracellular nucleotides hydrolysis via ecto-nucleotidases, but it is unclear whether the adenosine produced acts on adenosine A_1 or A_{2A} receptors. The Glu-induced stimulation of ecto-nucleotidase activities in neurons was mimicked by NMDA and was inhibited by dihydrokainate (DHK), an inhibitor of Glu uptake [1].

Since L- and D-Asp are agonists to NMDA receptor and substrates to EAATs, in the present study, we investigated if neurotoxic dose of L- and D-Asp mimic the stimulation of ecto-5'-nucleotidase activity via NMDA receptors activation and if the blocker to EAATs affects this stimulation. Moreover, we tested the potential neurotoxic effects of L- and D-Asp and if the NMDA receptors antagonists and the adenosine A_1 or A_{2A} receptors antagonist could be able to affect the L- and D-Asp-induced cellular damage in cerebellar granule cells.

MATERIALS AND METHODS

Materials

Unless otherwise stated, chemicals and reagents were obtained from Merck, Sigma or Life Technologies.

Cerebellar Granule Cell Culture

Primary cultures of cerebellar granule cells were prepared from 8-day-old Wistar rats [1]. Briefly, freshly dissected cerebella were incubated with 0.025% trypsin solution for 15 min at 37 °C and disrupted mechanically with a fire-polished Pasteur pipette in the presence of 0.08 mg/ml DNase and 0.05% trypsin inhibitor. Cells

were seeded at a density of 3.0 x $10^5/cm^2$ in a 24-well multiwell dish (Nunc) coated with 10 µg/ml poly-D-lysine in Eagle's basal medium (BME) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil), 50 µg/ml gentamicin and 25 mM KCl. The growth of non-neuronal cells was inhibited by an addition of 20 µM cytosine arabinofuranoside 18-20 h after seeding and the medium was maintained without any change during the culture period. Due to medium evaporation, water and 0.1% glucose were added to a final volume of 100 µL at 7 days in vitro (DIV). These cultures contain > 90% granule cells, 4-6% GABAergic neurons, and a small number of glial (2-3%) and endothelial cells (< 1%) [6].

Determination of ecto-5'-nucleotidase activity

After 8 DIV days *in vitro* (DIV) nutrient medium was carefully removed from dishes, and the cell monolayer was gently washed once with pre-warmed sodium containing HEPES-buffered saline (120 mM NaCl, 5 mM KCl, 2 mM CaCl2 and 10 mM glucose) [1]. Cells were pre-incubated for 30 min in the presence or absence of 1 mM L- or D-Asp. As specified, 500 µM dihydrokainate (DHK, EAATs blocker), 1 µM dizocilpine (MK-801, non-competitive NMDA receptor antagonist) or 100 µM (±)-2-amino-5-phosphonopentanoic acid (AP-5, competitive NMDA receptor antagonist) were present. Incubation of cerebellar granule cells started by the addition of 1 mM AMP (final concentration) and incubated for 10 min at 37°C. The reaction was stopped by the removal of aliquots, which were then mixed with 5% trichloroacetic acid (TCA, final concentration). After a centrifugation of 5 min at 12 000 X g at 4°C, the aliquots were taken for assay of released inorganic phosphate (Pi) [3]. Non-enzymatic Pi released from nucleotide into assay medium without cells

and Pi released from cells incubated without nucleotide was subtracted from the total Pi released during incubation, giving values for enzymatic activity. Enzymatic activity was expressed as nmol Pi/mg protein/min. We have observed, as previously described, that at the end of the experiment the survival and morphology of cells is maintained [1]. Protein was determined using bovine serum albumin as standard [2].

L- and D-Asp neurotoxicity and cellular damage

At 8 DIV, the nutrient medium was carefully removed from dishes, and the cell monolayer was gently washed twice with pre-warmed HEPES-buffered saline. Cells were incubated with or without 1 mM L- or D-Asp in the presence or absence of NMDA receptors antagonists (MK-801 or AP-5) or adenosine A_1 (100 nM CPT) or A_{2A} (50 nM ZM 241385) receptor antagonists for 30 min. After the neurotoxicity assay period, cells were carefully washed twice with pre-warmed HEPES-buffered saline and previously the saved sister culture medium (culture-conditioned medium) was replaced. Cell viability was monitored before and after each experiment by the inverted phase-contrast microscopy. Cells death was assessed 24 h post-neurotoxicity assay period using uptake of the fluorescent exclusion dye propidium iodide (PI), which is a polar compound that only enters into the dead or dying cells with damage membranes. Cells were incubated with 5 µg/mL PI for 30 min and then imaged on a standard inverted microscope (Nikon Eclipse TE 300) using a rhodamine filter set (excitation 540 nm; emission 617 nm). The PI uptake was quantified by a densitometric analysis using Scion Image software (Scion Corporation). The quantification of cellular death, which corresponds to PI uptake

into cells damaged, was expressed as percentage of maximal death induced by L- or D-Asp.

Statistics

Statistical analysis among groups was performed from independent cultures and the data were evaluated by a one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test. Differences were considered significant at < 95% confidence.

RESULTS

Since either Glu or NMDA increase the AMP hydrolysis, we investigated whether the Glu analogs and selective ligands for NMDA, L- and D-Asp, have the same effect on ecto-5'-nucleotidase activity. Cells were pre-incubated with either 1 mM L- or D-Asp for 30 min and the hydrolysis of AMP was measured. Fig. 1 shows the stimulatory effect of both L- and D-Asp on ecto-5'-nucleotidase activity from 28.5 ± 2 to 40.1 ± 6 nmol Pi/mg protein/min (40.9 ± 15 %) (Fig. 1A); or from 22.1 ± 1 to 29 ± 1 nmol Pi/mg protein/min (41.9 ± 22 %) (Fig. 1B), respectively. To determine whether the increase of AMP hydrolysis evoked by L- and D-Asp was mediated by NMDA receptors activation, MK-801 or AP-5 (NMDA receptor antagonists) were present during the pre-incubation with L- or D-Asp. Both antagonists prevented the stimulatory effect induced by L-Asp on AMP hydrolysis, but did not change the stimulatory effect evoked by D-Asp. Neither MK-801 nor AP-5 alone had any effect

on AMP hydrolysis. Moreover, we investigated the participation of EAAs transporter activity on the ecto-5'-nucleotidase activity stimulation, using DHK (EAAs transporter blocker) during the pre-incubation with L- or D-Asp. DHK inhibited the increase of AMP hydrolysis evoked by either L- or D-Asp, but had no effect on ecto-5'-nucleotidase activity when pre-incubated alone.

In order to investigate the involvement of NMDA receptors on the neurotoxicity induced by EAAs, cells were incubated with L- or D-Asp in the presence or absence of MK-801 or AP-5 during the neurotoxicity assay period. Following 24 hours the cellular damage was assayed by PI uptake (Fig. 2). L- and D-Asp induced cells in a way similar to that observed with Glu (data not shown). Both NMDA receptors antagonists prevented completely the neuronal death induced by L-Asp, but partially the D-Asp-induced cellular death.

Considering that the increase of the ecto-5'-nucleotidase activity induced by neurotoxic dose of L-and D-Asp leads to enhance in the extracellular adenosine levels, cells were exposed to adenosine A_1 or A_{2A} receptor antagonists to evaluate their effect on EAAs neurotoxicity. Cells were incubated with L- or D-Asp in the presence or absence of CPT or ZM 241385 for 30 min. Following 24 hours the cellular damage was assayed by PI uptake (Fig. 3). However, neither CPT nor ZM 241385 affected the excitotoxicity induced by EAAs.

DISCUSSION

The present report demonstrates that L- and D-Asp were able to stimulate, in a different manner, the ecto-5´-nucleotidase activity and leaded cerebellar granule

cells to damage. The stimulation evoked by L-Asp was mediated by NMDA receptors activation, since either non-competitive (MK-801) or competitive NMDA receptor antagonist (AP-5) were able to prevent the L-Asp effect upon ecto-5'-nucleotidase. Interestingly, the stimulation evoked by D-Asp was not prevented by either MK-801 or AP-5, what clearly keeps out the activation of NMDA receptors in the effect of D-Asp. Nevertheless, the stimulatory effect of the ecto-5'-nucleotidase by either L- or D-Asp were prevented by the EAAs uptake blocker, DHK, indicating that the uptake mechanism is fundamental to EAAs evoke the increase of the AMP hydrolysis. It corroborates with the observation that DHK inhibits the increase of the extracellular adenosine level evoked by Glu in synaptosomes [5]. Thus, a hypothesis arises from these results, is that probably the observed stimulation of the ecto-5'-nucleotidase was mediated by the electrogenic gradient through EAA transporters activation [14], whose co-transport of Na^+, K^+ and H^+ driven the EAAs uptake. In the present study we also observed the protective effect evoked by NMDA antagonists against cellular damage induced by L-Asp, but with partial effect upon D-Asp-induced neurotoxicity. Although, L- and D-Asp has been considered agonists to NMDA receptor, the results described herein indicated a different participation of NMDA receptors on the L-Asp or D-Asp actions in cerebellar granule cells. Others studies have observed atypical responses evoked by both EAAs on NMDA receptors [7]. Thus, our results corroborate with the studies that indicate L-Asp as agonist to NMDA receptors, but the NMDA receptor activation by D-Asp depends on the parameter analyzed. Also, high levels of D-Asp occur in tissues that do not posses functional NMDA receptors, such as testis and adrenal [13].

Since the activation of the AMP hydrolysis induced by high dose of EAAs enhances the extracellular adenosine levels and this adenosine could be acting on A_1 or A_{2A} adenosine receptors, we investigated the effect of the antagonist for both receptors on EAAs-induced neurotoxicity. Previous study has proposed that adenosine released leads to preferential adenosine A_1 receptors activation, whilst formed from ecto-nucleotidases pathway leads to favored adenosine A_{2A} receptors activation [4]. We could expect that the CPT or mainly ZM 241385 might affect the neurotoxicity induced by high concentrations of L- or D-Asp, enhancing or decreasing the neuronal death. However, in the present study we demonstrated that neither the adenosine A_1 (CPT) nor A_{2A} (ZM 241385) receptors antagonists affected the L- or D-Asp-induced cellular death. In neurotoxic situations that there are EAAs release, such as ischemia, CPT increases and ZM 241385 inhibits the cellular damage likely acting upon EAAs release mechanism, differently from our experimental design in which the neurons were directly incubated with each EAA.

In conclusion, the present study indicated a different involvement of NMDA receptors on the L-Asp or D-Asp actions in cerebellar granule cells. The neurotoxicity displayed by L- and D-Asp was due NMDA receptors activation, but only the stimulation of the ecto-5'-nucleotidase activity evoked by L-Asp occurred via NMDA receptors. However, further experiments need to clarify the role of adenosine formed from AMP hydrolysis stimulated by neurotoxic concentration of EAAs.

ACKNOWLEDGEMENTS

This research was supported by grants from FAPERGS, FINEP/CNPq and PRONEX. Carina R. Boeck was the recipient of a CAPES fellowship.

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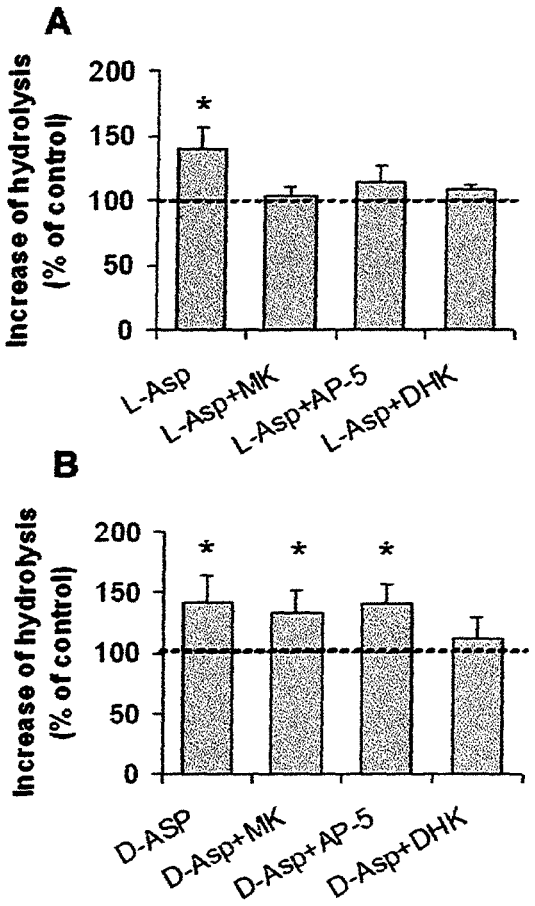

Fig.1. Stimulation of the ecto-5'-nucleotidase activity induced by EAAs in the presence or absence of antagonists of NMDA receptors (MK-801 or AP-5) or of the blocker of EAAs uptake (DHK) in cerebellar granule cells. A) L-Asp and B) D-Asp. Values are mean ± SD of independent cultures (n = 3-4).* $p < 0.001$ vs control, L-Asp+MK, L-Asp+Ap-5 and L-Asp+DHK groups; * $p < 0.001$ vs control and D-Asp+DHK groups.

