Universidade Federal do Rio Grande do Sul
Instituto de Ciências Básicas da Saúde
Departamento de Bioquímica

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica da Universidade Federal do Rio Grande do Sul como requisito parcial à obtenção do título de Doutor em Bioquímica.

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Dedico essa tese às pessoas mais
importantes da minha vida (por enquanto):
pai, mãe, Paula e Emílio.

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Lista de abreviaturas

ACh – acetilcolina
AChE – acetilcolinesterase
AGAT – arginina - glicina amidino transferase
ATP – adenosina trifosfato
CAT - catalase
CK – creatina quinase
CRTR – transportador de creatina
EIM – erros inatos do metabolismo
ERO – espécies reativas de oxigênio
$FADH_2$, flavina adenina dinucleotídeo, forma reduzida
GAA - guanidinoacetato
GABA – ácido γ-aminobutírico
GAMT – guanidino acetato metiltransferase
GPx – glutationa peroxidase
GSH – glutationa reduzida
L-NAME - N^{ϖ}-nitro-L-arginina metil Ester
LTP – potencial de longa duração
NADH, nicotinamida adenina dinucleotídeo
NMDA – *N*-metil-D-aspartato
SOD – superóxido dismutase
SNC – sistema nervoso central
TBARS – substâncias reativas ao ácido tiobarbitúrico
TRAP – potencial antioxidante total

Resumo

A deficiência de guanidino acetato metiltransferase (GAMT) é um erro inato do metabolismo da creatina caracterizado por hipotonia muscular, movimentos extrapiramidais involuntários e epilepsia. A doença é, bioquimicamente, evidenciada pelo acúmulo de guanidino acetato (GAA) e deficiência de creatina e fosfocreatina nos tecidos dos pacientes afetados. Os mecanismos da disfunção neurológica que ocorrem nessa doença ainda são desconhecidos. A Na^+,K^+-ATPase, a creatina quinase (CK) e a acetilcolinesterase (AChE) têm um papel fundamental no sistema nervoso central (SNC), e a alteração dessas enzimas, juntamente com a diminuição do metabolimo energético e a indução do estresse oxidativo têm sido associados com algumas doenças que afetam o SNC, como as doenças de Alzheimer, Parkinson e Huntington e isquemia cerebral. Propriedades neurotóxicas do GAA têm sido atribuídas, principalmente, à excitotoxicidade e ao estresse oxidativo. Dessa forma, esse trabalho tem como propósito ampliar o conhecimento sobre os mecanismos fisiopatológicos da deficiência de GAMT, investigando o efeito *in vitro* e *in vivo* (administração intra-estriatal de GAA), sobre as atividades da Na^+,K^+-ATPase, CK, AChE, metabolismo energético, bem como a recaptação de glutamato e a produção de espécies reativas de oxigênio (EROS) em estriado de ratos. Finalmente, investigamos o efeito da administração intra-estriatal de GAA sobre a memória/aprendizagem em estriado de ratos. Nossos resultados mostraram que a administração intraestriatal de GAA diminui as atividades da Na^+,K^+-ATPase, CK e complexo II e aumenta o TBARS e a atividade da AChE. Além destes, mostramos que o GAA inibe a recaptação de glutamato e induz estresse oxidativo em estriado de ratos. A administração de GAA, também é capaz de prejudicar a memória de ratos adultos. A inibição das atividades da Na^+,K^+-ATPase, da CK e do complexo II e o aumento do TBA e da atividade da AChE, causados pela administração intraestriatal de GAA, foram prevenidos pelo pré-tratamento (uma semana) com as vitaminas E e C ou taurina. Considerando os resultados apresentados, propõe-se que a inibição da atividade da Na^+,K^+-ATPase, da CK, do complexo II, bem como da inibição na recaptação de glutamato e de

indução de estresse oxidativo causados pelo GAA possam estar envolvidos na disfunção neuronal observada em pacientes com deficiência de GAMT.

Abstract

Guanidinoacetate methyltransferase deficiency (GAMT-deficiency) is an inborn error of creatine metabolism characterized by muscular hypotonia, involuntary extrapiramidal movements and epilepsy. The disease is biochemically characterized by accumulation of guanidinoacetate and deficiency of creatine and phosphocreatine in tissues of affected patients. However, the mechanisms underlying the neurological dysfunction of GAMT-deficiency patients are not well understood. Na^+,K^+-ATPase, creatine kinase (CK) and acetylcholinesterase (AChE) play a fundamental role in central nervous system (CNS). Alterations in these enzymes with reduction of energy metabolism and induction of oxidative stress have been associated to neurological diseases such as Alzheimer's, Parkinson's, Huntington's disease and brain ischemia. Neurotoxic properties of GAA have been mainly related to excitotoxicity and oxidative stress. In this study we investigated possible pathophysiologic mechanisms of GAMT-deficiency. We studied the *in vitro* effect of GAA and the effect of GAA intrastriatal administration on Na^+,K^+-ATPase, CK and AChE activities, on brain metabolism, on glutamate uptake and on reactive oxidative species (ROS) production in striatum of adult rats. Finally, we investigated the effect of intraestriatal administration of GAA on inhibitory avoidance in adult rats. Our results showed that the intrastriatal administration inhibits Na^+,K^+-ATPase, CK and complex II activities and increases TBARS and AChE activity. We also showed that GAA *in vitro* inhibits glutamate uptake and induces oxidative stress. GAA administration also impairs memory in adult rats. The inhibition of Na^+,K^+-ATPase, CK and complex II activities and increase of TBARS production caused by intrastriatal administration of GAA was prevented by vitamins E and C or taurine pretreatment (for one week). Our findings indicate that inhibition of Na^+,K^+-ATPase, CK and complex II activities and alterations in glutamate uptake and oxidative stress caused by GAA may contribute to the neurological dysfunction characteristic of GAMT-deficiency patients.

Sumário

1. Introdução

1.1. Erros Inatos do Metabolismo

Os erros inatos do metabolismo (EIM) são doenças causadas por genes mutantes que geralmente resultam em proteínas alteradas, mais freqüentemente enzimas. Os defeitos hereditários podem se expressar como uma perda total ou parcial da atividade enzimática. Sem tratamento, esses defeitos hereditários quase sempre resultam em retardo mental ou outras anormalidades do desenvolvimento, devido ao acúmulo prejudicial de metabólitos ou à falta de produtos essenciais (GIUGLIANI, 1988).

Dentre os EIM conhecidos, os mais freqüentes são os de aminoácidos e de ácidos orgânicos. Novos casos vêm sendo reconhecidos a cada dia e as novas técnicas de diagnóstico têm descoberto outros grupos de EIM. As síndromes da deficiência de creatina são um deles e a deficiência de guanidino acetato metiltransferase (GAMT), que é o alvo desse estudo, é um EIM desse tipo.

1.1.1 Síndromes da Deficiência de Creatina

As síndromes da deficiência de creatina são um grupo de EIM descritos recentemente que têm como característica comum a deficiência de creatina nos tecidos dos pacientes afetados. Três disfunções genéticas foram descobertas como causadores dessas síndromes: a deficiência de

guanidinoacetato metiltransferase (GAMT), a deficiência de arginina:glicina amidinotransferase (AGAT) e a deficiência no transportador de creatina (CRTR). Os sintomas clínicos comuns às três doenças são o retardo mental e a epilepsia, sugerindo assim um envolvimento importante SNC na patologia dessas doenças (SCHULZE, 2003; STROMBERGER *et al*., 2003).

1.1.2 Deficiência de GAMT

A deficiência de GAMT foi o primeiro EIM detectado das síndromes da deficiência de creatina (VON FIGURA *et al*, 2001). Diferente das outras duas doenças, a deficiência de GAMT é caracterizada pelo acúmulo de guanidino acetato (GAA) no cérebro e outros tecidos dos pacientes, e pela deficiência de creatina nos tecidos dos pacientes afetados, sendo assim considerada a mais severa das três doenças. O acúmulo de GAA nos tecidos é patognomônico para o diagnóstico de deficiência de GAMT e este parece ser o responsável pelas convulsões intratáveis e pelos movimentos hipotônicos encontrados exclusivamente em pacientes com a deficiência de GAMT (SCHULZE *et al.,*2003).

A GAMT é uma enzima importante que catalisa o segundo passo da biossíntese de creatina, transformando o GAA em creatina (Figura 1), principalmente no fígado e no pâncreas. Os achados laboratoriais e da deficiência de GAMT são heterogêneos. Os valores das concentrações plasmática, urinária e liquóricas de GAA em pacientes normais e portadores da deficiência estão demonstrados na tabela 1.

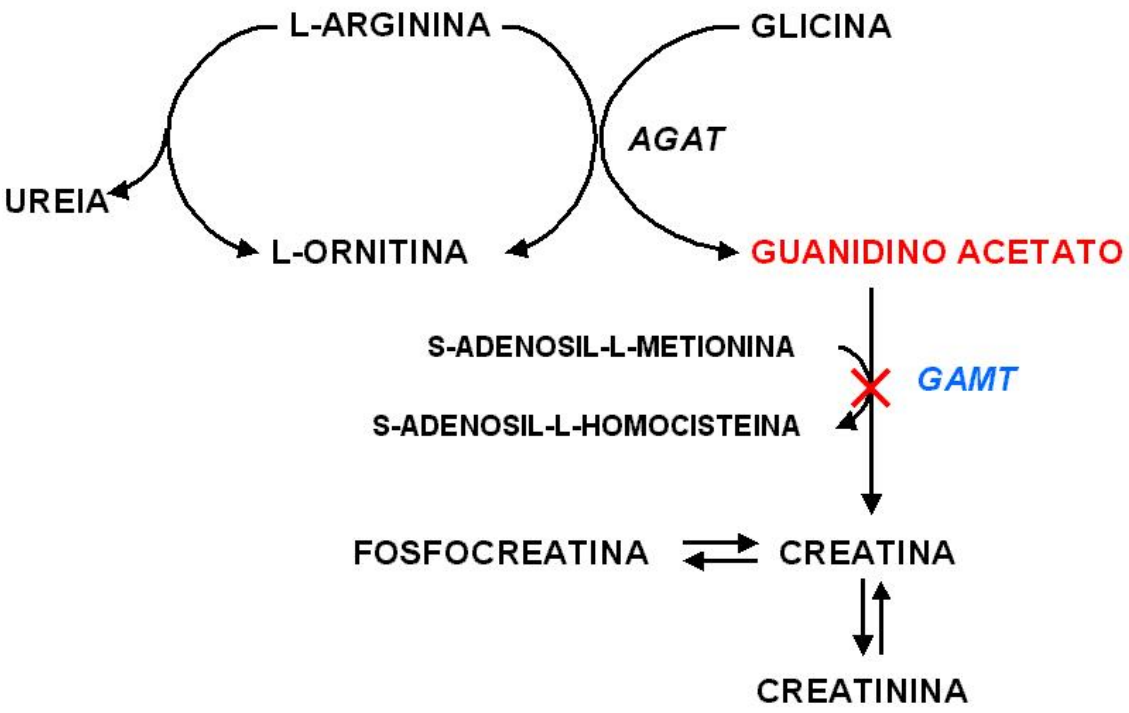

FIGURA 1. Metabolismo do guanidino acetato e deficiência da enzima guanidino acetato metiltransferase. (adaptado de ZUGNO *et al.*, 2003).