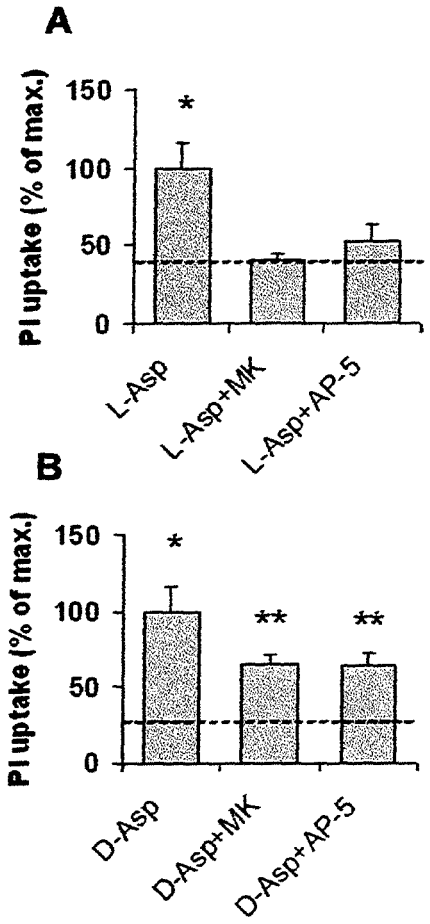

Fig. 2. Effect of NMDA receptors antagonist on neurotoxicity evoked by EAAs. Cells were treated with or without L- or D-Asp in the presence or absence of MK-801 or AP-5. Images were captured and then analyzed using Scion Image software. The area where Pi fluorescence was detectable above background was determined by densitometric analysis. Values of the quantification of L- or D-Asp-induced cellular damage are expressed as mean ± S.E.M. of independent cultures (n = 3). *Dashed line*, control group. * $p < 0.05$ vs control (40.1 ± 5%), L-Asp+MK and L-Asp+AP-5 groups; * $p < 0.05$ vs control (28.7 ± 7%), D-Asp+MK and D-Asp+AP-5 groups; ** $p < 0.05$ vs control and D-Asp groups.

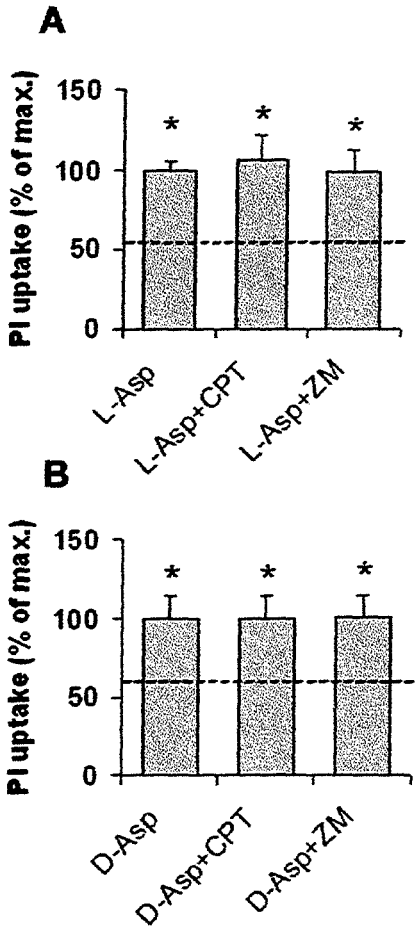

Fig. 3. Effect of selective adenosine A_1 and A_{2A} receptors antagonist on neurotoxicity evoked by EAAs. Cells were treated with or without L- or D-Asp in the presence or absence of CPT or ZM 241385. Cells were treated with or without L- or D-Asp in the presence or absence of MK-801 or AP-5. Images were captured and then analyzed using Scion Image software. The area where Pi fluorescence was detectable above background was determined by densitometric analysis. Values of the quantification of L- or D-Asp-induced cellular damage are expressed as mean ± S.E.M. of independent cultures (n = 3). *Dashed line*, control group. * $p < 0.05$ vs control (54.8 ± 7% vs L-Asp and 60.3 ± 5% vs D-Asp).

II.3. CAPÍTULO 3 - Carina R. Boeck, Eduardo H. Kroth, Mauro J. Bronzatto and *Deusa Vendite. **Adenosine receptors co-operate with NMDA preconditioning to protect cerebellar granule cells against glutamate neurotoxicity.** Submetido ao periódico **Neuroscience**.

Adenosine receptors co-operate with NMDA preconditioning to protect cerebellar granule cells against glutamate neurotoxicity

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Contract grant sponsor: FAPERGS, FINEP/CNPq and PRONEX.

Section Editor: **Neuropharmacology.**

ABSTRACT

N-methyl-D-aspartate (NMDA) preconditioning is evoked by subtoxic concentrations of NMDA (50 μM), which has been shown previously to lead transient resistance to subsequent lethal dose of glutamate or NMDA in cultured neurons. The purpose of this study was to investigate the participation of adenosine A_1 and A_{2A} receptors on NMDA preconditioning against glutamate-induced cellular damage in cerebellar granule cells. NMDA preconditioning prevented the stimulatory effect of glutamate on AMP hydrolysis, but not on ADP hydrolysis. The neuroprotection evoked by NMDA preconditioning against glutamate-induced cellular damage was prevented by the presence of adenosine A_1 receptor antagonist, 8-cyclopentyl-1,3-dimethylxanthine (CPT, 100 nM), but not by the adenosine A_{2A} receptors antagonist, (4-(2[7-amino-2-(2-furyl {1,2,4}-triazolo{2,3-a{1,3,5}triazian-5-yl-aminoethyl)phenol (ZM 241385, 50 nM). Interestingly, a long-term treatment with CPT or ZM 241385 alone protected cells against glutamate-neurotoxicity. Moreover, the functionality of adenosine A_1 receptor was not affected by NMDA preconditioning, but this treatment promoted adenosine A_{2A} receptor desensitization, measured by cAMP accumulation. Taken together, the results described herein suggest that the neuroprotection evoked by NMDA preconditioning against cellular damage elicited by glutamate occurred through mechanisms involving adenosine A_{2A} receptors desensitization co-operating with adenosine A_1 receptors activation in cerebellar granule cells.

Key words: NMDA preconditioning; neuroprotection; adenosine; cerebellar granule cells.

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system and mediates physiological pathways as well as contributes to neuronal damage (Olney and De Gubareff, 1978; Gasic and Hollmann, 1992). Excitotoxicity induced by glutamate involves at least in part intracellular increase of Ca^{2+} level through excessive stimulation of the N-methyl-D-aspartate (NMDA) receptors (Choi, 1988). Despite of neurotoxic actions of NMDA, neuroprotective effects evoked by NMDA receptors activation have been studied. Chemical preconditioning (e.g. NMDA preconditioning) is evoked by subtoxic concentrations of a substance, which leads transient resistance to its subsequent lethal dose (Rejdak et al., 2001). NMDA preconditioning prevents cellular death induced by glutamate or agonist to NMDA receptors in cultured neurons (Chuang et al., 1992; Dickie et al., 1996; Sei et al., 1998; Jonas et al., 2001), whose effect is prevented by the RNA synthesis inhibitor actinomycin D and the protein synthesis inhibitor cyclohexamide (Marini et al., 1992; Damschroder-Williams et al., 1995). The neuroprotection by NMDA in cerebellar granule cells requires the activated nuclear factor kappa B (NF-kB) and released brain-derived neurotrophic factor (BDNF) (Marini et al., 1998; Lipsky RH et al., 2001). In the retinal cells, besides BDNF, NT-4 is also released during NMDA pretreatment (Rocha et al., 1999). Additionally, systemically administered NMDA protects hippocampal neurons against damage induced by kainate through blockade of DNA fragmentation *in vivo* (Ogita et al., 2003) and against seizures and cellular damage quinolinic acid-induced (CR Boeck and D Vendite, in preparation). NMDA preconditioning has neuroprotective effects against oxygen-glucose deprivation-induced neuronal death (Grabb and Choi, 1999) with induction of HSP27 phosphorylation and expression (Valentim et al., 2003),

but the latest effect was not observed on HSP72 expression (Pringle et al., 1999). However, the mechanisms underlying the development of brain tolerance are not completely elucidated. It is well established that NMDA receptors activation promotes the increase of extracellular adenosine levels (Hoehn and White, 1990; Craig and White, 1993), an important endogenous neuromodulator. Adenosine can act through A_1 or A_3 receptors inhibiting adenylyl cyclase via $G_{i/o}$ proteins, decreasing cyclic AMP (cAMP) levels, or through A_{2A} and A_{2B} receptors with opposite effects via G_s proteins (Daly, 1985; Palmer and Stiles, 1995). The activation of adenosine A_1 receptors depress basal and evoked neuronal firing (Dunwiddie and Diao, 1994), decrease calcium uptake (Wu et al., 1982; Vacas et al., 2003) and inhibit release of excitatory neurotransmitters such as glutamate (Corradetti et al., 1984; Poli et al., 1991). The origins of extracellular adenosine can be via release by bi-directional transporter or formed from adenine nucleotides released, which are then degraded by an extracellular chain of ecto-nucleotidases. This enzymatic chain potentially includes ecto-ATP diphosphohydrolase, which hydrolyses ATP and ADP, resulting in the formation of AMP, which products adenosine by ecto-5'-nucleotidase activity. Ecto-5'-nucleotidase activity is a pivotal step of the extracellular adenosine production from enzymatic chain (James and Richardson, 1993). High doses of glutamate stimulate the AMP and ADP hydrolysis, probably to contribute the rise of extracellular adenosine to counteract the neurotoxicity induced by glutamate in cultured neurons (Boeck et al., 1999; Boeck et al., 2000).

Since NMDA preconditioning protects neurons from glutamate excitotoxicity *in vitro*, in the present study, we have investigated the potential participation of

adenosine at this mechanism of neuroprotection. We therefore investigated if ecto-ATP diphosphohydrolase and ecto-5'-nucleotidase activities stimulated by glutamate modifies when cells are preconditioned with NMDA. We also tested if ligands to adenosine A_1 or A_{2A} receptors have influence on neuronal survival and the functionality of these receptors through cAMP accumulation in cerebellar granule cells after NMDA preconditioning.

EXPERIMENTAL PROCEDURES

Materials

N-methyl-D-aspartate (NMDA), glutamic acid and adenosine deaminase were obtained from Sigma (Sto Louis, MO, USA). 8-phenyltheophylline (8-PT), N-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dimethylxanthine (CPT), 2-[4-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) and 4-[(3-[butoxy-4-methoxyphenyl]methyl]-2-imidazolidinone (Ro 20-1724) were obtained from RBI-Research Biochemical International (Natick, MA, USA). (4-(2[7-amino-2-(2-furyl{1,2,4}-triazolo{2,3-a{1,3,5}triazian-5-yl-aminoethyl)phenol (ZM 241385) was from Tocris Cookson (Bristol, UK). [^{3}H]-cyclic AMP (specific activity, 25 Ci/mmol) was from Amersham Pharmacia Biotech. Propidium Iodite (PI) was purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA).

Cerebellar Granule Cell Culture

Primary cultures of cerebellar granule cells were prepared from 8-day-old Wistar rats as described previously (Boeck et al., 2000). Briefly, freshly dissected cerebella were dissociated by mild trypsinization (0.025% trypsin (w/v), 37 °C, 15 min) and disrupted mechanically with a fire-polished Pasteur pipette in the presence of DNase (0.08 mg/ml) and trypsin inhibitor (0.05% (w/v). Cells were seeded at a density of 3.2 x 10^5/cm^2 in a 24-well or 96-well multiwell dish (Nunc) coated with 10 µg/ml poly-D-lysine in Eagle's basal medium (BME) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil), 25 mM KCl and 50 µg/ml gentamicin. The growth of non-neuronal cells was inhibited by an addition of 20 µM cytosine arabinofuranoside 18-20 h after seeding and the medium was maintained without any change during the culture period. Due to medium evaporation, glucose (0.1% in water (w/v)) was added to a final volume of 100 µL at 7 days in vitro (DIV) (Yan et al., 1994). These cultures contain > 90% granule cells, 4-6% GABAergic neurons, and a small number of glial (2-3%) and endothelial cells (< 1%) (Kingsbury et al., 1985).

NMDA Preconditioning and Neurotoxicity

According to Chuang et al. (1992), a long-term treatment with subtoxic concentrations of NMDA is a preconditioning stimulus, which leads to neuroprotection against subsequent cellular damage induced by glutamate. To investigate the participation of adenosine receptors on neuroprotection evoked by NMDA preconditioning, at 8 DIV, cells were pretreated with or without NMDA (50 µM) in the presence or absence of 8-PT (20 nM, non-selective adenosine A_1/A_{2A} receptor antagonist), CPT (100 nM, adenosine A_1 receptors antagonist)

or ZM 241385 (50 nM, adenosine A_{2A} receptors antagonist) for 24 hours. In another set of experiments, NMDA was replaced by CPA (100 nM, adenosine A_1 receptors) in the presence or absence of CPT or ZM 241385. After the preconditioning period (24 hours), the nutrient medium was carefully removed from dishes, and the cell monolayer was gently washed twice with pre-warmed HEPES-buffered saline (in mM: 120 NaCl, 5 KCl, 2 $CaCl_2$ and 10 glucose), pH 7.4. Cells were incubated in the presence or absence of glutamate (1 mM) for 30 min. When mentioned, at 9 DIV, cells without any pretreatment were incubated with glutamate in the presence or absence of CPT or ZM 241385 for 30 min. After the neurotoxicity assay period, cells were carefully washed twice with pre-warmed HEPES-buffered saline and previously the saved sister culture medium (culture-conditioned medium) was replaced. Cell viability was monitored before and after each experiment by the inverted phase-contrast microscopy.

Ecto-nucleotidase activities

At 9 DIV, the nutrient medium was carefully removed from dishes, and the cell monolayer was gently washed twice with pre-warmed HEPES-buffered saline. Cells with or without the NMDA preconditioning treatment were pre-incubated in the presence or absence of glutamate (1 mM) for 30 min. Ecto-nucleotidase activities was started by the addition of ADP or AMP (1 mM, final concentration) and incubated for 20 or 10 min at 37 °C, respectively. The nucleotides hydrolysis was stopped by the removal of an aliquot of assay medium that was mixed with trichloroacetic acid (TCA) to a final concentration of 5%. After a centrifugation of 5 min at 12000 x g at 4 °C, a sample of the supernatant was

taken for the assay of inorganic phosphate (Pi) release by colorimetrical determination (Chan et al., 1986). Non-enzymatic Pi released from nucleotide in the assay medium without cells and Pi released from cells incubated without nucleotide were subtracted from the total Pi released during nucleotide hydrolysis, giving values for enzymatic activity (nmol Pi/mg protein/min). We have previously described that at the end of the experiment, the survival and morphology of cells is maintained (Boeck et al., 2000). Protein was determined using bovine serum albumin as standard (Bradford, 1976).

Cellular viability

Cells death was assessed 24 h post-neurotoxicity assay period with high doses of glutamate, using uptake of the fluorescent exclusion dye propidium iodide (PI), which is a polar compound that only enters into the dead or dying cells with damage membranes. Once inside the cells, PI complexes with DNA inducing intense red fluorescence (630 nm), when excited by green light (495 nm). Cells were incubated with 5 µg/mL PI for 30 min and then imaged on a standard inverted microscope (Nikon Eclipse TE 300) using a rhodamine filter set. The PI uptake was quantified by a densitometric analysis using Scion Image software (Scion Corporation) (Porciúncula et al., 2001).

Accumulation of cAMP

Cyclic AMP accumulation was investigated using the method previously described (Sanz et al., 1996) with minor modifications. Briefly, the nutrient

medium from cells with or without NMDA preconditioning treatment was gently removed, and cells were washed twice with pre-warmed HEPES-buffered saline. Cells were pre-incubated for 10 min at 37 °C with Ro 20-174 (25 μM, phosphodiesterase inhibitor) and 2 U/mL adenosine deaminase (ADA), to remove the endogenous adenosine. In experiments investigating the functionality of adenosine A_1 receptors, cells were incubated with or without CPA (100 nM) for 10 min followed by forskolin (10 μM, adenylyl cyclase activator) for 15 min; and to study the functionality of adenosine A_{2A} receptors, cells were incubated with or without ZM 241385 (50 nM for 10 min) followed by CGS 21680 (100 nM, adenosine A_{2A} receptors agonist) for 10 min. The reaction was stopped by the aspiration of the assay medium and the addition of ice-cold 5% TCA. Samples were kept 30 min at 4 °C and then were neutralized with 2 M KOH. cAMP was quantified by a radioimmunoassay using protein kinase A (PKA) as the binding protein (Brown et al., 1971).

Statistical Analysis

Statistical analysis among groups was performed from independent cultures and the data were evaluated by a one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test. Differences were considered significant at < 95% confidence.

RESULTS

Effect of NMDA preconditioning on Ecto-nucleotidase activities

We have demonstrated that a neurotoxic concentration of glutamate stimulates the hydrolysis of ADP and AMP in cultured cerebellar granule cells after 8 DIV. Thus, the ecto-nucleotidase activities modulated by glutamate were examined following pretreatment with a subtoxic concentration of NMDA during 24 hours. NMDA preconditioning did not affect the basal nucleotides hydrolysis in cerebellar granule cells (8.8 ± 1.4 nmol Pi/min/mg protein for ADP and 23.2 ± 7.9 nmol Pi/min/mg protein for AMP) (Fig. 1A and 1B). However, NMDA preconditioning blocked the glutamate-induced stimulation of ecto-5'-nucleotidase activity, but not the ecto-ATP diphosphohydrolase activity ($n = 3$-4, $p < 0.05$). Thus, the extracellular production of adenosine through glutamate-induced stimulation of AMP hydrolysis by ecto-5'-nucleotidase was prevented by NMDA preconditioning.

Effect of ligands to adenosine A_1 or A_{2A} receptors on neuroprotection evoked by NMDA preconditioning against glutamate-induced neurotoxicity

Studies have demonstrated that the pretreatment with subtoxic concentrations of NMDA protects cerebellar granule cells against subsequent Glu-induced neurotoxicity. We confirm that the protection evoked by NMDA preconditioning against Glu-induced cellular death estimating the survival through conversion of MTT to a blue formazan product by viably cells (data not shown) and exclusion test with PI fluorescence (Fig. 2). To investigate the participation of adenosine receptors on NMDA preconditioning, the non-selective adenosine A_1/A_{2A}

receptor antagonist, 8-PT (Fredlhom et al., 1994), was tested to modify the protection evoked by NMDA preconditioning against glutamate-induced neurotoxicity. A long-term pretreatment with a dose of 8-PT was enough to lead to cellular damage and had no effect on glutamate-induced cellular death (Fig. 2A and 2B).

To directly demonstrate the participation of each adenosine receptors, selective adenosine receptor antagonists were used. The presence of the adenosine A_1 receptors antagonist, CPT, during NMDA preconditioning abolished the neuroprotective effect evoked by NMDA against glutamate-induced cellular damage (Fig. 3A and 3B). But, the adenosine A_{2A} receptors antagonist, ZM 241385, was devoid of the effect, since the protective effect of NMDA preconditioning against glutamate-induced neurotoxicity was maintained. Differently from 8-PT effect, a long-term treatment with either CPT or ZM 241385 alone protected cells against glutamate-induced cellular death independently from NMDA preconditioning.

Effect of long-term activation of adenosine A_1 receptors against glutamate-induced neurotoxicity

With the possibility that adenosine receptor antagonists, CPT and ZM 241385, presented the protective effects against glutamate-induced neuronal damage due an action on adenosine A_1 receptors, cells were pretreated with CPA, an agonist to adenosine A_1 receptors, in the presence or absence of CPT or ZM 241385 for 24 hours before glutamate neurotoxicity assay. CPA alone did not show any effect on cellular viability neither against glutamate-induced

cellular damage (Fig. 4A and 4B). The long-term treatment with CPA prevented the protective effect of CPT against glutamate-induced cellular death. But, the protective effect of ZM 241385 against glutamate-induced cellular death was maintained.

In order to determine whether CPT and ZM 241385 had any effect when applied acutely, the ligands to adenosine receptors were added just during the glutamate neurotoxicity assay (see Materials and Methods). Neither CPT nor ZM 241385 was able to protect glutamate-induced cellular damage in this experimental design (Fig. 5A and 5B), indicating that a long-term treatment with these ligands is necessary for their protective effect.

Effect of NMDA preconditioning on cAMP accumulation

We investigated the functionality of the adenosine A_1 or A_{2A} receptors on NMDA preconditioning through inhibition or activation of adenylyl cyclase, respectively. NMDA preconditioning did not change the inhibitory effect of CPA on forskolin-stimulated cAMP accumulation when compared to control cells (Fig. 6A). However, NMDA preconditioning prevented the increase in cAMP levels evoked by CGS 21680 (Fig. 6B). The basal cAMP accumulation did not change with NMDA preconditioning.

DISCUSSION

In according with several studies, subtoxic dose of NMDA provided a chemical preconditioning that leads to cellular tolerance to subsequent toxic doses of

glutamate. The present study indicates an important evidence to the participation of the adenosine A_1 receptors activation during the NMDA preconditioning. It is well established that NMDA increases the extracellular levels of adenosine via bi-directional adenosine transporter or formed from adenine nucleotides released, which are degraded by a chain of ecto-nucleotidases (Craig and White, 1993; Cunha et al., 1996). Adenosine released leads to preferential adenosine A_1 receptors activation, whilst formed from ecto-nucleotidases pathway leads to favored adenosine A_{2A} receptors activation (Cunha et al., 1996). Cerebellar granule cells in the presence of neurotoxic concentration of glutamate show a stimulation of the ecto-nucleotidase activities (Boeck et al., 2000). However, the present results demonstrate that NMDA preconditioning prevents the ecto-5'-nucleotidase activation evoked by glutamate, but has no effect on the ecto-ATP diphosphohydrolase activation. The observed lack of stimulatory effect of glutamate on AMP hydrolysis after the NMDA preconditioning treatment suggests a decrease on the adenosine production to act on adenosine A_{2A} receptors during a neurotoxic insult. Regarding the neuroprotection evoked by NMDA preconditioning, the presence of the non-selective adenosine A_1 and A_{2A} receptors antagonist, 8-PT, during this period has no effect on the protection against glutamate-induced neurotoxicity. Although the acute administration of another non-selective adenosine A_1 and A_{2A} receptors antagonist (8-pSPT) prevents ischemic preconditioning (Reshef et al., 2000), the 8-PT neurotoxicity has been demonstrated under chronic treatment with 5 μM 8-PT in cultured neurons (Brooke and Sapolsky, 2000); indicating it inadequate for a long-term treatment in the present experiment design. Thereby, the NMDA preconditioning was

assayed in the presence of selective adenosine receptor antagonists. The adenosine A_1 receptors antagonist, CPT, prevented the protection evoked by NMDA preconditioning against glutamate-induced cellular damage, whereas the adenosine A_{2A} receptors antagonist, ZM 241385, was ineffective in preventing neuroprotection. These findings demonstrate that the neuroprotection occurred at least in part through activation of adenosine A_1 receptors in cerebellar granule cells during the NMDA preconditioning period.

As strategy to investigate the adenosine receptors functionality on NMDA preconditioning, the cAMP accumulation was investigated. The NMDA preconditioning had no effect on the inhibition induced by CPA on forskolin-cAMP accumulation, whereas it was effective in preventing CGS 21680-evoked cAMP accumulation through adenosine A_{2A} receptors activation. These interestingly observations in the present results could be due a desensitization of adenosine A_{2A} receptors evoked by NMDA preconditioning. This differential behavior of adenosine receptors toward NMDA preconditioning corroborate with previous studies demonstrating a cross talk between adenosine A_1 and A_{2A} receptors (Lopes et al., 1999). Adenosine A_{2A} receptors activation decrease the functionality of adenosine A_1 receptors (Dixon et al., 1997; Dunwiddie et al., 1997; O'Kane and Stone, 1998; Lopes et al., 2002) and the inhibitory activity of adenosine A_1 receptors is suppressed by an increase of the function of adenosine A_{2A} receptors induced by NMDA (Nikbakht et al., 2001). Thus, the NMDA preconditioning pathway favored adenosine A_1 receptors activation when adenosine A_{2A} receptors are inactivated. It was also supported by the preventive effect of NMDA preconditioning on adenosine production from AMP hydrolysis and on the functionality of adenosine A_{2A} receptors measured by cAMP

accumulation. Thus, the present study clearly includes a lower functionality of adenosine A_{2A} receptors co-operating with adenosine A_1 receptors activation on NMDA preconditioning-mediated neuroprotection.