AGAT – arginina-glicina amidino transferase; GAMT – guanidino acetato metiltransferase

Tabela 1 – Concentrações de guanidino acetato no plasma, na urina e no líquor de indivíduos normais e afetados pela deficiência de GAMT (LEUZZI *et al.*, 2000; VON FIGURA *et al.* 2001; NEU *et al.*, 2002; VERHOEVEN *et al.*, 2005).

	Plasma (μM)	Urina (μM)	Líquor (μM)
Indivíduos Normais	0,83 – 1,1	65 – 430	0,05 – 0,08
Deficiência de GAMT	10 – 30	2.220 – 4.000	10,6 – 12,7

O primeiro caso relatado de deficiência de GAMT foi de um paciente que nasceu após uma gestação normal, com tamanho, peso e desenvolvimento neonatal normais. Nas cinco semanas seguintes ao nascimento, o paciente começou a apresentar hipotonia e, com o passar das semanas, o quadro evoluiu para um atraso de desenvolvimento global, incluindo retardo mental, movimentos irregulares dos olhos, hipotonia e fraqueza muscular (STÖCKLER *et al*., 1994). A combinação de altos níveis de GAA e a deficiência de creatina sugerem um bloqueio na síntese de creatina e na enzima que converte GAA em creatina. A herança genética é do tipo autossômica recessiva (VON FIGURA *et al*., 2001) e a alteração da GAMT ocorre devido a um defeito molecular presente na região telomérica do cromossomo 19 humano. Este gene codificador foi identificado como 19p 13.3, Quatro alelos já foram identificados até agora: 327G → A/309ins13/c.491insG/IVS5-3C → G (STÖCKLER *et al*., 1996; CARDUCCI *et al*., 2000).

O papel do GAA sobre as alterações do SNC é incerto e ainda não se sabe quais as verdadeiras causas da doença. No entanto, as manifestações neurológicas encontradas nos pacientes associadas ao acúmulo dessa substância no cérebro, sugerem uma ação neurotóxica (SCHULZE *et al.*, 2001). Além disso, a creatina também parece ter importância significativa, principalmente nos sintomas musculares (SCHULZE *et al.*, 1997). Muitos estudos estão sendo feitos na tentativa de elucidar os mecanismos envolvidos na disfunção cerebral da deficiência de GAMT (NEU *et al.*, 2002; SCHIMIDT *et al.*, 2004).

O potencial neurotóxico de muitos compostos guanidínicos foi estudado e sugere-se uma possível interação dos mesmos com receptores $GABA_A$ (DE

DEYN *et al.*, 1991; D'HOOGE *et al.*, 1999). Mais recentemente, Neu e colaboradores (2002) mostraram que o GAA também atua nesses receptores como agonista, sugerindo um possível mecanismo de indução de disfunção neuronal em pacientes com deficiência de GAMT. A estimulação prolongada desses receptores pode causar uma desestabilização dos mesmos e aumentar a excitabilidade neuronal (NEU *et al.*, 2002). Além disso, propriedades epileptogênicas do GAA foram recentemente demonstradas por Schulze e colaboradores (2001).

Acredita-se também que o estresse oxidativo possa estar envolvido na fisiopatologia desta doença. Dados mostram que os compostos guanidínicos induzem convulsões em ratos e coelhos e que são capazes de produzir radicais livres (MORI *et al.*, 1996; HIRAMATSU, 2003). Nesse contexto, vem sendo proposto que o GAA possa estar envolvido na neuropatologia da deficiência de GAMT (VON FIGURA *et al.*, 2001).

O diagnóstico é feito durante os primeiros meses de vida, de acordo com a suspeita clínica, observando-se os sinais e sintomas característicos da doença. A baixa concentração de creatina parece estar intimamente ligada a sintomas como fraqueza muscular e hipotonia, já que a suplementação de creatina como adjunto no tratamento, ameniza essas alterações, porém, o mesmo não ocorre na disfunção neurológica encontrada nesses pacientes (STÖCKLER *et al*., 1996).

A suplementação alimentar de creatina tem sido utilizada e corrige parcialmente a deficiência desse composto no cérebro, além de normalizar a excreção urinária de creatinina. Além disso, uma dieta restrita de arginina combinada com suplementação de ornitina vem sendo utilizada e parece

mostrar melhores resultados na diminuição nos níveis de GAA no plasma levando assim, a uma melhora nas crises de convulsão. Esse dado sugere que o acúmulo de GAA parece estar relacionado com os sintomas neurológicos da deficiência de GAMT (SCHULZE, 2003).

1.2 Na^+,K^+-ATPase

A Na^+,K^+-ATPase, enzima descoberta por Jean C. Skou (1957), também conhecida como bomba de sódio-potássio, é uma enzima que catalisa o transporte ativo de sódio e potássio através da membrana celular. Esse transporte é de grande importância já que mais de um terço do ATP consumido é utilizado para bombear tais íons (APERIA, 2007). A Na^+,K^+-ATPase está presente em praticamente todas as células, incluindo o cérebro e os músculos esquelético e cardíaco (APERIA, 2007).

A Na^+,K^+-ATPase é constituída de três subunidades, α, β e γ. Na subunidade α encontram-se os sítios de ligação de ATP, íons e ouabaína, e é responsável pelas propriedades catalíticas e de transporte da enzima (BLANCO e MERCER, 1998). A subunidade β está ligada à estrutura, bem como à afinidade dos íons pela enzima (CHOW e FORTE, 1995; BLANCO e MERCER, 1998). A subunidade γ, descrita mais recentemente, é uma proteína pequena com cerca de 12 kDa de massa molecular, associada às subunidades α e β. Embora essa subunidade não seja essencial para a função da enzima, ela parece ter uma ação regulatória na atividade da Na^+,K^+-ATPase (THERIEN e BLOSTEIN, 2000).

A Na^+,K^+-ATPase desempenha um papel fundamental no SNC, sendo responsável pela manutenção dos gradientes iônicos e pela propagação do impulso nervoso já que as funções neuronais, responsáveis por geração, transmissão e propagação do impulso nervoso dependem da atividade do neurônio e esta está intimamente ligada aos íons e seus potenciais de ação através da membrana (KAPLAN, 2002).

Considerando a importância da Na^+,K^+-ATPase para o funcionamento normal do organismo e do SNC, e seu papel fundamental na manutenção do gradiente elétrico da membrana celular, a inibição dessa enzima tem sido associada a fisiopatologia de diversas doenças, como a isquemia cerebral (WYSE *et al.*, 2000), epilepsia (GRISAR, 1984), crises convulsivas (RENKAWEK *et al.*, 1992) e doença de Alzheimer (HATTORI *et al.,* 1998).

Estudos realizados em nosso laboratório mostram que metabólitos acumulados em alguns EIM inibem a atividade da Na^+,K^+-ATPase em cérebro de ratos. Alguns aminoácidos, tais como fenilalanina (WYSE *et al.*, 1995; WYSE *et al*., 1999), prolina (PONTES *et al.*, 1999), arginina, (REIS *et al*., 2002a) homocisteína (STRECK *et al.*, 2002a; STRECK *et al.*, 2002b) e metionina (STEFANELLO *et al.,* 2005) inibiram a atividade dessa enzima em cérebro de ratos *in vitro* e *in vivo*. Além dos aminoácidos, alguns compostos guanidínicos, os quais estão acumulados na hiperargininemia, também inibem a atividade da Na^+,K^+-ATPase (SILVA *et al*., 1999). O GAA também foi capaz de inibir a atividade da Na^+,K^+-ATPase *in vitro* em estriado de ratos (ZUGNO *et al.*, 2003).

A Na^+,K^+-ATPase é sensível à ação de radicais livres (LEES, 1993). Estudos mostram que essa enzima é inibida por radicais livres em modelos

animais *in vitro* e *in vivo* de EIM tais como hiperprolinemia e hiperhomocisteinemia, (WYSE *et al.,* 2002; FRANZON *et al.*, 2003; STRECK *et al.*, 2003; MATTE *et al.,* 2004). Corroborando com esses resultados, dados da literatura demonstram que a inibição da Na^+,K^+-ATPase é prevenida pela adição de antioxidantes tais como L-cisteína, glutationa, α-tocoferol e L-NAME (KURELLA *et al.*, 1999; TSAKIRIS *et al.*, 2000, ZUGNO *et al.*, 2004).

1.3 Creatina Quinase

A creatina quinase (CK) consiste de um grupo de isoenzimas com um papel central no metabolismo energético em tecidos com alta demanda energética, tais como cérebro, músculos cardíaco e esquelético. A CK é uma enzima citoplasmática e mitocondrial responsável pela fosforilação reversível da creatina com formação de ATP mantendo assim, os níveis dos substratos fosforilados. (BESSMAN e CARPENTER, 1985; SCHNYDER *et al.*, 1991; WALLIMANN *et al.*, 1992).