Surprisingly, independent of NMDA preconditioning, a long-term treatment with either CPT or ZM 241385 alone was efficient to protect cells against glutamate-induced damage. Regarding adenosine A_1 receptors, these findings suggest an up-regulation of adenosine A_1 receptors due the long-term treatment with CPT (Jacobson et al., 1996), since the long-treatment with adenosine A_1 receptors agonist, CPA, prevented the neuroprotection evoked by CPT. In opposite, CPA did not affect the neuroprotective effect displayed by ZM 241385. Previous study has demonstrated that chronic administration of adenosine A_{2A} receptor antagonists improves neuronal survival after ischemic insult (von Lubitz et al., 1995). Other studies have indicated that a chronic treatment with adenosine A_{2A} receptor antagonists does not result in changes of either receptor density or their dissociation constants (Lupica et al., 1991; Abbracchio et al., 1992). Moreover, chronic activation of adenosine A_{2A} receptor agonists protects retinal neurons against glutamate-induced neurotoxicity (Ferreira and Paes-de-Carvalho, 2001). The neuroprotection evoked by a long-term treatment with ZM 241385 against glutamate-induced cellular damage needs additional experiments to clarify its effect. Additionally, when CPT or ZM 241385 was added just during glutamate neurotoxicity assay in cells without any pretreatment, the cellular damage was not prevented, indicating that a long-term treatment with these ligands is necessary for their protective effect.

In conclusion, NMDA preconditioning protected cerebellar granule cells against damage elicited by glutamate through mechanisms involving adenosine

A_{2A} receptors desensitization co-operating with adenosine A_1 receptors activation. The underlying mechanisms involved in this neuroprotective effects need to be further investigated in order to delineate a better understanding upon the interaction between adenosine A_1 and A_{2A} receptors on neuromodulatory process in cerebellar granule cells.

Acknowledgment - This research was supported by grants from FAPERGS, FINEP/CNPq and PRONEX. Carina R. Boeck was the recipient of a CAPES fellowship.

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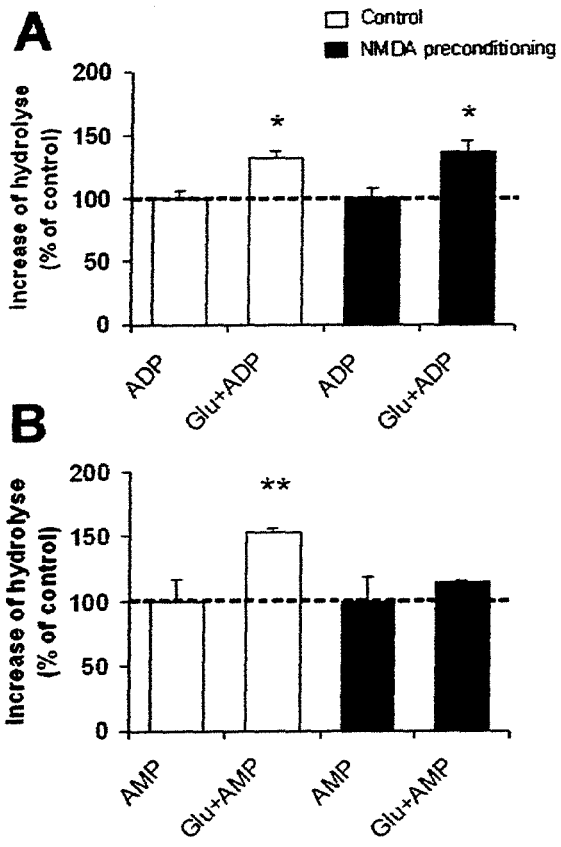

Fig. 1. Effect of NMDA preconditioning on ecto-nucleotidase activities stimulated by Glu. After NMDA preconditioning (see Materials and Methods), A) ADP hydrolysis and B) AMP hydrolysis were measured following pre-incubation with 1 mM Glu for 30 min. Values are mean ± SD of independent cultures (n = 3-4).* $p < 0.05$ compared to control groups; ** $p < 0.05$ compared to other groups.

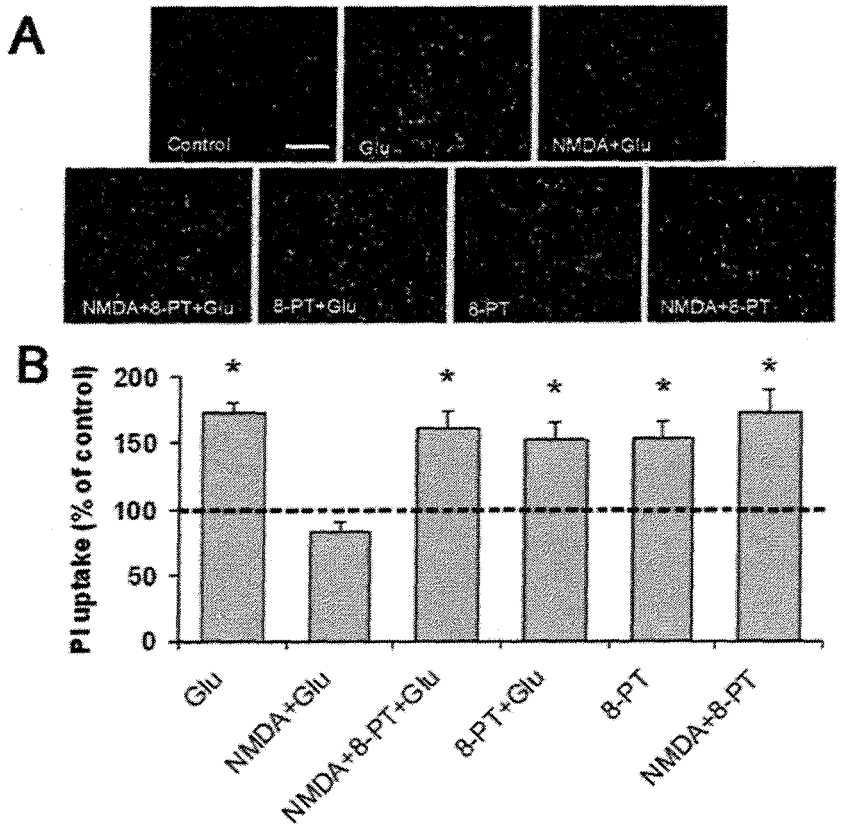

Fig. 2. Effect of non-selective adenosine A_1 and A_{2A} receptors antagonist on neuroprotection evoked by NMDA preconditioning against Glu-induced cellular damage. Cells were pretreated with or without NMDA in the presence or absence of 20 nM 8-PT. A) Photomicrographs show PI uptake following Glu neurotoxicity (see Materials and Methods). The fluorescence area corresponds to the cellular death at the different groups of treatment. A typical experiment is shown. 8-PT had no effect on the neuroprotection evoked by NMDA preconditioning. Scale bar, 100 µm. B) Quantification of Glu-induced cellular damage after NMDA preconditioning period. Images were captured and then analyzed using Scion Image software. The area where Pi fluorescence was detectable above background was determined by densitometric analysis. Values are expressed as mean ± S.E.M. of independent cultures (n = 5). * $p < 0.001$ compared to control group and NMDA + Glu.

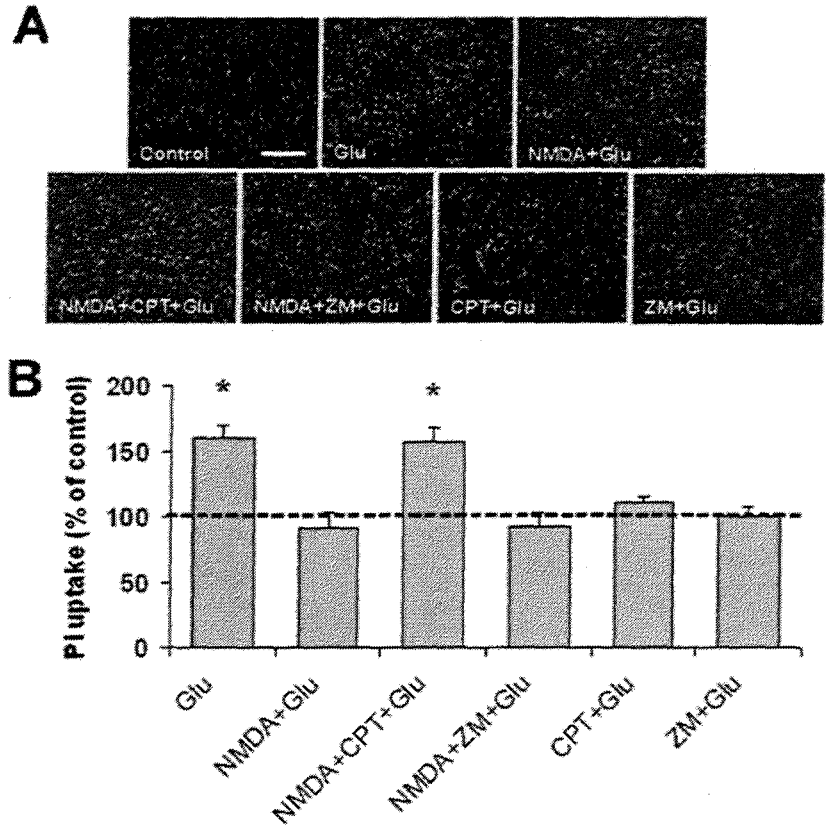

Fig. 3. Effect of selective adenosine A_1 (CPT) or A_{2A} (ZM 241385) receptor antagonists on neuroprotection evoked by NMDA preconditioning against Glu-induced cellular damage. Cells were pretreated with or without NMDA in the presence or absence of 100 nM CPT or 50 nM ZM 241385. A) Photomicrographs show PI uptake following Glu neurotoxicity (see Materials and Methods). The fluorescence area corresponds to the cellular death at the different groups of treatment. A typical experiment is shown. CPT prevented the neuroprotection evoked by NMDA preconditioning. Scale bar, 100 μm. B) Quantification of Glu-induced cellular damage after NMDA preconditioning period. Images were captured and then analyzed using Scion Image software. The area where Pi fluorescence was detectable above background was determined by densitometric analysis. Values are expressed as mean ± S.E.M. of independent cultures (n = 4-5). * $p < 0.01$ compared to control other groups.

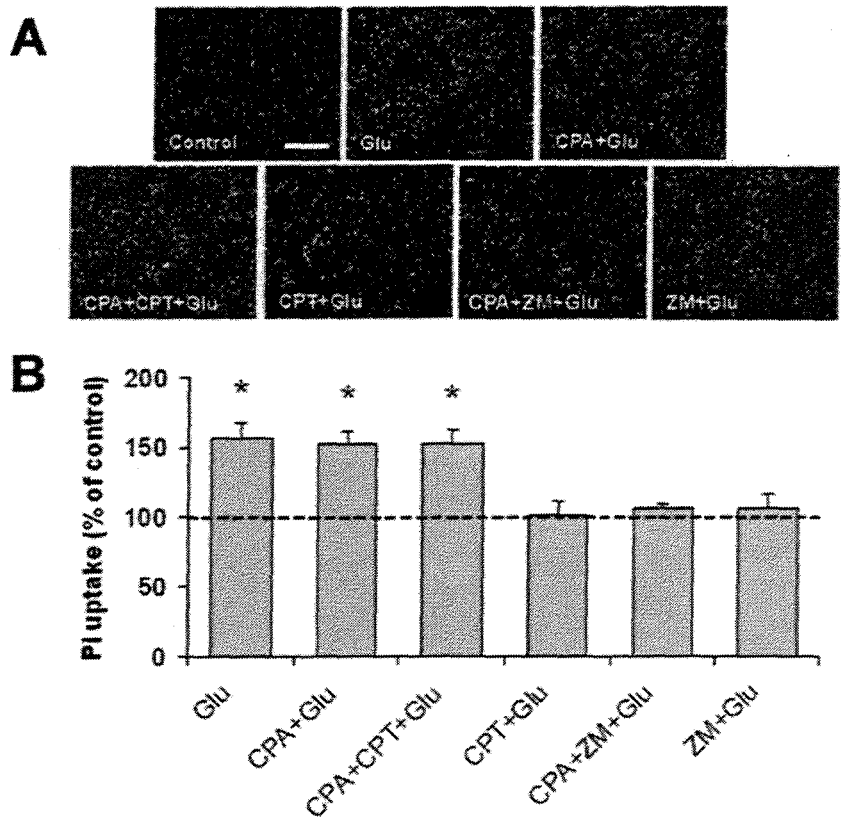

Fig. 4. Effect of a long-term treatment with selective ligands to adenosine receptors on neuroprotection evoked by NMDA preconditioning against Glu-induced cellular damage. Cells were pretreated with or without adenosine A_1 receptors agonist, 100 nM CPA, in the presence or absence of 100 nM CPT or 50 nM ZM 241385. A) Photomicrographs show PI uptake following Glu neurotoxicity (see Materials and Methods). The fluorescence area corresponds to the cellular death at the different groups of treatment. A typical experiment is shown. CPA had no effect on Glu-induced cellular damage. Scale bar, 100 μm. B) Quantification of Glu-induced cellular damage after a long-term treatment with ligands to adenosine receptors. Images were captured and then analyzed using Scion Image software. The area where Pi fluorescence was detectable above background was determined by a densitometric analysis. Values are expressed as mean ± S.E.M. of independent cultures (n = 5). * $p < 0.01$ compared to other groups.