As isoformas da CK estão presentes em locais estratégicos de demanda e produção energética. A isoforma citosólica consiste de três dímeros específicos: cerebral (CK-BB), muscular esquelética (CK-MM) e um heterodímero muscular cardíaca (CK-MB) (SCHNYDER *et al.*, 1991; WALLIMANN *et al.*, 1992; HAMMAN *et al.*, 1995; O'GORMAN *et al.*, 1997). As formas mitocondriais da CK são dispostas em octâmeros e são compostas da isoforma sarcomérica músculo-específica Mib-CK e da forma ubíqua Mia-CK, que as quais encontrada nas mitocôndrias do tecido cerebral (SAKS *et al.*,

1985; SCHELEGEL *et al.*, 1988; WALLIMANN *et al.*, 1992; GROSS *et al.*, 1996).

Diminuição na atividade da CK vem sendo associada com perda neuronal seguida de isquemia e com doenças neurodegenerativas (TOMIMOTO *et al.*, 1993; DAVID *et al.*, 1998; AKSENOV *et al.*, 2000).

Dados obtidos em nosso grupo mostram que metabólitos acumulados em EIM tais como o ácido etilmalônico são capazes de inibir a atividade da CK tanto *in vivo* quanto *in vivo* (SCHUCK *et al.*, 2002; LEIPNITZ *et al.*, 2003).

1.4. Metabolismo Energético

O cérebro possui uma alta atividade metabólica e dessa forma, necessita de uma quantidade considerável de mitocôndrias. As mitocôndrias são organelas intracelulares, responsáveis essencialmente pela produção de ATP no metabolismo aeróbico, além disso, desempenham outras funções intracelulares tendo um papel crítico no processo de apoptose e servindo como um tampão de cálcio (ORTH e SCHAPIRA, 2001).

A cadeia respiratória é formada por uma série de complexos protéicos, onde ocorre a transferência de elétrons doados por NADH e $FADH_2$ Essa transferência leva ao bombeamento de prótons da matriz para o lado citosólico da membrana mitocondrial interna produzindo assim, o gradiente de prótons, que é usado para impulsionar a síntese de ATP (ORTH e SCHAPIRA, 2001). A cadeia respiratória é composta de quatro complexos protéicos (I, II, III e IV): NADH desidrogenase (complexo I), sucinato: ubiquininona oxirredutase (complexo II), complexo citocromo b-c_1 (complexo III) e citocromo c oxidase

(complexo IV), além de elementos móveis que se localizam entre os complexos e por fim, o complexo ATP sintase (WALLACE, 1999).

Deficiências no funcionamento normal da cadeia respiratória mitocondrial levam à diminuição da síntese de ATP (HEALES *et al.*, 1999). Essa diminuição está amplamente associada a diversos comprometimentos cerebrais tais como morte celular (ANKARCRONA *et al.*, 1995), diminuição da síntese de neurotransmissores (acetilcolina, glutamato, aspartato e GABA) e lipídios (DI DONATO, 2000) e ainda é capaz de diminuir a captação de glutamato pelos astrócitos e pelos neurônios, causando acúmulo excessivo de glutamato na fenda sináptica, que pode levar excitotoxicidade glutamatérgica (LUDOLPH *et al.*, 1993, DANBOLT, 2001).

A diminuição do metabolismo energético cerebral está associada a inúmeras doenças neurodegenerativas. Nesse contexto, Maurer e colaboradores (2000) descrevem uma redução do complexo IV da cadeia respiratória em pacientes com a doença de Alzheimer. Também observada uma diminuição na atividade dos complexos I, III e IV da cadeia respiratória em mitocôndrias em pacientes com esclerose lateral amiotrófica (SWERDLOW *et al*. 1997). Dados pré-clínicos mostram que ratos *knockout* para a deficiência de GAMT apresentam um aumento na atividade do complexo ATP sintase, sugerindo um mecanismo compensatório para o decréscimo de energia (SCHIMIDT, *et al.,* 2004).

1.5. Estresse Oxidativo

Radicais livres são definidos como espécies químicas que contenham um ou mais elétrons desemparelhados. Essa definição abrange vários tipos de espécies, entre elas, as mais importantes para os processos biológicos são as espécies reativas de oxigênio (ERO). Esses radicais são normais no organismo e exercem funções importantes, tais como fagocitose, sinalização celular e regulação de proteínas (HALLIWELL e GUTTERIDGE, 2006). No entanto, quando formados em excesso, os radicais livres geram o estresse oxidativo, ou seja, podem provocar reações em cadeia e causar alteração a um grande número de moléculas. As ERO são muito reativas e podem provocar danos em moléculas, como lipídios, proteínas e DNA (HALLIWELL, 2006). As mais importantes são o ânion superóxido ($O_2^{\bullet -}$), radical hidroxila ($OH^{\bullet}$), peróxido de hidrogênio (H_2O_2), ânion hipoclorito (OCl^-) o oxigênio *singlet* ($^1O_2$), o óxido nítrico ($NO^{\bullet}$) e o peroxinitrito ($ONOO^-$) (HALLIWELL, 2006).

Todas as células aeróbicas sofrem algum tipo de dano oxidativo, sendo que o cérebro é um tecido especialmente sensível, além disso, esse órgão apresenta altos níveis de ferro, que pode catalisar reações de formação de radicais livres (HALLIWELL, 2001). As defesas antioxidantes são meios importantes que mantém os níveis de oxigênio no cérebro o mais reduzido possível a fim de diminuir o dano oxidativo (HALLIWELL, 2006).

As principais defesas enzimáticas antioxidantes são a superóxido dismutase (SOD), catalase (CAT) e a glutationa peroxidase (GPx). Além dessas, existem defesas antioxidantes não-enzimáticos, como a glutationa

reduzida (GSH), o tocoferol (vitamina E) e o ácido ascórbico (vitamina C) e a taurina (FANG *et al.*, 2002; HALLIWELL e GUTTERIDGE, 2006).

O estresse oxidativo é um importante evento que vem sendo relacionado a diversas neuropatologias, tais como epilepsia, esclerose mútilpla e demência (HALLIWELL, 2001, WAJNER, *et al.,* 2004, MOREIRA, *et al.,* 2005)

Estudos realizados em nosso laboratório mostram que o estresse oxidativo também está aumentado em modelos experimentais químicos de EIM, como hiperprolinemia tipo II (DELWING *et al.*, 2003), hiperargininemia (WYSE *et al*., 2001), hiperhomocisteinemia (STRECK *et al.*, 2003) e hipermetioninemia (STEFANELLO *et al.*, 2005). Além disso, dados mostram que o GAA é um agente formador de radicais livres (MORI et al., 1996; HIRAMATSU, 2003).

1.6. Glutamato

O glutamato, neurotransmissor excitatório mais importante do SNC, tem um papel fundamental no desenvolvimento, plasticidade cerebral, aprendizado e memória, além de outras inúmeras funções em todo o organismo (DANBOLT, 2001; SEGOVIA *et al.*, 2001). O glutamato é armazenado em vesículas e liberado pelo neurônio pré-sináptico. Na fenda sináptica pode seguir por caminhos distintos: ligar-se aos receptores no neurônio pós-sináptico ou pode ser recaptado pela glia através de transportadores sódio-dependente específicos localizados na membrana dessas células. Esse glutamato recaptado é convertido a glutamina e volta novamente para o neurônio pré-

sináptico onde é reconvertido a glutamato e armazenado em vesículas para ser novamente liberado na fenda sináptica (DANBOLT, 2001).

O cérebro contém grande quantidade de glutamato, mas somente uma pequena fração é normalmente encontrada no meio extracelular. O glutamato em altas concentrações extracelulares é uma potente neurotoxina. A liberação e principalmente a recaptação de glutamato pelas células gliais deve ser finamente regulada evitando a excitotoxicidade glutamatérgica na fenda sináptica. Sua toxicidade tem sido relacionada com morte celular e neurodegeneração observada em pacientes com epilepsia, doença de Huntigton e Alzheimer (FRIEDLER, 2003; MARAGAKIS e ROTHSTEIN, 2004).

Estudos indicam que alterações nos transportadores glutamatérgicos em humanos e em ratos levam a estados epiléticos (TANAKA *et al.,* 1997; MARAGAKIS e ROTHSTEIN, 2004). Além disso, já foi descrito que uma concentração menor que 1 μM de glutamato já é suficiente para matar 50% de neurônios em cultura de córtex cerebral (FRANDSEN e SCHOUSBOE, 1990).

1.7. Vitaminas E e C

Estudos na literatura indicam que o estresse oxidativo afeta a função normal do organismo, agindo como co-fator importante em doenças neurodegenerativas e aterosclerose (CARR *et al.*, 2000; HALLIWELL, 2006). Uma dieta rica em antioxidantes vem sendo estudada como uma possível prevenção dos danos causados por ERO (HUANG et al., 2002). A vitamina E (α-tocoferol) é um dos mais importantes antioxidantes lipofílicos em humanos, sendo essencial para o funcionamento normal do cérebro (VATASERRY, *et al.,*

1998). A deficiência de α-tocoferol pode ocorrer em síndromes de má absorção de lipídios e tem como conseqüência danos neurológicos graves (SOKOL, 1989). A vitamina C é descrita como a vitamina solúvel mais importante. Dessa forma, as vitaminas E e C compõem os maiores seqüestradores de radicais livres e conseqüentemente, protetores de lipoperoxidação (AMES *et al.*, 1993; ZAIDI e BANU, 2004). A oxidação do α-tocoferol gera o radical tocoferil, regenerado pela vitamina C, justificando a importância da utilização concomitante dessas duas vitaminas (CARR e FREI,1999).

Estudos na literatura mostram que a suplementação de vitamina C é capaz de diminuir o dano oxidativo causado ao DNA em células mononucleares sangüíneas de fumantes (MOLLER *et al.*, 2004). O potencial antioxidante dessa substância é descrito em vários estudos (HUANG *et al*., 2002; ZAIDI e BANU, 2004) e, além disso, dados mostram um potencial antiaterogênico dessas vitaminas (CARR *et al.*, 2000).

A vitamina E tem sido usada como terapia adjuvante em pacientes com doença de Alzheimer, mostrando uma melhora cognitiva desses pacientes (GRUNDMAN, 2000). Estudos em modelos animais em nosso laboratório mostram que o tratamento de uma semana com as vitaminas E e C é capaz de prevenir o prejuízo de memória (REIS *et al.*, 2002b; MONTEIRO *et al.*,2005), bem como o efeito inibitório da administração de homocisteína e prolina sobre a atividade da Na^+,K^+-ATPase (WYSE *et al*., 2002; FRANZON *et al*., 2003),

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1.8. Taurina

A taurina, ou ácido 2-aminoetanosulfônico, é um dos aminoácidos livres mais abundantes do SNC. Em algumas fases do desenvolvimento, pode chegar a uma concentração maior que do glutamato, sugerindo assim, um importante papel no amadurecimento de células neurais (OJA e SARANSAARI, 2000).

A taurina vem sendo descrita como um potente agente de proteção neurotóxica participando de funções como regulação do volume celular, manutenção da integridade da membrana neuronal e controle da homeostasia do cálcio (FOOS e WU, 2002; SERGEEVA *et al.,* 2003).

Dados na literatura mostram que a taurina foi capaz de evitar a apoptose e diminuir danos por estresse oxidativo na isquemia/reperfusão do miocárdio (ORIYANHAN, *et al.,* 2005). Além disso, a taurina parece estimular a indução de formação de potencial de longa duração (LTP), processo importante para a formação de memória (OLMO, *et al.*, 2003) e atuar como estabilizador de membrana prevenindo lipoperoxidação (QI, *et al.*, 1995). Estudos em nosso grupo mostram que a taurina previne a inibição da Na^+, K^+-ATPase causada pelo GAA em hipocampo de ratos (ZUGNO *et al*., 2004).

Um dos papéis mais importantes da taurina vem sendo relacionado a excitoxicidade glutamatérgica. A taurina atua como um neurotransmissor inibitório e é liberada na fenda sináptica após a hiperestimulação de receptores glutamatérgicos, diminuindo os efeitos excitatórios do glutamato e agindo assim como protetor contra a excitotoxicidade (OJA e SARANSAARI, 2000). Diversos

estudos mostram a interação da taurina com receptores glutamatérgicos no SNC (OJA e SANSAARI, 2000; ZIELINSKA *et al.,* 2002; HILGIER, *et al.*, 2003)

1.9. Acetilcolinesterase (AChE)

A acetilcolina (ACh) é um neurotransmissor excitatório que atua na junção neuromuscular, é armazenada em vesículas no neurônio pré-sináptico e, com a despolarização, a vesícula migra e libera a ACh para a fenda sináptica, onde liga-se a receptores muscarínicos ou nicotínicos localizados na membrana pré e pós-sináptica. Na fenda ainda situa-se a acetilcolinesterase (AChE), enzima que hidrolisa a ACh em colina e acetato, permitindo a transmissão normal regulando a concentração do neurotransmissor na fenda sináptica (SOREQ e SEIDMAN, 2001).

A AChE, enzima mais importante das colinesterases, é uma serina hidrolase que catalisa a hidrólise da ACh inativando-a e regulando a concentração do neurotransmissor na fenda sináptica. Essa enzima contém uma unidade catalítica conhecida por tríade, composta por serina, histidina e glutamato, que se localiza no interior da enzima (SOREQ e SEIDMAN, 2001). A AChE apresenta duas formas moleculares: forma assimétrica, localizada na junção neuromuscular e a forma globular, existente como monômeros, dímeros ou tetrâmeros catalíticos e localizam-se no SNC (TALESA, 2001). O papel da AChE está sendo relacionado com doenças neurodegenerativas (CUMMINGS, 2000; LAW *et al.*, 2001). Além disso, dados apontam uma relação entre AChE e memória (DAS, *et al*., 2005). Já foi mostrado que a diminuição da transmissão colinérgica contribui para o déficit cognitivo e distúrbios de comportamento em

pacientes com a doença de Alzheimer. Além disso, dados mostram um envolvimento do estresse oxidativo no aumento da atividade da AChE em cultura de células de pacientes com Alzheimer (MELO *et al*., 2002). Estudos na literatura mostram um papel neuroprotetor da ACh, sugerindo que a diminuição desse neurotransmissor no SNC esteja relacionada ao aumento da neurodegeneração observada em pacientes com a doença de Alzheimer (PILLAY *et al.*, 2003;).

Sendo assim, inibidores da AChE representam um tratamento promissor em doenças com comprometimento cognitivo, tal como a doença de Alzheimer (GIACOBINI, 2004; BORLONGAN *et al.*, 2005).

1.10. Memória

O aprendizado pode ser definido como o processo por meio do qual os seres adquirem o conhecimento e a memória nada mais é do que o processo pelo qual esse conhecimento é armazenado. Assim sendo, os processos de aprendizagem e memória são propriedades fundamentais do SNC (IZQUIERDO, 2002). A memória é um processo dinâmico e pode ser dividida em três etapas: aquisição (obtida através de experiências), consolidação (quando a informação obtida na aquisição é processada) e evocação (que é a recordação em si) (IZQUIERDO, 1989).

Os mecanismos diversos tais como o de aprendizagem e memória, tanto de longa como de curta duração, bem como os mecanismos de extinção de memória e os fenômenos de plasticidade sináptica, característicos do SNC,

estão envolvidos nas alterações fisiológicas, morfológicas e bioquímicas verificadas no SNC (IZQUIERDO e MEDINA, 1997; IZQUIERDO, 2002).

O mecanismo de formação de memória ainda não é totalmente compreendido, mas sabe-se que ocorre a ativação do sistema glutamatérgico seguida de mudanças em segundos mensageiros com ativação de proteínas quinases (IZQUIERDO, 1993).

Diversos métodos são utilizados em laboratórios a fim de avaliar a memória. Assim sendo, vários parâmetros podem ser avaliados, tais como memória aversiva, memória espacial, memória de trabalho, dentre outras (IZQUIERDO e MEDINA, 1997). O teste de esquiva inibitória é um tipo de avaliação de memória aversiva em modelos animais que envolve uma resposta do animal a determinado estímulo, no caso da esquiva inibitória, um choque nas patas ao descer de uma plataforma. Com essa técnica é possível avaliar a memória de aquisição, consolidação e evocação adquiridas (IZQUIERDO e MEDINA, 1997).

Os pacientes com a deficiência de GAMT apresentam um grau variado de atraso cognitivo além de outras disfunções neurológicas (VON FIGURA *et al.,* 2001). Trabalhos realizados em nosso laboratório mostraram uma diminuição de memória na tarefa de esquiva inibitória em alguns EIM como homocistinúria (REIS *et al,* 2002b), hiperargininemia (REIS *et al.,* 2002a) e na doença do xarope do bordo (VELASQUES, *et al.*, 2005). Recentemente foi descrito que ratos *knockout* para a deficiência de GAMT apresentam um déficit cognitivo moderado (TORREMANS *et al.,* 2005).

2. Objetivos

2.1 Objetivo Geral

Com a finalidade de compreender melhor os mecanismos da deficiência de GAMT, e considerando os danos neurológicos encontrados na doença, bem como a importância do estriado devido às alterações nos gânglios da base, observado nos pacientes com essa deficiência, o objetivo desse trabalho foi investigar o efeito do GAA sobre alguns parâmetros bioquímicos e comportamentais em estriado de ratos adultos e verificar uma possível prevenção com vitaminas e taurina.

2.2. Objetivos Específicos

O presente estudo foi dividido em capítulos de acordo com os artigos desenvolvidos.

Capítulo I

Objetivos específicos

1. Investigar o efeito da administração intraestriatal de GAA sobre a atividade da Na^+,K^+-ATPase.
2. Investigar o efeito da administração intraestriatal de GAA sobre a atividade da CK.
3. Investigar o mesmo efeito nas substâncias reativas ao ácido tiobarbitúrico-TBARS (uma medida de lipoperoxidação) em estriado de ratos.

Capítulo II

Objetivos Específicos

1. Investigar o efeito *in vitro* do GAA sobre alguns parâmetros do metabolismo energético, tais como a produção de CO_2, a atividade dos complexos II, II-III, III e IV da cadeia respiratória em estriado de ratos.
2. Investigar o efeito da administração intra-estriatal do GAA sobre os mesmos parâmetros do metabolismo energético avaliados *in vitro* em estriado de ratos.
3. Estudar o efeito de substâncias antioxidantes e com conhecido papel neuroprotetor, tais como as vitaminas E e C e taurina sobre e inibição causada pelo GAA sobre as atividades dos complexos da cadeia respiratória e de enzimas importantes para o funcionamento normal do SNC como Na^+,K^+-ATPase e CK.

Capítulo III

Objetivos Específicos

1. Investigar o efeito *in vitro* do GAA, sobre alguns parâmetros não enzimáticos de estresse oxidativo, tais como TRAP e estudar também as defesas enzimáticas antioxidantes, tais como a catalase, glutationa peroxidase (GPx) e superóxido dismutase (SOD). Estudar ainda os marcadores de oxidação de proteínas como quantidades de grupos carbonil e sufidril formados em estriados de ratos.
2. Investigar o efeito da administração intraestriatal de GAA sobre os mesmos parâmetros de estresse oxidativo avaliados *in vitro* em estriados de ratos.

Capítulo IV

Objetivos Específicos

1. Verificar o efeito *in vitro* de GAA sobre a captação de glutamato em fatias de estriado de ratos jovens e adultos.
2. Verificar o efeito da administração intraestriatal de GAA sobre a captação de glutamato em fatias de estriado de ratos adultos.

Capítulo V

Objetivos Específicos

1. Estudar o efeito da administração intraestriatal de GAA sobre a atividade da AChE em estriado de ratos adultos.
2. Avaliar memórias de aquisição, consolidação e evocação na tarefa aversiva de esquiva inibitória em ratos após a administração intra-estriatal de GAA em estriado de ratos.

3. Materiais e Métodos

Ratos Wistar de 60 dias de idade foram utilizados obtidos do biotério do departamento de bioquímica da Universidade Federal do rio Grande do Sul. Os animais são mantidos em ciclos de claro/escuro alternados de 12 em 12 horas com água e alimentação à vontade.