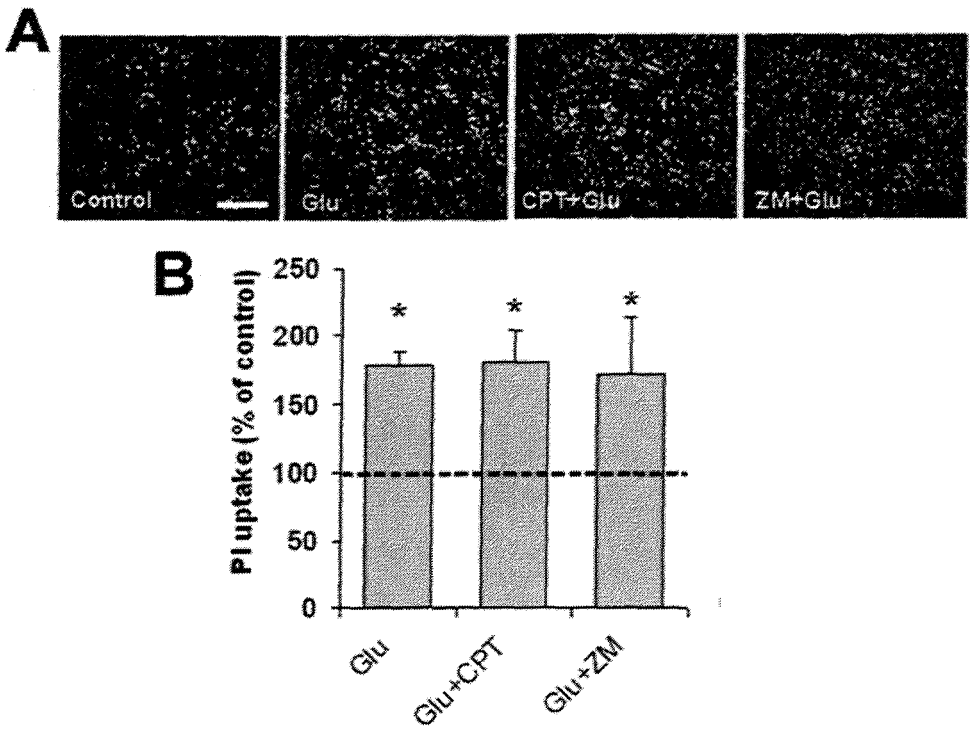

Fig.5. Effect of an acute treatment with a selective adenosine A_1 or A_{2A} receptor antagonist on neurotoxicity induced by Glu. Cells were incubated with Glu in the presence or absence of 100 nM CPT or 50 nM ZM 241385. A) Photomicrographs show PI uptake following Glu neurotoxicity (see Materials and Methods). The fluorescence area corresponds to the cellular death at the different groups of treatment. A typical experiment is shown. Either CPT or ZM 241385 had no effect on Glu-induced cellular damage. Scale bar, 100 µm. B) Quantification of Glu-induced cellular damage in the presence or absence of CPT or ZM 241385. Images were captured and then analyzed using Scion Image software. The area where Pi fluorescence was detectable above background was determined by a analysis. Values are expressed as mean ± S.E.M. of independent cultures (n = 3-4). * $p < 0.01$ compared to control groups.

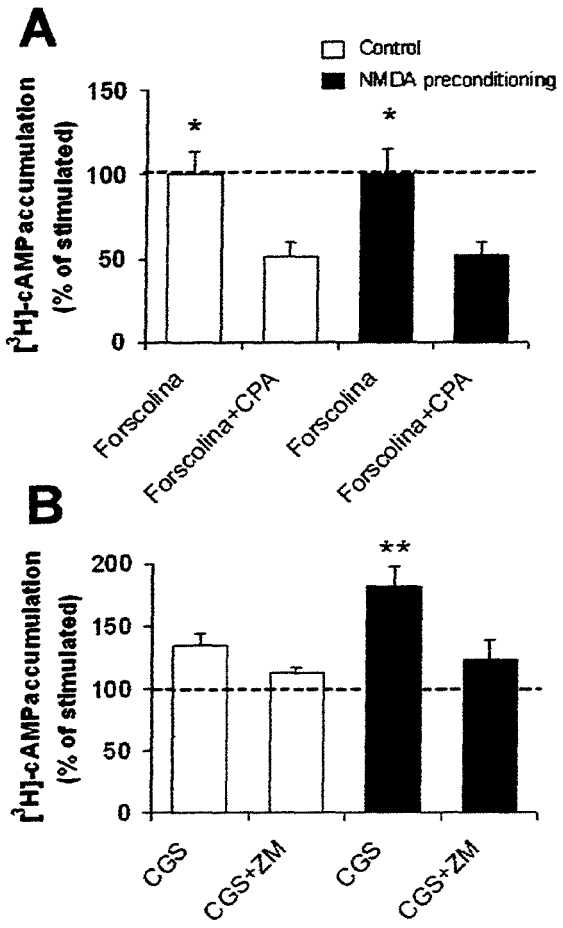

Fig. 6. Effect of NMDA preconditioning on cAMP accumulation. A) Cells were incubated with or without 10 μM forskolin in the presence or absence of adenosine A_1 receptors agonist, 100 nM CPA, following the NMDA preconditioning period. cAMP accumulation was measured after inhibition of CPA on forskolin stimulation (n = 5-6). CPA alone had no effect on cAMP accumulation. B) Cells were incubated with or without adenosine A_{2A} receptors agonist, 100 nM CGS 21680, in the presence or absence of ZM 241385. cAMP accumulation was measured after CGS 21680 stimulation (n = 3). ZM 241385 alone had no effect on cAMP accumulation. Values are expressed as mean ± S.E.M. of independent cultures, expressed as percentage of cAMP levels obtained in the presence of forskolin or CGS 21680. * $p < 0.001$ compared to forskolin + CPA groups. * $p < 0.05$ compared to other groups.

II.4. CAPÍTULO 4 - *Carina R Boeck, Marcelo Ganzella, Amilcar Lottermann, and Deusa Vendite. **NMDA preconditioning protects against seizures and hippocampal neurotoxicity induced by quinolinic acid in mice.** Em fase de publicação no periódico **Epilepsia**.

Epilepsia, 45(7):1–6, 2004
Blackwell Publishing, Inc.

NMDA Preconditioning Protects against Seizures and Hippocampal Neurotoxicity Induced by Quinolinic Acid in Mice

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Summary: *Purpose:* *N*-methyl D-aspartate (NMDA) preconditioning has been used to prevent cellular death induced by glutamate or NMDA in cultured neurons. Quinolinic acid (QA)-induced seizures are used to average NMDA receptors–evoked neurotoxicity in animal models. The purpose of this study was to investigate the potential neuroprotective effects of NMDA preconditioning against QA-induced seizures and hippocampal damage in vivo.

Methods: Mice were pretreated with nonconvulsant doses of NMDA for different times before i.c.v. QA infusion and observed for the occurrence of seizures. Hippocampal slices from mice were assayed to measure cellular viability.

Results: NMDA preconditioning presented 53% protection against QA-induced seizures, as well as QA-induced cellular death in the hippocampus. The NMDA-receptor antagonist, MK-801, prevented the protection evoked by NMDA preconditioning. The adenosine A_2-receptor antagonist, CPT, prevented the protection evoked by NMDA preconditioning against QA-induced seizures, but not against QA-induced hippocampal cellular damage. The adenosine A_1-receptor agonist, CPA, did not mimic the NMDA preconditioning–evoked protective effects.

Conclusions: These results suggest that in vivo preconditioning with subtoxic doses of NMDA protected mice against seizures and cellular hippocampal death elicited by QA, probably through mechanisms involving NMDA receptors operating with adenosine A_1 receptors. **Key Words:** Quinolinic acid—Seizures—NMDA preconditioning—Neurotoxicity—Neuroprotection.

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS) and mediates physiologic pathways as well as contributes to neuronal damage (1,2). Excitotoxicity induced by glutamate involves at least in part intracellular increase of Ca^{2+} level through excessive stimulation of the *N*-methyl-D-aspartate (NMDA) receptors (3). Despite the neurotoxic actions of NMDA, the neuroprotective effects evoked by NMDA receptors activation have been studied. Chemical preconditioning (e.g., NMDA preconditioning) is evoked by subtoxic concentrations of a substance that evokes transient resistance to its subsequent lethal dose (4). NMDA preconditioning prevents cellular death induced by glutamate or NMDA in cultured neurons (5,6), whose effect is prevented by the RNA synthesis inhibitor actinomycin D and the protein synthesis inhibitor cyclohexamide (7).

Additionally, systemically administered NMDA protects hippocampal neurons against damage induced by kainate through blockade of DNA fragmentation in vivo (8). Short nonlethal ischemic insult protects neural cells from damage induced by severe global ischemia; this brain-tolerance process is known as "ischemic preconditioning" (9,10). Recently, NMDA preconditioning was reported to have neuroprotective effects on oxygen and glucose deprivation (OGD) in hippocampal organotypic cultures (11,12). However, the mechanisms underlying the development of brain tolerance are not completely elucidated. Quinolinic acid (QA), an endogenous tryptophan metabolite (13,14), is a selective NMDA-receptor agonist and displays similar potency found to NMDA in producing neurotoxicity (15). The increase of the QA level in the CNS induces seizures (16) and has been proposed to be involved in the etiology of epilepsy in humans (17). Q1

Because seizures induced by QA can be used to average NMDA receptors–evoked neurotoxicity in vivo, in the present study, we investigated the potential neuroprotective effects of NMDA preconditioning against seizures and cellular hippocampal damage induced by QA in mice.

MATERIALS AND METHODS

Materials

NMDA and QA were obtained from Sigma (St. Louis, MO, U.S.A.). 5-Methyl-10-11-dihydro-5H-dibenzo

Accepted February 29, 2004.

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[*a,b*]cyclohepta-5-10-imine maleate (MK-801 or dizocilpine), 8-cyclopentyl-1,3-dimethylxanthine (CPT), and *N*-cyclopentyladenosine (CPA) were obtained from RBI-Research Biochemicals International (Natick, MA, U.S.A.). Propidium iodide was purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA, U.S.A.). The anesthetic sodium thiopental was obtained from Cristalia (Itapira, SP, Brazil).

Animals

Male adult Swiss albino mice (30–40 g) were kept on a 12-h light/dark cycle (light on at 07.00 a.m.) at a constant temperature of 22 ± 1°C. They were housed in plastic cages with tap water and commercial food ad libitum. All procedures were carried out according to the institutional policies on animal experimental handling, designed to minimize suffering and limit the number of animals used. All experiments were performed at the same time during the day to avoid circadian variations.

Surgical procedure

Animals were anesthetized with sodium thiopental (60 mg/kg) intraperitoneally (i.p.) and placed in a stereotaxic apparatus. The intracerebroventricular (i.c.v.) guide cannula for infusion was implanted as previously described (18). Stereotaxis coordinates were 1 mm posterior to bregma, 1 mm right of the midline, and 1 mm above the lateral brain ventricle. The guide cannula was implanted 1.5 mm ventral to the superior surface of the skull and fixed with jeweler's acrylic cement. The tip of the 30-gauge infusion cannula protruded 1 mm beyond the guide cannula, aiming the lateral brain ventricle.

Treatments and chemical convulsant test

The pretreatments were performed 48 h after surgery. Animals were pretreated with NMDA in a low nonconvulsant dose (75 mg/kg, i.p.) (19) or vehicle (saline, 0.9%) at different times (30 min, 1, 8, 24, 48, or 72 h) before QA administration and observed for 30 min. When mentioned, the NMDA-receptor antagonist, MK-801, was administered (0.5 mg/kg, i.p.) 30 min before NMDA injection. Both drugs were dissolved in saline and adjusted to pH 7.4 with NaOH. The adenosine A_1-receptor antagonist, CPT (1 μl, 100 n*M* i.c.v.) or vehicle (DMSO) was infused immediately before NMDA injection. For experiments with MK-801 and CPT, QA was infused only 24 h after NMDA administration. The adenosine A_1-receptor agonist, CPA (1 μL, 100 n*M* i.c.v.) or vehicle (ethanol) was infused 24 h before QA administration (20). The chemical seizures were induced by QA infusion (4 μl, 9.2 m*M* i.c.v.) (18). Mice were observed for 10 min in Plexiglas boxes for the occurrence of wild running, clonic, tonic, or tonic–clonic seizures lasting >5 s. Animals not displaying seizures were considered protected. All drugs administered i.p. were carried out at 10 ml/kg.

Cellular viability

Methylene blue (4 μl) was injected through the cannula 24 h after QA infusion, and animals without contrast in the lateral brain ventricle were discarded. Mice were decapitated, the brain removed, and the hippocampus rapidly dissected into ice-cold phosphate-buffered saline (PBS) supplemented with 10 m*M* glucose. Hippocampus was cut in 400-μm-thick transverse slices with a McIlwain tissue chopper, followed by transfer of the sections to PBS, and separated into individual slices. On average, six slices from the middle of hippocampus were obtained and placed in a 96-well multiwell dish (Nunc) containing prewarmed HEPES-buffered saline (in m*M*: 120 NaCl, 5 KCl, 2 $CaCl_2$, and 10 glucose), pH 7.4. The slices were incubated for 1 h before cell-viability assay. Cell death was assessed by using uptake of the fluorescent exclusion dye propidium iodide (PI), which is a polar compound that enters only the dead or dying cells with damaged membranes. Once inside the cells, PI complexes with DNA, inducing intense red fluorescence (630 nm) when excited by green light (495 nm). The slices were incubated with 7.5 μg/ml PI for 1 h and then imaged on a standard inverted microscope (Nikon Eclipse TE 300) by using a rhodamine Q2 filter set. The PI uptake was quantified by densitometric analysis with Scion Image software (Scion Corporation) (12).

Statistical analysis

Statistical analysis among groups was performed with the Fisher's exact test for the occurrence of seizures or animal death. PI-uptake assay in hippocampal slices was evaluated by a one-way analysis of variance (ANOVA) followed by Duncan's post hoc test. Differences were considered significant at <95% confidence.

RESULTS

Effect of NMDA preconditioning on QA-induced seizures

Mice were observed during 30 min after i.p. administration of a nonconvulsant dose of NMDA (75 mg/kg) to evaluate the behavioral changes. As previously described by Melani et al. (19), the systemic administration of subconvulsant doses of NMDA decreased the initial motor activation, followed by hyperactivity. Mice were infused i.c.v with QA after 30 min, 1, 8, 24, 48, or 72 h of NMDA or saline pretreatment (Fig. 1). After saline pretreatment, QA infusion induced seizures in all animals (n = 28). NMDA pretreatment for 30 min or 1 or 8 h before QA infusion did not affect the QA-induced seizures (n = 7–10). However, NMDA administered 24 or 48 h before QA infusion gave 53% protection against the incidence of seizures (n = 28 and 15, respectively; $p < 0.0001$). In animals in which

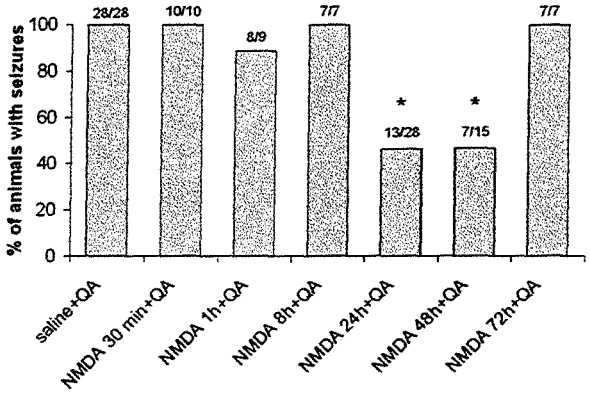

FIG. 1. Effect of pretreatment with *N*-methyl-D-aspartate (NMDA) for different times before quinolinic acid (QA)-induced seizures in mice. Mice received NMDA (75 mg/kg, i.p.) 30 min, 1, 8, 24, 48, or 72 h before QA infusion (36.8 n*M*, i.c.v), and the incidence of seizures was observed for 10 min. The numbers on top of bars represent the number of the animals with seizures/total number of animals per group. *$p < 0.0001$ compared with saline plus QA group.

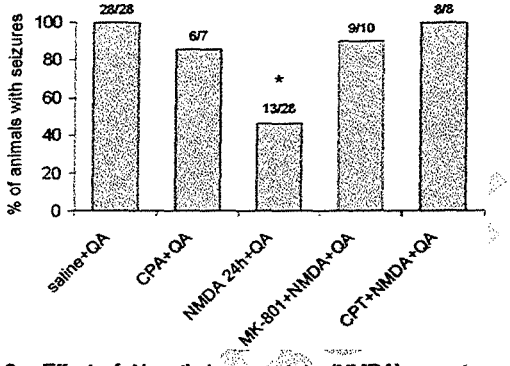

FIG. 2. Effect of *N*-methyl-D-aspartate (NMDA) receptors antagonist or adenosine A_1 receptor ligands on NMDA preconditioning against quinolinic acid (QA)-induced seizures. Mice received MK-801 (0.5 mg/kg, i.p.) 30 min or CPT (adenosine A_1 receptor antagonist) (100 n*M*, i.c.v.) immediately before NMDA administration. QA was infused 24 h after NMDA or CPA (adenosine A_1 receptor agonist) (100 n*M*, i.c.v.), and the incidence of seizures was observed for 10 min. The numbers on top of bars represent: the number of the animals with seizures/total number of animals per group. *$p < 0.0001$ compared with saline-plus-QA group.

NMDA did not provide protection, NMDA had no effect either on latencies for seizures (mean ± SD, in s: 27.9 ± 15, 27.7 ± 16, and 33.3 ± 0.6 for control, NMDA 24 h, and NMDA 48 h, respectively) or on duration of seizures (20.9 ± 10, 16.2 ± 5, and 12.7 ± 7 for control, NMDA 24 h, and NMDA 48 h, respectively). This protection was not observed when animals were treated with NMDA 72 h before QA infusion (n = 7).

Effect of MK-801 or adenosine-receptor ligands on NMDA preconditioning

The NMDA antagonist, MK-801 (0.5 mg/kg) administered 30 min before a nonconvulsant dose of NMDA prevented the changes in motor activity induced by NMDA (data not shown). The MK-801 dose was selected according to preliminary experiments in which MK-801 prevented the seizures when administered 30 min before QA infusion (18). Thus pretreatment with MK-801 prevented the protection evoked by NMDA preconditioning, because 90% of animals had seizures induced by QA infusion (n = 10; Fig. 2).

Regarding the participation of adenosinergic system in the NMDA-preconditioning mechanism, mice were administered i.c.v with CPT, 100 n*M* (an adenosine A_1-receptor antagonist) immediately before NMDA injection. CPT prevented NMDA preconditioning, because all animals had QA-induced seizures (n = 8). To determine whether adenosine A_1 receptors were able to mimic the NMDA preconditioning, mice were pretreated with CPA (adenosine A_1-receptors agonist; 100 n*M*; i.c.v.), instead of NMDA, 24 h before QA infusion. CPA was not able to protect against the QA-induced seizures in 86% of mice (n = 7). None of the vehicles demonstrated any effect on QA-induced seizures (data not shown).

Effect of NMDA preconditioning on QA-induced cellular death

To determine whether NMDA preconditioning protects cells against damage evoked by QA-induced seizures, the cellular death in the hippocampal slices was quantified by using a PI incorporation assay. Mice had the hippocampi removed and sliced 24 h after the QA infusion. Figure 3 shows the cellular death in hippocampal slices from different groups of animals, and Figure 4 shows the quantification of PI fluorescence. Animals that received saline i.p. and i.c.v. represent the control group. The PI uptake observed in the control group probably represents the damage due to the cut of the hippocampal tissue (n = 10). The injury of the cells due to QA-induced seizures was widely distributed in mouse hippocampus, as shown by PI uptake into the nonviable cells (n = 12; p = 0.0001; Figs. 3 and 4A). Besides the protective effect of the NMDA pretreatment against convulsions, NMDA preconditioning had a widespread protection against QA-induced cellular death in the hippocampus from mice (n = 9; Figs. 3 and 4A), whereas NMDA itself was not different from the control group (n = 9). The protection with NMDA preconditioning against QA-induced cellular death was maintained even in those animals that had QA-induced seizures.

The pretreatment with MK-801 blocked the neuroprotection induced by NMDA preconditioning on cellular death (n = 4; Figs. 3 and 4B). Although CPT prevented the protection evoked by NMDA preconditioning against QA-induced seizures, it was ineffective in preventing the neuroprotective effect of NMDA on cellular death, because this effect was preserved (n = 4; Figs. 3 and 4B). The pretreatment with CPA (adenosine A_1-receptors

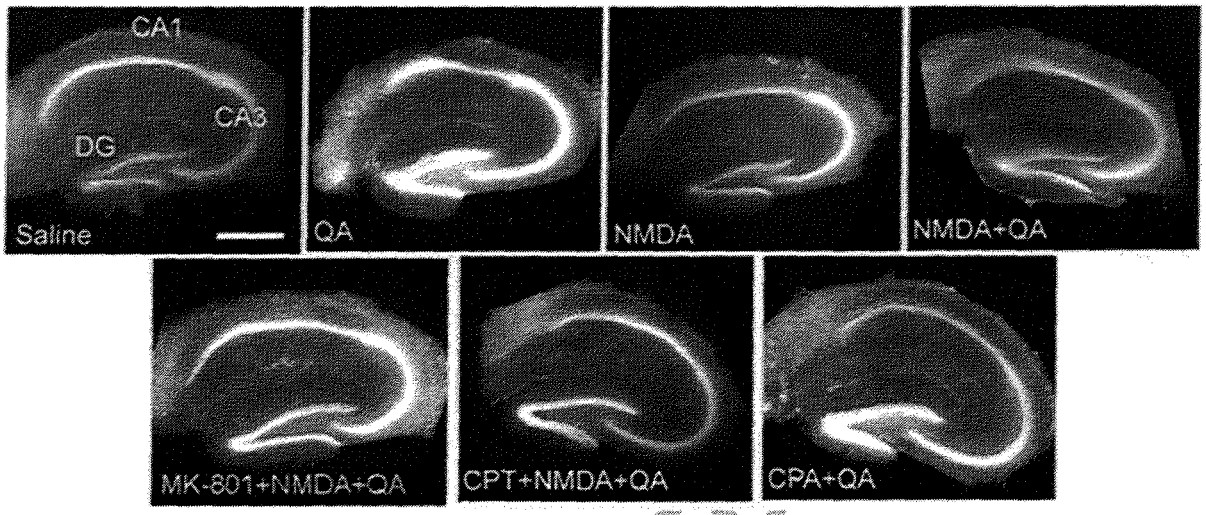

FIG. 3. Effect of *N*-methyl-D-aspartate (NMDA) receptor antagonist or adenosine A_1 receptor ligands on NMDA preconditioning against quinolinic acid (QA)-induced cellular death in hippocampal slice from mice. Photomicrographs show propidium iodide (PI) uptake after the treatments. The fluorescence area corresponds to the cellular death in the different groups of animals. Mice received MK-801 30 min or 8-cyclopentyl-1,3-dimethylxanthine (CPT) immediately before NMDA administration. QA was infused 24 h after NMDA or *N*-cyclopentyladenosine (CPA) administration. The CA1, CA3, and DG (dentate gyrus) subfields are indicated. Scale bar, 100 μm.

agonist) was ineffective in protecting against QA-induced cellular death (n = 6; Figs. 3 and 4B).

DISCUSSION

The main finding of the present study was that systemic pretreatment with subconvulsant doses of NMDA led to protection against seizures and cellular death induced by administration of an NMDA agonist in mice. Subtoxic doses of NMDA provide a chemical preconditioning that leads to cellular tolerance to subsequent toxic doses of glutamate-receptor agonists. The neuroprotective effects of NMDA against damage induced by glutamate-receptor agonists have been observed with cultured rat neurons in vitro (5,6) and in murine hippocampal neurons (8), beyond neuroprotection against ischemia in organotypic hippocampal cultures (12) and in hippocampal slices (21).

To date, the protection against QA-induced cellular death and seizures has been attributed to NMDA-receptor antagonists (e.g., MK-801) (18,22). However, in this study, based on potent neuroprotective action of NMDA in different models of excitotoxicity in vitro, NMDA was used as an anticonvulsant agent against seizures induced through activation of an NMDA receptor by QA in vivo. Additionally, the preconditioning evoked by the pretreatment with nonconvulsant doses of NMDA was time dependent. The efficacy of preconditioning was observed when mice received QA infusion 24 to 48 h after NMDA administration. Previous studies demonstrated cellular damage after QA-induced seizures (23,24), and we observed that NMDA preconditioning was largely effective in preventing QA-induced cellular death in hippocampal slices, even in animals displaying seizures after QA administration.