Para a avaliação dos parâmetros bioquímicos e comportamentais deste trabalho, a cirurgia intraestriatal foi feita de acordo com o método de Folbergová e colaboradores (2001). Os animais foram anestesiados com cetamina e xilazina (100 mg/Kg e 14 mL/Kg, respectivamente). A cabeça dos animais foi fixada no aparelho de cirurgia esteriotáxica, e preparada para a introdução da cânula de 9 mm posicionada na parte superior do estriado, de acordo com as coordenadas (AP: -0,5 mm; L: -2,5 mm; DV: -2,5 mm). Quarenta e oito horas após a cirurgia, uma microseringa Hamilton foi utilizada para a administração das soluções de GAA ou NaCl 0,9%. O volume administrado intra-estriatalmente foi de 2 µL. Os animais foram divididos em 2 grupos: grupo controle: ratos que sofreram cirurgia e foram tratados com solução de NaCl 0,9% e grupo GAA: ratos que sofreram cirurgia e foram tratados com solução de GAA (0,02 nmol/estriado). Os animais foram decapitados sem anestesia 30 minutos ou 3 horas após a administração de GAA.

As técnicas específicas de cada trabalho foram descritas nos artigos apresentados.

4. Resultados

Artigo 1

Intrastriatal administration of guanidinoacetate inhibits Na^+, K^+-ATPase and creatine kinase activities in rat striatum.

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ORIGINAL PAPER

Intrastriatal Administration of Guanidinoacetate Inhibits Na^+, K^+-ATPase and Creatine Kinase Activities in Rat Striatum

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Abstract Guanidinoacetate methyltransferase deficiency (GAMT deficiency) is an inherited neurometabolic disorder clinically characterized by epilepsy and mental retardation and biochemically by accumulation of guanidinoacetate (GAA) and depletion of creatine. Although this disease is predominantly characterized by severe neurological findings, the underlying mechanisms of brain injury are not yet established. In the present study, we investigated the effect of intrastriatal administration of GAA on Na^+, K^+-ATPase activity, total (tCK), cytosolic (Cy-CK), and mitochondrial (Mi-CK) creatine kinase (CK) activities in rat striatum. We verified that Na^+, K^+-ATPase, tCK, and Mi-CK activities were significantly inhibited by GAA, in contrast to Cy-CK which was not affected by this guanidino compound. Since these enzyme activities can be affected by reactive species, we also investigated the effect of intrastriatal administration of GAA on thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation in rats. We found that this metabolite significantly increased this oxidative stress parameter. Considering the importance of Na^+, K^+-ATPase and CK activities for brain metabolism homeostasis, our results suggest that the inhibition of these enzymes by increased intracerebral levels of GAA may contribute to the neuropathology observed in patients with GAMT-deficiency.

Keywords Guanidinoacetate · Na^+, K^+-ATPase · Creatine kinase · Intrastriatal administration

Introduction

Guanidinoacetate methyltransferase deficiency (GAMT deficiency) is an inborn error of creatine biosynthesis characterized by severe neurological symptoms including mental retardation, speech impairment, involuntary extrapyramidal movements, and epilepsy. Affected

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patients also present other important symptoms such as muscular hypotonia. The disease is biochemically characterized by accumulation of guanidinoacetate (GAA) and deficiency of creatine/phosphocreatine in blood fluids and brain (Von Figura *et al*., 2001).

Although neurological dysfunction is commonly found in human GAMT deficiency and has been mainly attributed to a decreased intracellular creatine or/and increased GAA levels (Leuzzi *et al*., 2000; Schulze *et al*., 2001), the exact pathomechanisms of brain dysfunction, particularly epilepsy and mental retardation are poorly understood in this disease. However, Schulze *et al*. (2001) have suggested that GAA may be epileptogenic in man. In this scenario, it has been demonstrated that GAA may alter neurotransmission (Shiraga *et al*., 1986; Neu *et al*., 2002) and decrease membrane fluidity (Hiramatsu *et al*., 1992).

Na^+, K^+-ATPase (EC 3.6.1.37) is a membrane enzyme responsible for the generation of the membrane potential through the active transport of sodium and potassium ions in the central nervous system (CNS) necessary to maintain neuronal excitability. It is present at high concentrations in brain, consuming about 40–50% of the ATP generated in this tissue (Erecinska and Silver, 1994). Na^+, K^+-ATPase is decreased in cerebral ischemia (Wyse *et al*., 2000), in epilepsy (Grisar, 1984), and in various neurodegenerative disorders (Lees, 1993). It has been shown that some guanidino compounds, such as methylguanidine, argininic acid, N-acetylarginine, and homoarginine significantly inhibit Na^+, K^+-ATPase activity in rat brain (Matsumoto *et al*., 1976; Silva *et al*., 1999). We have recently demonstrated that GAA inhibits *in vitro* Na^+, K^+-ATPase activity in rat striatum (Zugno *et al*., 2003).

Creatine Kinase (CK) comprises a group of isoenzymes with a central role in energy metabolism especially for high-energy consuming tissues such as brain, skeletal muscle, and heart (Bessman and Carpenter, 1985). A functional interplay between mitochondrial and cytosolic isoforms of CK is thought to be important for regulation of cellular energy homeostasis (Wallimann *et al*., 1992). CK isoforms are located at places of energy demand and production. The cytosolic CK isoforms (Cy-CK) consist of dimers that are expressed in a tissue-specific manner, that is, brain-specific (BB-CK), skeletal muscle-specific (MM-CK), and cardiac muscle-specific (MB-CK) isoenzymes (Wallimann *et al*., 1992; Hamman *et al*., 1995; O'Gorman *et al*., 1996). The mitochondrial forms of CK (Mi-CK) are thought to be octamers *in vivo* and consist of the muscle-specific sarcomeric isoform Mib-CK and Mia-CK, the ubiquitous isoform, which is mainly found in brain tissue mitochondria (Wallimann *et al*., 1992; Saks *et al*., 1985; Schegel *et al*., 1988; Gross *et al*., 1996). It has been shown that CK activity is one of the markers of cell damage in diseases affecting CNS such as Azheimer's disease and ischemia (Tomimoto *et al*., 1993; Aksenov *et al*., 2000). Besides, there is no data showing the effect of GAA on CK activity in the literature.

In the present study, we investigated the *in vivo* effect of intrastriatal administration of GAA on Na^+, K^+-ATPase and CK activities, two important enzymes for neurotransmission and energy metabolism respectively, in rat striatum. We also tested the effect of GAA administration on thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation, in the same cerebral structure. Striatum was used because have been shown basal ganglia abnormalities in patients with GAMT deficiency (Von Figura *et al*., 2001).

Material and Methods

Animals and reagents

Wistar rats weighing 180–200 g obtained from the Central Animal House of Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil, were used

in the experiments. Animals were maintained on a 12/12 h light/dark cycle (lights on from 7 A.M. to 7 P.M.) in an air-conditioned constant temperature (22°C) colony room, and had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Society for Experimental Biology and was approved by the Ethics Committee of the Federal Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical, St. Louis, MO, USA.

Surgery and intrastriatal administration

Surgery and intrastriatal infusion were performed according to Folbergovà *et al*. (2001). Animals were anesthetized with a mixture i.p. injection of ketamine and xylazine (100 mg/kg and 14 mg/kg, respectively). The head of the animals were fixed in a stereotaxic apparatus, the skin of the skull was removed and a 27-gauge 9-mm guide cannula was then placed above the striatum (AP: − 0.5 mm; L: − 2.5 mm; DV: − 2.5 mm). The cannula was fixed with acrylic cement. Experiments were performed 48 h after surgery. A 30-gauge cannula was fitted into the guide cannula and connected by polyethylene tube to a 5 μL Hamilton microsyringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula towards the striatum. The animals were divided into three groups: Group 1 (naive group), rats that did not suffer surgery; group 2 (sham group), rats that suffered surgery and received saline; and group 3 (GAA-treated), rats that received 10 μM of GAA solution (0.02 nmol/striatum). The volume administered intrastriatally (saline or GAA solution) was 2 μL. The animals were killed by decapitation without anesthesia at 30 min or 3 h after inserction.

Tissue preparation

The brain was quickly removed and the striatum was dissected. For preparation of synaptic plasma membrane and determination of Na^+, K^+-ATPase activity, striatum was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4 and for the TBARS assay, striatum was homogenized 10 volumes of 1.15% KCl.

For CK assays whole striatum homogenates were used to measure total CK activity and was homogenized in 10 volumes of saline solution with pH adjusted to 7.5. For mitochondrial and cytosolic fractions, they were prepared according to Ramirez and Jiménez (2000). Striatum homogenates obtained from other animals were centrifuged at 800 × *g* for 10 min at 4°C and the pellet discarded. The supernatant was then centrifuged at 27,000 × *g* for 30 min at 4°C in a Sorval DC-2B centrifuge. The pellet containing the mitochondria was washed three times with saline solution and used as the mitochondrial fraction for the enzymatic assay (Mi-CK). The supernatant was further centrifuged at 125,000 × *g* for 60 min at 4°C in a OTD-65B Sorvall centrifuge, the microsomal pellet discarded, and the cytosol (supernatant) was used for the enzymatic assay (Cy-CK). The cytosolic fraction did not contain contaminating Mi-CK. Cytosolic and mitochondrial fractions were prepared on the day of the experiment and immediately used for the determination of Cy-CK and Mi-CK activities.

Preparation of synaptic plasma membrane from striatum

Synaptic plasma membranes were prepared according to the method of Jones and Matus (1974), with some modifications (Wyse *et al*., 1995). The homogenate was centrifuged at 1000 × *g* for 20 min and the supernatant removed and centrifuged at 12,000 × *g* for a further 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris–HCl buffer, pH 8.1), incubated at 0°C for 30 min, and applied on a discontinuous sucrose density gradient

consisting of successive layers of 0.3, 0.8, and 1.0 M. After centrifugation at 69,000 × *g* for 2 h, the fraction at the 0.8–1.0 M sucrose interface was taken as the membrane enzyme preparation.

Na^+, K^+-ATPase activity assay

The reaction mixture for Na^+, K^+-ATPase assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 μL. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays according to the method of Wyse *et al*. (2000). Released inorganic phosphate (Pi) was measured by the method of Chan *et al*. (1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein.

Determination of creatine kinase activities

The reaction mixture for CK assay contained the following final concentrations: 60 mM Tris–HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$, and approximately 0.4–1.2 μg protein in a final volume of 0.1 mL. For enzymatic analysis in mitochondrial fractions, 0.625 mM lauryl maltoside was added to the medium. After 5 min of pre-incubation at 37°C, the reaction was started by the addition of 3.2 mM ADP plus 0.8 mM reduced glutathione. After 10 min of incubation at 37°C, the reaction was stopped by the addition of 1 μmol *p*-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls containing all components of the incubation medium except homogenates, were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL 2% α-naphthol and 0.1 mL 0.05% diacetyl in a final volume of 1 mL and read at 540 nm after 20 min. Results were expressed as μmol of creatine formed per min per mg protein.