The noncompetitive NMDA-receptor antagonist, MK-801, prevented the NMDA protection against seizures as well as cellular damage induced by QA, strongly indicating that preconditioning arises from NMDA-receptor activity. Adenosine is a neuromodulator with anticonvulsant and neuroprotective activity, mainly through adenosine A_1 receptors (25). Because stimulation of NMDA receptor induces an increase on extracellular adenosine levels (26–28), we administered the adenosine A_1-receptor antagonist, CPT, to evaluate adenosine A_1 receptors role on the neuroprotective mechanism of NMDA. CPT prevented the protection evoked by NMDA preconditioning against QA-induced seizures, whereas it was ineffective in preventing neuroprotection from cellular death. By contrast, studies have observed that systemic administration of CPT has an anticonvulsant effect against GABA/benzodiazepine receptor complex–induced seizures in mice (29) and prevents neuroprotection mediated by NMDA pretreatment against kainate-induced neuronal loss (8). The lack of CPA effect (adenosine A_1-receptors agonist) on QA-induced seizures or cellular death probably was due to the time of CPA administration (24 h before QA infusion) in the present study. Studies have observed that short administration of CPA has anticonvulsant effect, whereas prolonged administration for 9 days showed the opposite effect (30,31). In addition, these differential effects have been discussed elsewhere (30–32). Nevertheless, our results suggest that in vivo NMDA preconditioning occurred, at least in part, through activation of adenosine A_1 receptors, because an adenosine A_1-receptor antagonist

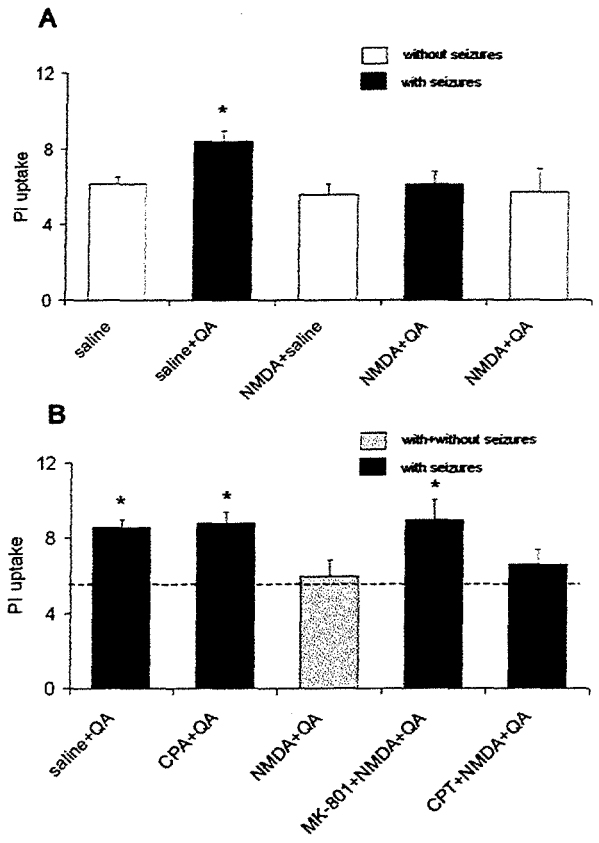

FIG. 4. Quantification of quinolinic acid (QA)-induced cellular death after *N*-methyl-D-aspartate (NMDA) preconditioning. Images were captured and then analyzed with Scion Image software. The area where propidium iodide (PI) fluorescence was detectable above background was determined by densitometric analysis. A: Mice were pretreated with saline or a nonconvulsant dose of NMDA and received QA infusion 24 h later. B: Mice received MK-801 30 min or 8-cyclopentyl-1,3-dimethylxanthine (CPT) immediately before NMDA administration. QA was infused 24 h after NMDA or *N*-cyclopentyladenosine (CPA) administration. *Dashed line*, saline group. Values are expressed as mean ± SEM. *$p < 0.0001$ compared with saline group.

prevented the protection evoked by NMDA preconditioning against QA-induced seizures but not cellular death. Probably the NMDA preconditioning involves different neuroprotective pathways: one depends on NMDA-receptor activation, and the other cooperates with adenosine A_1 receptors.

In conclusion, in vivo preconditioning with subconvulsive doses of NMDA protected mice against seizures and cellular hippocampal death elicited by QA through mechanisms involving the NMDA receptor cooperating with adenosine A_1 receptors. The underlying mechanisms involved in these neuroprotective effects must be further investigated to delineate a better understanding of brain tolerance evoked by NMDA preconditioning.

Acknowledgment: This research was supported by grants from FAPERGS, FINEP/CNPq, and PRONEX. Carina R. Boeck was the recipient of a CAPES fellowship.

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III. DISCUSSÃO

Os aminoácidos excitatórios glutamato e aspartato participam de importantes processos fisiológicos assim como patológicos no SNC. As regiões cerebrais, cerebelo e hipocampo são mais susceptíveis ao insulto excitotóxico provocado pelo aumento nos níveis extracelulares destes aminoácidos. Com isto, é de grande importância para o sistema que o nível extracelular dos aminoácidos sejam mantidos baixos para que não haja excessiva ativação de seus receptores. Os transportadores de aminoácidos são as proteínas responsáveis em regular os níveis dos aminoácidos, regulando assim a transmissão sináptica e seu efeito excitatório.

A adenosina é um nucleosídeo purinérgico que desempenha importante função inibitória na transmissão sináptica. A adenosina além de inibir a atividade sináptica e a excitabilidade no neurônio via ativação dos receptores A_1, inibe a liberação de aminoácidos excitatórios, tais como glutamato e aspartato (POLI et al., 1991). Porém, tem se observado que a adenosina ativando receptores A_{2A} também pode promover a liberação de glutamato e condições normais e durante um insulto isquêmico (O'REGAN et al., 1992; CHEN et al., 1999).

No presente trabalho estudou-se a interação entre os sistemas glutamatérgico e purinérgico em situações de excitotoxicidade celular, bem

como, mecanismos que participam da regulação dos níveis extracelulares de glutamato ou adenosina e a sua participação no processo de neuroproteção.

Os transportadores EAATs realizam a retirada do neurotransmissor do meio extracelular, finalizando suas ações e assim regulando as sinalizações fisiológicas e patológicas. O glutamato é um importante neurotransmissor excitatório, mas a sua concentração na fenda sináptica é crítica para determinar a sua neurotoxicidade. Desde que excessiva a ativação dos receptores glutamatérgicos desencadeia sinalizações que resultam na morte celular, a retirada de glutamato da fenda sináptica pelos EAATs contribui para o processo de neuroproteção. No presente trabalho, neurônios granulares do cerebelo foram pré-incubados com altas doses dos aminoácidos excitatórios glutamato, L-aspartato ou D-aspartato para induzir a neurotoxicidade. Após o período de pré-incubação, os aminoácidos foram retirados e o transporte de glutamato medido. Tanto glutamato, quanto L-aspartato ou D-aspartato inibiram a captação do glutamato nos neurônios. Os aminoácidos L-aspartato e D-aspartato são conhecidos inibidores competitivos do transporte de glutamato em neurônios do cerebelo, pois são inibidores substrato para o transportador (DREJER et al., 1982; GORDON et al., 1983). Contudo, estudos têm mostrado em cultura de astrócitos e de neurônios com alta densidade de astrócitos um aumento na captação do glutamato pelos aminoácidos (LONGUEMARE et al., 1995; DUAN et al., 1999; MUNIR et al., 2000). No presente estudo, provavelmente, os aminoácidos foram captados pelos neurônios durante o

período de pré-incubação, e liberados durante o período de captação, inibindo competitivamente a captação do glutamato. Esta hipótese é reforçada pela presença de um bloqueador do transporte durante o período de pré-incubação, pois o DHK bloqueou o efeito inibitório do L-aspartato na captação do glutamato. Provavelmente, o DHK impediu a captação do L-aspartato durante o período de pré-incubação, e conseqüentemente a sua posterior liberação. Além disso, também é possível que a inibição da captação do glutamato observada por L-aspartato, D-aspartato e glutamato tenha ocorrido devido à união dos aminoácidos ao sítio de ligação no lado intracelular do transportador, num processo conhecido como 'trans-inibição' (DEBERNARDI et al., 1999), diminuindo assim a velocidade da captação extracelular do aminoácido. Então, os resultados sugerem que a inibição induzida pelos aminoácidos no transporte do glutamato se deve a sua liberação durante o período da captação, o que acarretaria em uma inibição competitiva em ambos sítios de ligação intracelular e extracelular do transportador. Observou-se, além disso, uma regulação do transporte de glutamato por agonistas de receptores glutamatérgicos. Estudos prévios mostraram um aumento na captação de glutamato induzido por NMDA (agonista dos canais-receptores NMDA) ou por *t*-ACPD (agonista não-específico de receptores metabotrópicos) (DUAN et al., 1999; MUNIR et al., 2000). Os dados apresentados aqui confirmam estes resultados, porém observou-se que a ativação no transporte de glutamato induzida pelos agonistas ocorre por diferentes mecanismos de regulação. O aumento na

captação observada com SR-ACPD foi bloqueado em presença do antagonista dos receptores metabotrópicos, L-AP3; o que não ocorreu para a estimulação induzida por NMDA, pois nem o antagonista competitivo AP-5, ou não-competitivo MK-801, foram eficazes contra o aumento na captação de glutamato induzido por NMDA. Além disso, o antagonista MK-801, que é um bloqueador do canal no receptor NMDA, por si só estimulou a captação. Sabe-se que MK-801 também afeta a captação de outros neurotransmissores como: adrenalina, serotonina e dopamina, mas esse efeito é independente dos canais-receptores NMDA e sim do processo de despolarização induzido pelo antagonista (LONGUEMARE et al., 1996; PUBILL et al., 1996; CALLADO et al., 2000). A estimulação induzida por NMDA ou MK-801 é desconhecida em neurônios, mas é provável que um componente iônico esteja envolvido. Ambas as substâncias levam ao aumento extracelular dos íons K^+ e conseqüentemente, elevam os níveis intracelulares dos íons Na^+, favorecendo assim o transporte eletrogêneo do glutamato. Estudos indicam uma inter-relação funcional entre os receptores metabotrópicos ativados por SR-ACPD e os transportadores glutamatérgicos, o que poderia contribuir para a retirada do glutamato do meio extracelular e conseqüentemente, finalizar a excitabilidade no neurônio (BRASNJO et al., 2001). Então, durante a neurotoxicidade glutamatérgica pode haver uma regulação diferencial dos receptores e dos transportadores do glutamato em neurônios. Isto sugere, que a captação é um mecanismo importante na 'limpeza' do glutamato do meio extracelular,

principalmente em eventos de excitotoxicidade por receptores glutamatérgicos. Estes resultados contribuem para o entendimento da regulação celular durante eventos neurotóxicos induzidos pelo excesso de glutamato extracelular.

Em situações de insulto celular, como por exemplo, durante um trauma mecânico, uma isquemia ou convulsão, o glutamato e/ou aspartato são liberados (MELANI et al., 1999, MELDRUM et al., 1999; MELDRUM, 2000), ativando excessivamente os receptores glutamatérgicos, principalmente os canais-receptores NMDA. O glutamato por meio da ativação de seus receptores provoca a liberação do nucleotídeo ATP de neurônios (INOUE et al., 1992) e de astrócitos (QUEIROZ et al., 1998), além de aumentar os níveis extracelulares do nucleosídeo adenosina (HOEHN & WHITE, 1990; CRAIG & WHITE, 1993; DELANEY & GEIGER, 1998). O aumento nos níveis de adenosina se deve à sua liberação como tal pelo transportador bi-direcional de nucleosídeo ou, pela degradação de nucleotídeos liberados para o meio extracelular pela ação da cascata de ecto-enzimas (POLI et al., 1991; BRUNDEGE & DUNWIDDIE, 1997). Doses neurotóxicas de glutamato ou NMDA estimulam a atividade das ecto-enzimas ATP difosfoidrolase e 5'-nucleotidase de neurônios em cultura. Este efeito do glutamato é prevenido por DHK, bloqueador dos transportadores de aminoácido, indicando que parte da adenosina extracelular, observada durante a excitotoxicidade, é proveniente da hidrólise dos nucleotídeos (BOECK et al., 1999; BOECK et al., 2000). Os aminoácidos L-aspartato e D-aspartato mimetizam quase todas as ações do glutamato, pois são ligantes para os

canais-receptores NMDA e para os transportadores EAATs. No presente estudo também se observou uma estimulação da atividade da ecto-5'-nucleotidase por esses aminoácidos. A estimulação induzida por L-aspartato, foi revertida em presença dos antagonistas dos receptores NMDA, o MK-801 e o AP-5, porém, os antagonistas não tiveram efeito sobre estimulação enzimática induzida por D-aspartato. Ambos os aminoácidos são considerados ligantes de alta afinidade para os canais-receptores NMDA, mas possuem ações consideradas atípicas, pois as respostas induzidas por ambos ocorrem mesmo em presença dos íons Mg^{2+}, que na célula em repouso bloqueia os canais-receptores NMDA (YUZAKI et al., 1996; KUBRUSLY et al., 1998). Mas, certamente o efeito de ambos aminoácidos sobre a atividade da ecto-5'-nucleotidase ocorreu devido a sua captação pelos EAATs, pois o bloqueador do transporte DHK reverteu suas ações, indicando a fundamental participação do processo de captação para que a estimulação ocorra. É possível que este efeito se deva ao gradiente eletrogêneo acoplado ao transporte de aminoácido (ZERANGUE & KAVANAUGH, 1996). O aumento na expressão da ecto-5'-nucleotidase (BRAUN et al., 1998) e na atividade, também tem sido observado na epilepsia (BONAN et al., 2000). Tem sido observado, um aumento extracelular de adenosina em situações neurotóxicas, como por exemplo, durante um insulto ocasionado por glutamato (HOEHN & WHITE, 1990; CRAIG & WHITE, 1993), por insulto induzido por hipóxia (KOBAYASHI et al., 2000) ou isquemia (MELANI et al., 1999; WARDAS, 2002). Este aumento nos níveis