TBARS assay

TBARS is a measurement of lipid peroxidation and were determined according to Ohkawa (1979). Briefly, homogenates in 1.15% KCl were mixed with 20% trichloroacetic acid and 0.8% thiobarbituric acid and heated in a boiling water bath for 60 min. TBARS were determined by the absorbance at 535 nm. The results were reported as nmol of malonaldehyde per mg protein.

Protein determination

Protein was measured by the method of Bradford (1976) or Lowry (1951) using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by the Student's *t*-test or by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the *F*-test was significant. Pearson linear regression coupled to ANOVA was also used to verify dose-dependent effects. All

analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $p < 0.05$ was considered to be significant.

Results

Figure 1 shows the effect of intrastriatal injection of GAA on Na^+, K^+-ATPase activity in synaptic plasma membranes of rat striatum decapitated at 30 min and 3 h after administration. As can be seen, GAA significantly inhibited (40%) Na^+, K^+-ATPase activity at 30 min after injection when compared to naive and sham groups [$F(5, 18) = 7{,}550$; $p < 0.01$]. However, at 3 h after injection, GAA did not alter this enzymatic activity [$F(5, 18) = 0.652$; $p > 0.05$].

We also examined the effect of intrastriatal injection of GAA on CK activity in striatum of rats at 30 min and 3 h after administration. As can be observed in Fig. 2, GAA significantly inhibited CK (30%) in total homogenates after 30 min of GAA administration [$F(2, 17) = 4.352$; $p < 0.05$] when compared to naive and sham groups. In contrast, GAA did not alter CK activity at 3 h after the injection (naive $= 8.77 \pm 0.97$; sham $= 9.43 \pm 0.55$; GAA $= 8.37 \pm 1.08$ μmol creatine min^{-1} mg^{-1} protein). Then, we investigated the effect of intrastriatal administration of GAA on CK activity in the Mi-CK

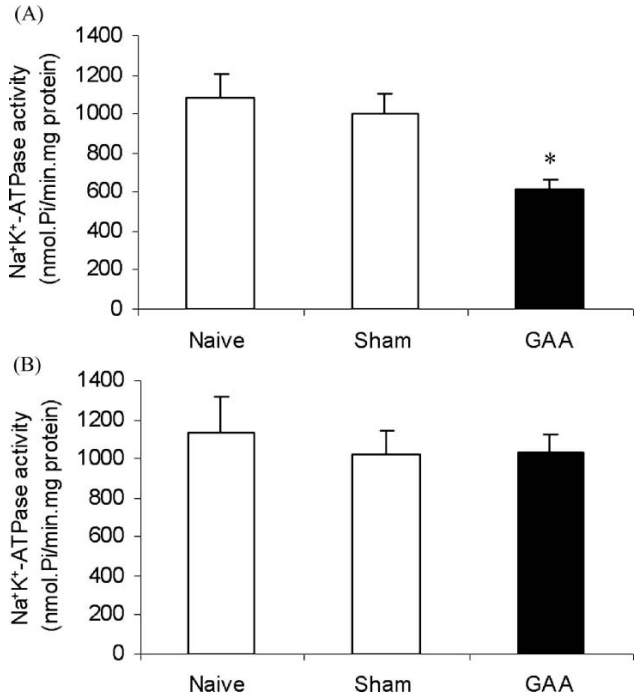

Fig. 1 Effect of intrastriatal administration of GAA on Na^+, K^+-ATPase activity in synaptic plasma membranes from rat striatum. Results shows the effect at 30 min (A) and 3 h (B) after GAA administration. Data are expressed as mean ± SD for four independent experiments (animals) performed in duplicate. $^*p < 0.05$ compared to naive and sham groups (Duncan's multiple range test)

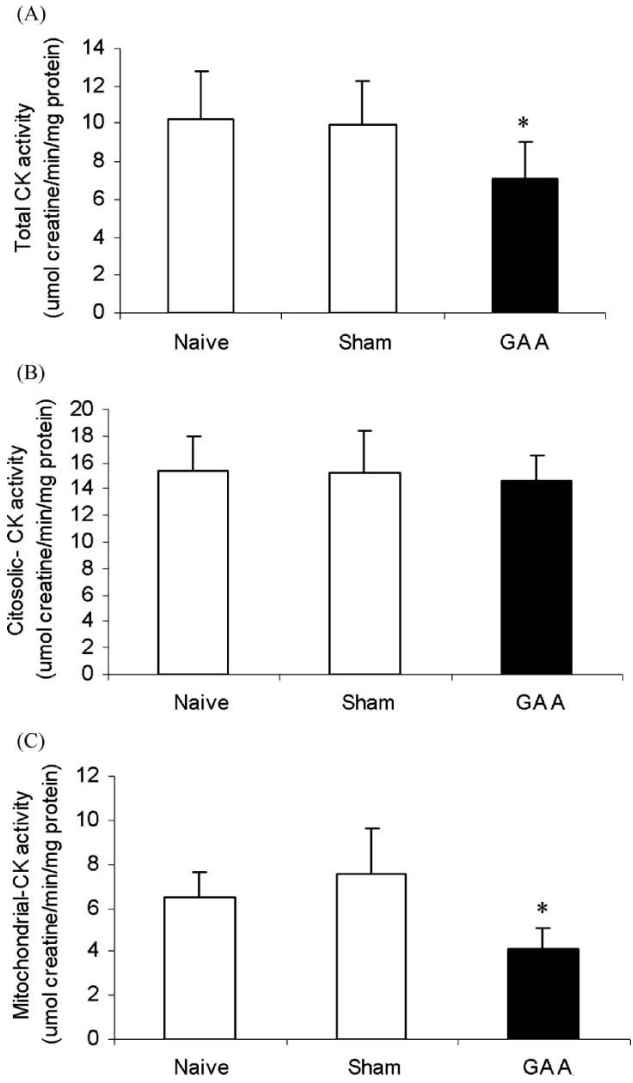

Fig. 2 Effect of intrastriatal administration of GAA on creatine kinase activity homogenates (A), in cytosolic preparations (B), and in mitochondrial preparations (C) after 30 min of GAA administration. Data are expressed as mean ± SD for six independent experiments (animals) performed in duplicate. $^{*}p < 0.05$ compared to naive and sham groups (Duncan's multiple range test)

and Cy-CK fractions of rat striatum. It can be seen that GAA significantly inhibited Mi-CK [$F(2, 15) = 8.411$; $p < 0.05$], but did no affect Cy-CK [$F(2, 12) = 0.138$; $p > 0.05$]. The contralateral striatum was also examinated in all experiments and showed no alterations in Na^+, K^+-ATPase and CK activities or lipid peroxidation (TBARS) at all times tested (data not shown).

Considering that Na^+, K^+-ATPase and CK activities can be inhibited by free radicals, we tested the effect of intrastriatal injection of GAA on TBARS in rat striatum 30 min and 3 h after administration. Figure 3 shows that GAA significantly increased (47%) TBARS

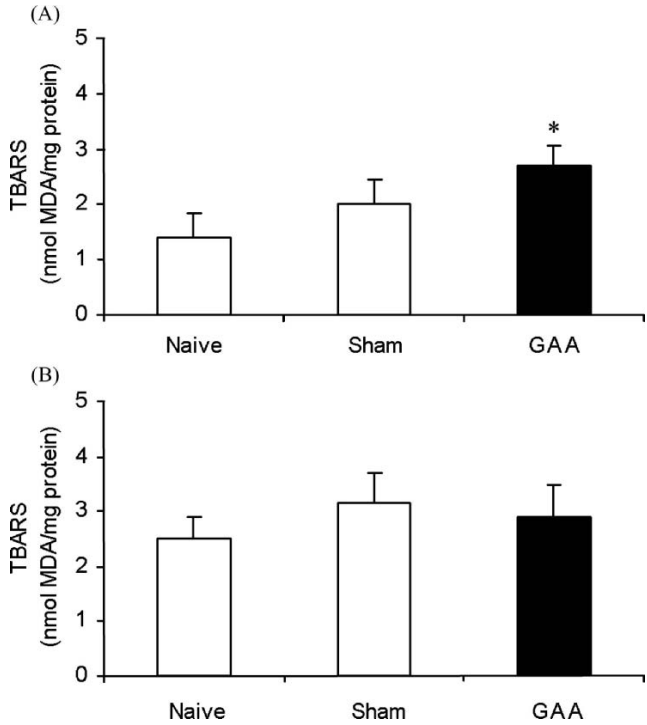

Fig. 3 Effect of intrastriatal administration of GAA on TBARS in rat homogenates at 30 min (A) and 3 h (B) after GAA administration. Results are expressed as mean ± SD for four independent experiments (animals) performed in duplicate. $^{*}p < 0.05$ compared to naive and sham groups (Duncan's multiple range test)

$[F(2, 9) = 0.123; p < 0.05]$ at 30 min after injection, but did not alter this oxidative stress parameter 3 h after GAA administration $[F(2, 12) = 2.214; p > 0.05]$ when compared to naive and sham groups.

Discussion

Chronic neurologic disability and seizures are observed in most patients with GAMT-deficiency (Von Figura *et al*., 2001). However, the pathomechanisms responsible for brain damage in this disease are still poorly understood. There is some data in the literature suggesting that GAA is a potentially epileptogenic compound (Schulze *et al*., 2001) and other studies showing that oral supplementation of creatine does not ameliorates epilepsy (Stöckler *et al*., 1996). Therefore, it is feasible that GAA may be neurotoxic and possibly responsible, at least in part, for the brain dysfunction in GAMT-deficiency.

In this study, we investigated the effect of GAA administration on Na^+, K^+-ATPase and CK activities in rat striatum. The maintenance of Na^+, K^+-ATPase and CK activities is critical for normal brain function and a reduction of these activities may lead to selective neuron damage in the brain (Lees, 1993; Tomimoto *et al*., 1993).

Na^+, K^+-ATPase is a transmembrane enzyme responsible for the transport of three Na^+ from the cell while moves two K^+ into the cell. It is responsible for the generation of the membrane potential to maintain neuronal excitability. It has been demonstrated that this

enzyme is decreased in neurodegenerative disorders (Yu, 2003) and is inhibited by free radicals (Yousef *et al*., 2002). CK plays an important role in brain energy homeostasis. Damage of CK function can be a neurodegenerative pathway that leads to neuronal loss in brain (Tomimoto *et al*., 1993). In addition, organic acids, which are accumulated in some inborn errors of metabolism, such as methylmalonic acid and ethylmalonic acid, have been shown to inhibit Na^+, K^+-ATPase and CK activities (Wyse *et al*., 2000; Leipnitz *et al*., 2003; Schuck *et al*., 2002). Our results showed that administration of GAA provoked a significant inhibition (40%) of Na^+, K^+-ATPase activity at 30 min, but not 3 h after intrastriatal injection. Although we did not measure the intrastriatal concentrations of GAA at these periods after its administration, it is tempting to speculate that this transitory effect occurred when high amounts of this guanidino compound was present in the striatum. This is in accordance with our findings related to CK activities. We found that GAA administration provoked a significant reduction of total and mitochondrial CK activities at 30 min but not after 3 h of administration of this compound. On the other hand, our present results suggest that GAA administration did not elicit neural death at the periods where the enzyme activities were tested since, if this was the case, the reduction of these activities would persist.

It has been shown that GAA increases reactive oxygen species production (Mori *et al*., 1996) and decreases membrane fluidity (Hiramatsu *et al*., 1992). Considering that Na^+, K^+-ATPase activity can be inhibited by free radicals formation (Lees, 1993) and that fluidity is an important physical property of cell membranes in which this enzyme is imbibed and that this enzyme is crucial to maintenance of normal cellular function (Schreier *et al*., 1978), it may be suggested that these effects caused by GAA could be involved in the inhibition of the Na^+, K^+-ATPase activity. Similarly, we presume that the inhibition of Mi-CK activity may be mediated by oxidation of sulfhydryl or other essential groups of the enzyme, since that Mi-CK molecule has cysteine residues in its structure, which can be a target for nitric oxide and other free radicals (Wolosker *et al*., 1996).

In order to test this hypothesis and to evaluate the mechanisms through which GAA inhibits Na^+, K^+-ATPase and CK activities, we investigated the effect of intrastriatal administration of GAA on TBARS, an index of lipid peroxidation occurring secondarily due to free radical generation, in rat striatum. In this context, we had recently demonstrated that Na^+, K^+-ATPase activity is inhibited by GAA *in vitro* and this action was prevented by some antioxidants (Zugno *et al*., 2004). In this work, we showed that 30 min but not 3 h after GAA intrastriatal administration, TBARS were increased, indicating that this compound induced lipid peroxidation. Although not conclusive, these data suggest that lipid peroxidation and/or oxidative stress, may at least partially explain the inhibition provoked by GAA on Na^+, K^+-ATPase and CK activities.

Therefore, we observed in this work that GAA alters the activities of Na^+, K^+-ATPase and CK, as well as TBARS production only at 30 min after its administration, but not at 3 h, suggesting that GAA may was taken up by glial cells or neurons and subsequently metabolized to creatine by GAMT, which is also expressed in brain (Braissant *et al*., 2001).

Although it is difficult to extrapolate our results to the human condition, it is conceivable that the inhibition of Na^+, K^+-ATPase and CK activities, which are crucial enzymes to normal CNS function, might be involved in the pathophysiology of the neurological features present in patients with GAMT-deficiency. However, more studies are necessary to investigate additional mechanisms involved in GAMT deficiency.

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Artigo 2

Evidence that the inhibitory effects of guanidinoacetate on the activities of the respiratory chain, Na^+,K^+-ATPase and creatine kinase can be differentially prevented by taurine and vitamins E and C administration in rat striatum *in vivo*

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Evidence that the inhibitory effects of guanidinoacetate on the activities of the respiratory chain, Na^+,K^+-ATPase and creatine kinase can be differentially prevented by taurine and vitamins E and C administration in rat striatum *in vivo*

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Abstract

Guanidinoacetate methyltransferase (GAMT) deficiency is an inherited neurometabolic disorder biochemically characterized by tissue accumulation of guanidinoacetate (GAA) and depletion of creatine. Affected patients present epilepsy and mental retardation whose etiopathogeny is unclear. In a previous study we showed that instrastriatal administration of GAA caused a reduction of Na^+,K^+-ATPase and creatine kinase (CK) activities, as well as an increase in TBARS (an index of lipid peroxidation). In the present study we investigated the *in vitro* and *in vivo* effects of GAA on glucose uptake from [U-^{14}C] acetate (citric acid cycle activity) and on the activities of complexes II, II–III, III and IV of the respiratory chain in striatum of rats. Results showed that 50 and 100 μM GAA (*in vitro* studies) and GAA administration (*in vivo* studies) significantly inhibited complexes II and II–III, respectively, but did not alter complexes III and IV, as well as CO_2 production. We also studied the influence of taurine or vitamins E and C on the inhibitory effects caused by intrastriatal administration of GAA on complexes II and II–III, Na^+,K^+-ATPase and CK activities, and on TBARS in rat striatum. Pre-treatment with taurine and vitamins E and C revealed that taurine prevents the effects of intrastriatal administration of GAA on the inhibition of complex II, complex II–III, and Na^+,K^+-ATPase activities. Vitamins E and C prevent the effects of intrastriatal administration of GAA on the inhibition of CK and Na^+,K^+-ATPase activities, and on the increase of TBARS. The data suggest that GAA *in vivo and in vitro* treatment disturbs important parameters of striatum energy metabolism and that oxidative damage may be mediating these effects. It is presumed that defects in striatum bioenergetics might be involved in the pathophysiology of striatum damage characteristic of patients with GAMT-deficiency.

Keywords: GAMT-deficiency; Metabolic disease; Guanidinoacetate; Respiratory chain; Taurine; Vitamins E and C

1. Introduction

Guanidinoacetate (GAA) is the immediate precursor of creatine, being mainly synthesized in the kidney from arginine and glycine by glycine amidinotransferase. It is then transported by creatine transporters to various tissues, including brain and liver, where it is methylated to creatine by guanidinoacetate methyltransferase (GAMT). GAMT-deficiency is an inherited metabolic disease biochemically characterized by tissue accumulation of GAA and depletion of creatine. Affected patients present neurological symptoms, including muscular hypotonia, involuntary extrapyramidal movements and epilepsy [1]. The diagnosis is based on excessive levels of GAA in body fluids of the affected patients, which is a pathognomonic feature of GAMT-deficiency [1].

Neurological dysfunction is commonly found in human GAMT-deficiency, but the exact mechanisms underlying brain damage in this disease are not well established. However, at the present the neurological symptoms of the affected patients have been mainly attributed to creatine deficiency, although GAA accumulation may play a role in this process. This is in accordance

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with the findings of Schulze and colleagues [2] showing that GAA is probably epileptogenic in man. In this context, it was demonstrated that GAA alters neurotransmission [3] and decreases membrane fluidity [4]. It should be however emphasized that creatine deprivation is highly neurotoxic *per se*, as evidenced by the serious neurological dysfunction affecting patients with AGAT deficiency and creatine transporter deficiency in which there is no accumulation of GAA [5,6].

Tissues with high energy requirement, such as the brain, are more susceptible to damage under situations of reduced aerobic metabolism [7]. In this context, impairment of energy metabolism caused by mitochondrial dysfunction has been implicated in the pathogenesis of a number of neurological conditions, including dementia, cerebral ischemia, Alzheimer's disease and Parkinson's disease [8–12]. To the best of our knowledge, there is little information associating GAA and energy metabolism. Schmidt and colleagues [13] recently showed that the knockout mice for GAMT presented an increase of complex V activity, suggesting that this effect was a compensation mechanism for energy impairment. In addition, recent reports from our laboratory showed that intrastriatal administration of GAA inhibits Na^+,K^+-ATPase and creatine kinase (CK) activities in rat striatum [14].

On the other hand, the central nervous system (CNS) is highly sensitive to oxidative stress due to its high oxygen consumption, its high iron and lipid contents, especially polyunsaturated fatty acids, and the low activity of antioxidant defenses [15]. In this context, we have previously demonstrated that GAA induced lipid peroxidation in the brain [14].

Therefore, in the present study the main objective was to investigate the *in vitro* and *in vivo* effects of GAA on CO_2 production from [U-^{14}C] acetate and on the activities of complexes II, II–III, III and IV of the respiratory chain in rat striatum. Since vitamins E and C and taurine have antioxidant properties [16–18] and taurine is neuroprotective [19,20] and has an antiepileptic action in experimental models [21] and in humans [22], we also evaluated the *in vivo* influence of taurine and vitamins E and C on the inhibition of the activities of some complexes of the respiratory chain caused by GAA, as well as of Na^+,K^+-ATPase and CK, which were previously shown to be inhibited by GAA. Striatum was used because patients with GAMT-deficiency may present basal ganglia abnormalities [1].

2. Materials and methods

2.1. Animals and reagents

Sixty-day-old Wistar rats were obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. The animals were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22 °C± 1 °C) colony room, with food and water *ad libitum*. All reagents used were of analytical grade and purchased from Sigma Co. (St Louis, MO, USA).

2.2. In vivo studies

2.2.1. Guanidinoacetate administration

For the *in vivo* studies, surgery and intrastriatal infusion were performed as described in Zugno et al. [14]. Sixty day-old rats were anesthetized with an intraperitoneal injection of ketamine plus xylazine (100 mg/kg and 14 mg/kg, respectively). The head of the animals was fixed in a stereotaxic apparatus, the skin of the skull was removed and a 27-gauge 9-mm guide cannula was then placed above the striatum (AP: −0.5 mm; L: −2.5 mm; DV: −2.5 mm) [23]. The cannula was fixed with jeweler acrylic cement. Experiments were performed 48 h after surgery. A 30-gauge cannula was fitted into the guide cannula and connected by polyethylene tube to a 5 μL Hamilton microsyringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula towards the striatum. The animals were divided into two groups: group 1 (control group), rats that received saline solution; group 2 (GAA-treated), rats that received 10 μM of GAA solution (0.02 nmol/striatum). Two μL of saline or GAA solution was administered intrastriatally during 2 min. The needle was left in place for another 1 min before being softly removed, so that the total procedure lasted 3 min. The correct position of the needle was tested by injecting 0.5 μL of methylene blue (4% in saline solution). The animals were killed by decapitation without anesthesia 30 min after injection. The experimental protocol was approved by the Ethics Committee for animal research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil and followed the NIH *Guide for the Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

2.2.2. Taurine and vitamins E and C administration

In some experiments the animals were pretreated during 7 days with daily intraperitoneal administration of alpha-tocopherol (40 mg/kg) and ascorbic acid (100 mg/kg) or taurine (50 mg/kg). Controls animals received saline. Doses of vitamins E and C and taurine were chosen according to Wyse et al. [24] and Sener [18], respectively. Animals were sacrificed by decapitation without anesthesia 30 min after the intrastriatal injection of GAA.