extracelulares de adenosina tem sido considerado uma resposta neuroprotetora, pois agindo nos receptores A_1, a adenosina inibe a liberação de aminoácidos excitatórios, como glutamato e aspartato (POLI et al., 1991). A ativação dos receptores A_{2A}, por outro lado, aumenta a liberação de glutamato sobre condições normais e durante insulto isquêmico, potencializando assim a neurotoxicidade (O'REGAN et al., 1992; SIMPSON et al., 1992; ONGINI & SCHUBERT, 1998). A ativação de um ou outro receptor de adenosina depende da concentração extracelular do nucleosídeo, pois a afinidade da adenosina para os receptores A_1 é na faixa de nanomolar e para os receptores A_{2A} é de micromolar. Tem-se proposto que baixas concentrações extracelulares de adenosina são originadas da sua liberação como tal e assim ativa preferencialmente os receptores A_1. Enquanto mais altas concentrações são originadas da sua formação pela cascata das ecto-enzimas, o que favorece a ativação dos receptores A_{2A} (CUNHA et al., 1996). Apesar dos aminoácidos L-aspartato e D-aspartato estimularem a atividade da ecto-5´-nucleotidase, a morte celular induzida por esses aminoácidos não foi afetada pela presença dos ligantes CPT ou ZM 241385, antagonistas dos receptores A_1 e A_{2A}, respectivamente. As expectativas eram de que a neurotoxicidade por L-aspartato ou D-aspartato pudesse ser aumentada ou diminuída com a presença de um ou outro antagonista. Provavelmente, a falta de efeito de ambos os antagonistas CPT e ZM 241385 pode ser um sinal de que o máximo de neurotoxicidade por L-aspartato ou D-aspartato (e também glutamato) tenha

sido atingido. Estudos têm mostrado que o tratamento agudo com antagonista do receptor A_{2A} potencialmente protege contra neurotoxicidade induzida por hipóxia/isquemia (von LUBITZ et al., 1995). Nesta metodologia, o antagonista foi pré-incubado antes do insulto, impedindo a ativação dos receptores A_{2A} e a conseqüente liberação de glutamato, protegendo assim da excitotoxicidade. Com isto, provavelmente, a ausência de efeito do antagonista dos receptores A_{2A} (ZM 241385) seja devido ao fato de que no presente desenho experimental as células foram incubadas diretamente com o glutamato. Então, outros experimentos devem ser realizados para esclarecer a função de adenosina formada pela estimulação da ecto-5'-nucleotidase. O pré-condicionamento é um mecanismo de proteção celular devido à indução de tolerância ao dano a partir de um estímulo subtóxico. Estudos têm demonstrado que a ativação dos receptores A_1 de adenosina por um tratamento agudo com a adenosina ou agonistas é eficaz contra a morte celular, mimetizando o pré-condicionamento na isquemia (RESHEF et al., 2000).

O pré-condicionamento com doses subtóxicas do NMDA induz a tolerância celular e os mecanismos envolvidos na proteção são alvo de muitas investigações (CHUANG et al., 1992; MARINI & PAUL, 1992; DAMSCHTODER-WILLIAMS et al., 1995; MARINI et al., 1998). No presente trabalho, investigou-se a participação dos receptores de adenosina no pré-condicionamento com NMDA em modelos de neurotoxicidade glutamatérgica *in vitro*, em cultura de neurônios granulares do cerebelo, e *in vivo*, em convulsões

induzidas por ácido quinolínico em camundongos. O modelo de pré-condicionamento com NMDA em cultura de neurônios é o mais investigado, no entanto, ainda não havia sido realizado um estudo sobre a participação do sistema adenosinérgico neste processo. No presente estudo, observou-se que o pré-condicionamento com NMDA evitou a estimulação da atividade da ecto-5'-nucleotidase induzida por doses neurotóxicas de glutamato, mas a estimulação da atividade da ecto-ATP difosfoidrolase não foi modificada. Desde que a ecto-5'-nucleotidase é a enzima marca-passo na produção de adenosina pela degradação de nucleotídeos, e os aminoácidos excitatórios aumentam a sua atividade, este resultado indica que a produção de adenosina estimulada por glutamato foi afetada, provavelmente para diminuir a atividade do receptor A_{2A}, que poderia potencializar a resposta excitotóxica. Desde que os dados apresentados aqui reproduzem a neuroproteção com o pré-condicionamento com NMDA contra o dano causado por altas doses de glutamato, se investigou a participação dos receptores de adenosina nesta neuroproteção. O 8-PT, antagonista não-seletivo dos receptores A_1 e A_{2A}, não reverteu a proteção pelo pré-condicionamento com NMDA contra a morte celular induzida por glutamato, além de apresentar um efeito neurotóxico por si só. Isto indica que o uso de 8-PT por longos períodos é inadequado como medida da participação dos receptores de adenosina no processo de tolerância pelo pré-condicionamento. Com isto, os antagonistas CPT e ZM 241385 foram utilizados por serem específicos para os receptores A_1 e A_{2A}, respectivamente. O antagonista CPT

reverteu a neuroproteção induzida pelo pré-condicionamento com NMDA contra a morte celular, mas o ZM 241385 não teve efeito. Após o período do pré-condicionamento, a funcionalidade dos receptores foi investigada e observou-se uma diminuição na funcionalidade dos receptores A_{2A}, verificada através da medida da formação intracelular de AMPc induzida pela ativação de ambos receptores de adenosina. Juntos estes dados reforçam a hipótese de que na neuroproteção pelo pré-condicionamento com NMDA é fundamental a participação dos receptores A_1: dessensibilizando os receptores A_{2A} e assim favorecendo os receptores A_1. Sabe-se que há uma inter-relação funcional entre os receptores A_1 e A_{2A}, pois a ativação dos receptores A_{2A} diminui a atividade dos receptores A_1 (DIXON et al., 1997; DUNWIDDIE et al., 1997; O'KANE & STONE, 1998; LOPES et al., 2002), assim como, o aumento nas respostas mediadas pelos receptores A_{2A} induzidas pelos receptores NMDA suprime a funcionalidade dos receptores A_1 (NIKBAKHT et al., 2001). Provavelmente, o que se observou foi uma regulação dos receptores A_{2A} pelo pré-condicionamento favorecendo a atividade dos receptores A_1 por adenosina, e com isto co-operando com a neuroproteção contra a morte celular induzida por glutamato. Outro achado interessante do presente estudo foi a neuroproteção observada após o longo período de pré-tratamento com os antagonistas CPT e ZM 241385 sozinhos. Provavelmente, os receptores A_1 foram sensibilizados pelo antagonista (von LUBITZ et al., 1994), pois em presença do agonista dos receptores A_1, CPA, o efeito protetor do CPT foi

bloqueado. Mas CPA não teve efeito sobre a neuroproteção induzida por ZM 241385. A neuroproteção devido ao tratamento prolongado com ZM 241385 tem sido observada contra danos induzidos pela isquemia (von LUBITZ et al., 1995); assim como, o tratamento prolongado com agonistas dos receptores A_{2A} protege da excitotoxicidade glutamatérgica (FERREIRA & PAES-DE-CARVALHO, 2001). Então, como discutido anteriormente, a funcionalidade e a regulação dos receptores A_{2A} em neurônios granulares do cerebelo são menos estudadas, mas provavelmente, no processo do pré-condicionamento com NMDA a sua dessensibilização auxilia as ações de adenosina nos receptores A_1.

O potencial neuroprotetor do NMDA no pré-condicionamento tem sido pouco estudado em experimentos realizados *in vivo*. Mas, recentemente, foi observado que o pré-condicionamento com NMDA protege contra a excitotoxicidade induzida pela administração de cainato em camundongos e esta neuroproteção foi revertida em presença do antagonista CPT, sugerindo a participação dos receptores A_1 (OGITA et al., 2003). No presente estudo, verificou-se a eficácia do pré-condicionamento contra as convulsões induzidas por ácido quinolínico e sobre a morte celular decorrente deste processo no hipocampo de camundongos. O modelo de convulsão pelo ácido quinolínico é utilizado como medida de excitotoxicidade provocada pelos receptores NMDA, pois o antagonista do canal-receptor NMDA, o MK-801, bloqueia as convulsões induzidas pelo ácido quinolínico (SCHMIDT et al., 2000) e, além disso, impede

a peroxidação de lipídeos devido a este evento (SANTAMARÍA & RÍOS, 1993). Os camundongos foram pré-tratados com doses sub convulsivas de NMDA por tempos diferentes antes da injeção intra-cerebroventricular do ácido quinolínico. A convulsão por ácido quinolínico foi evitada em 53 % dos animais tratados com NMDA 24 ou 48 horas antes da administração do ácido quinolínico. Mesmo naqueles animais que apresentaram comportamento convulsivo, o pré-condicionamento reverteu totalmente a morte celular induzida pelo ácido quinolínico no hipocampo. O antagonista MK-801 bloqueou a proteção pelo pré-condicionamento, tanto sobre a convulsão, quanto sobre a morte celular, indicando que os receptores NMDA precisam estar disponíveis para que o mecanismo de proteção seja desencadeado. Além disso, observou-se a participação dos receptores A_1 de adenosina neste processo, pois o antagonista CPT preveniu a proteção do pré-condicionamento contra as convulsões. Porém, CPT não afetou a neuroproteção contra a morte celular, efeito esse oposto àquele observado contra a excitotoxicidade por cainato (OGITA et al., 2003). Em convulsões induzidas por um antagonista do complexo GABA/benzodiazepina, o CPT mostrou pronunciada ação anticonvulsiva; efeito inesperado e similar ao do agonista CPA (KLITGAARD et al., 1993). Contudo, no presente estudo, CPA não protegeu contra os danos causados pelo ácido quinolínico, mostrando sua ineficiência em mimetizar o pré-condicionamento com NMDA. O tratamento crônico com CPA foi observado ser pró-convulsivo em modelo de convulsão por NMDA (von LUBITZ et al., 1994), contrário ao

efeito do tratamento agudo, onde se mostrou anticonvulsivo (von LUBITZ et al., 1993; KLITGAARD et al., 1993). Isto sugere que, nos mecanismos de neuroproteção induzidos pelo processo de pré-condicionamento com NMDA *in vivo* está associada diferentes vias de proteção: uma dependente exclusivamente da ativação do receptor NMDA e outra dependente da cooperação dos receptores A_1 de adenosina.

A utilização dos ligantes de receptores da adenosina como agentes neuroprotetores depende, principalmente, se a administração é crônica ou aguda, pois estudos mostram que o tratamento prolongado com agonistas ou antagonistas de receptores para adenosina podem levar a uma hipofunção ou hiperfunção dos receptores, respectivamente (JACOBSON et al., 1996). Então, os dados apresentados nesta tese indicam a participação dos receptores de adenosina no pré-condicionamento com NMDA, tanto em modelos de toxicidade *in vitro*, quanto em *in vivo*.

III.1. CONCLUSÕES

A presente tese abordou possíveis mecanismos de neuroproteção contra a excessiva ativação do sistema glutamatérgico.

Uma das vias estudada foi a modulação da captação de glutamato em neurônios do cerebelo. Foi observado que a pré-incubação com altas doses dos aminoácidos glutamato, L-aspartato e D-aspartato inibiu a posterior captação de

glutamato. Esta inibição ocorreu, provavelmente, devido a união destes aminoácidos ao sítio extracelular e/ou intracelular de ligação no transportador. Por outro lado, os agonistas glutamatérgicos NMDA e SR-ACPD estimularam a captação de glutamato, mas somente a estimulação induzida por SR-ACPD foi dependente da ativação do seu receptor. Estes dados indicam diferentes possibilidades de regulação do transportador, o que pode contribuir para a neuroproteção ou para a neurotoxicidade glutamatérgica.

A outra via estudada foi a participação de adenosina em mecanismos de neuroproteção. Observou-se que uma dose neurotóxica do L-aspartato ou D-aspartato estimulam a atividade da ecto-5'-nucleotidase, enzima responsável em produzir adenosina no espaço extracelular. Contudo, não se observou qualquer efeito dos antagonistas de receptores de adenosina na neurotoxicidade induzida por ambos os aminoácidos em neurônios granulares cerebelo.

A neuroproteção pelo pré-condicionamento com NMDA envolveu a participação dos receptores A_1 de adenosina e uma dessensibilização dos receptores A_{2A} em cultura de neurônios do cerebelo.

Então, observou-se que, o pré-condicionamento com NMDA protege contra as convulsões e morte celular induzidas pelo ácido quinolínico. Neste processo houve a cooperação dos receptores A_1, contudo, somente no processo que previne contra as convulsões em camundongos.

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