2.2.3. Tissue preparation

The brain was rapidly excised on a Petri dish placed on ice and the striatum was dissected, weighed and kept chilled until homogenization with a ground glass type Potter–Elvehjem homogenizer in the specific buffer used for each technique.

For the determination of the electron transfer chain complexes and CK activities, the striatum was homogenized in 20 volumes of SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, and 50 IU/mL heparin). The homogenates were centrifuged at 800×*g* for 10 min at 4 °C. The pellet was discarded and the supernatants, a suspension of mixed and preserved organelles, including mitochondria, kept at −70 °C until determination of the enzyme activities. The maximal period between supernatant preparation and enzyme analysis was always less than 7 days.

For Na^+,K^+-ATPase activity measurement, synaptic plasma membranes were prepared according to the method of Jones and Matus [25], with some modifications [26]. The homogenate was centrifuged at 1000×*g* for 20 min and the supernatant removed and centrifuged at 12,000×*g* for a further 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris–HCl buffer, pH 8.1), incubated at 0 °C for 30 min, and applied on a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 M. After centrifugation at 69,000×*g* for 2 h, the fraction at the 0.8–1.0 M sucrose interface was taken as the membrane enzyme preparation.

For the $^{14}CO_2$ production assay, the striatum was homogenized (1:10, w/v) in Krebs–Ringer bicarbonate buffer pH 7.4 using an ice-chilled glass homogenizing vessel at 900 rpm. Total homogenates were used in these experiments.

2.3. In vitro studies

For the *in vitro* studies, striatum from 60-day-old non-treated rats was dissected and homogenized in a similar manner as that for the *in vivo* experiments to obtain supernatants. The supernatants were then incubated in the presence of 10, 30, 50 or 100 μM GAA.

2.4. Determination of the respiratory chain complexes activities

Mitochondrial respiratory chain enzyme activities (complexes II, III, II–III and IV) were measured in striatum supernatants 30 min after GAA or NaCl intrastriatal injection. The protein concentration in the supernatants varied from 1.5 to 4.0 mg protein/mL in all experiments. The activities of succinate: DCIP-oxidoreductase (complex II) and succinate: cytochrome *c* oxidoreductase

(complex II–III) were determined according to the method of Fischer et al. [27]. The activity of ubiquinol: cytochrome *c* oxidoreductase (complex III) was assayed according to the method described by Birch-Machin et al. [28] and that of cytochrome *c* oxidase (complex IV) according to Rustin et al. [29]. The methods described to measure these activities were slightly modified, as described in details in previous reports [30,31]. The activities of the respiratory chain complexes were expressed as nmol/min mg protein.

For the *in vitro* studies, 10, 30, 50 or 100 μM GAA was supplemented to the incubation medium, whereas the control group did not contain the metabolite.

2.5. Creatine kinase (CK) activity assay

CK activity was measured in striatum supernatants 30 min after GAA or NaCl intrastriatal injection in a reaction mixture consisting of 60 mM Tris–HCl, pH 7.5, containing 7 mM phosphocreatine, 9 mM $MgSO_4$, 0.625 mM lauryl maltoside and approximately 0.4–1.2 μg protein in a final volume of 100 μL. For the *in vitro* studies, 10, 30, 50 or 100 μM GAA was supplemented to the incubation medium, whereas the control group did not contain the metabolite. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of 0.3 μmol ADP. The reaction was stopped after 10 min by addition of 1 μmol of *p*-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes [32] with slight modifications as described previously [33]. The color was developed by the addition of 100 μL 2% α-naphtol and 100 μL 0.05% diacetyl in a final volume of 1 mL and read spectrophotometrically at 540 nm after 20 min. Results were expressed as μmol creatine/min/mg protein.

2.6. Na^+,K^+-ATPase activity assay

The reaction mixture for Na^+, K^+-ATPase assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 μL. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays according to the method of Wyse and colleagues [34]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. [35]. Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein.

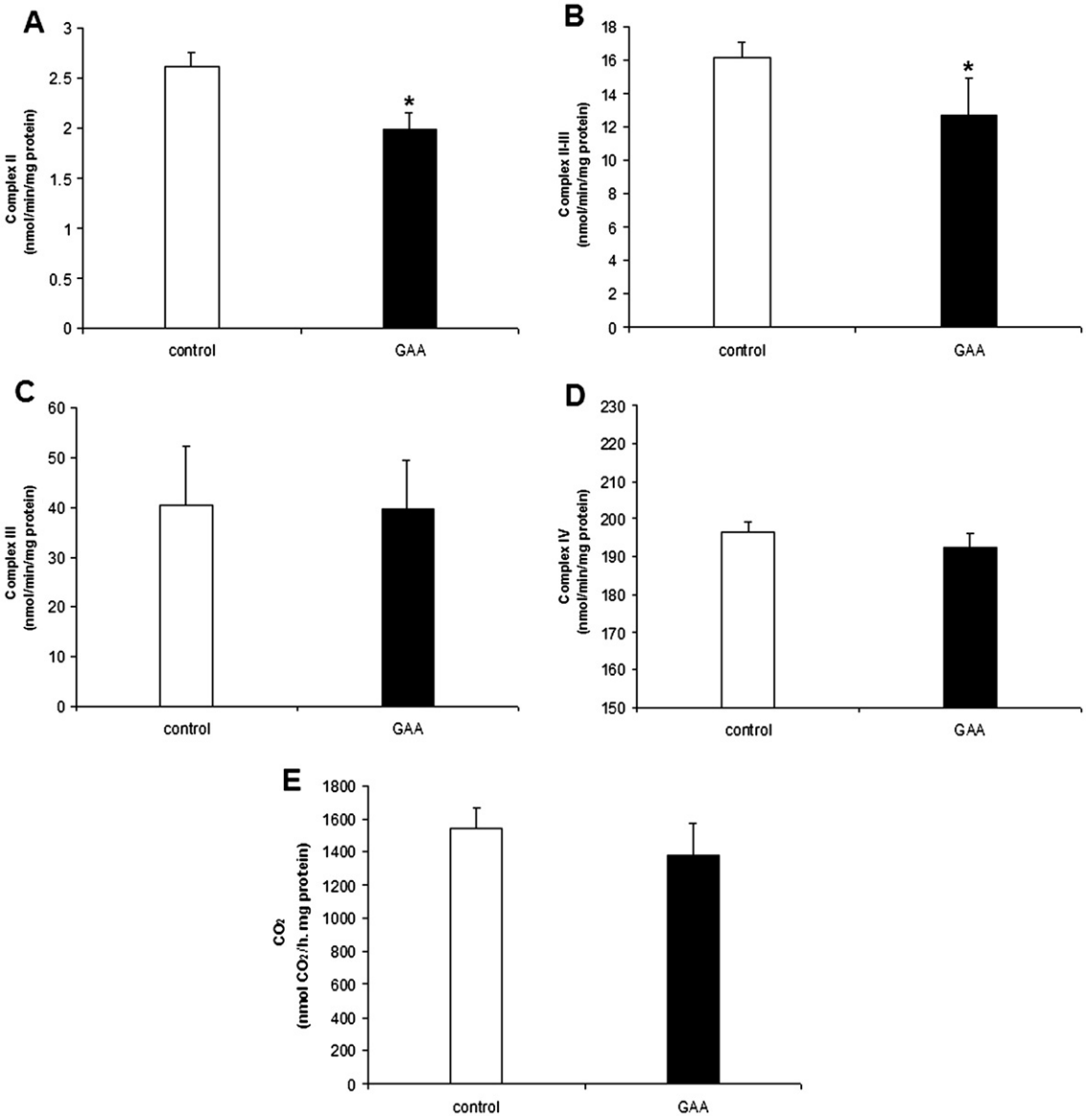

Fig. 1. Effect of intrastriatal administration of guanidinoacetate on complex II (A), complex II–III (B), complex III (C) and complex IV (D) activities and on CO_2 production (E) in striatum from 60-day-old rats. Values are mean±standard deviation for five to six independent experiments per group. *Different from control, $p<0.01$ (Duncan's multiple range test).

2.7. $^{14}CO_2$ production from [1-^{14}C]acetate

For the experiments designed to evaluate $^{14}CO_2$ production, the animals were sacrificed 30 min after intrastriatal GAA or NaCl injection. The striatum homogenates containing 1.5–2 mg protein were added to small flasks (11 cm^3). Flasks were pre-incubated in a metabolic shaker at 37 °C for 15 min. After pre-incubation, 0.1 μCi [1-^{14}C]-acetate and 1.0 mM of unlabeled acetate were added to the incubation medium. The flasks were gassed with a O_2:CO_2 (95:5) mixture and sealed with rubber stoppers and Parafilm M. Glass center wells containing a folded 65 mm/5 mm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min of incubation at 37 °C, 0.1 mL of 50% trichloroacetic acid was added to the medium and 0.1 mL of benzethonium hydroxide was added to the center wells with a needle introduced through the rubber stopper. The flasks were left to stand for 30 min to complete $^{14}CO_2$ trapping and then opened. The filter papers were removed and added to vials containing scintillation fluid, and radioactivity was measured [36]. Results correspond to pmol $^{14}CO_2$/h/mg protein and were expressed as percentage of controls.

2.8. Protein determination

Protein content was measured by the method of Lowry et al. [37], using bovine serum albumin as standard.

2.9. Statistical analysis

Data were analyzed by the Student's *t*-test or by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the F-test was significant. Pearson linear regression coupled to ANOVA was also used to verify

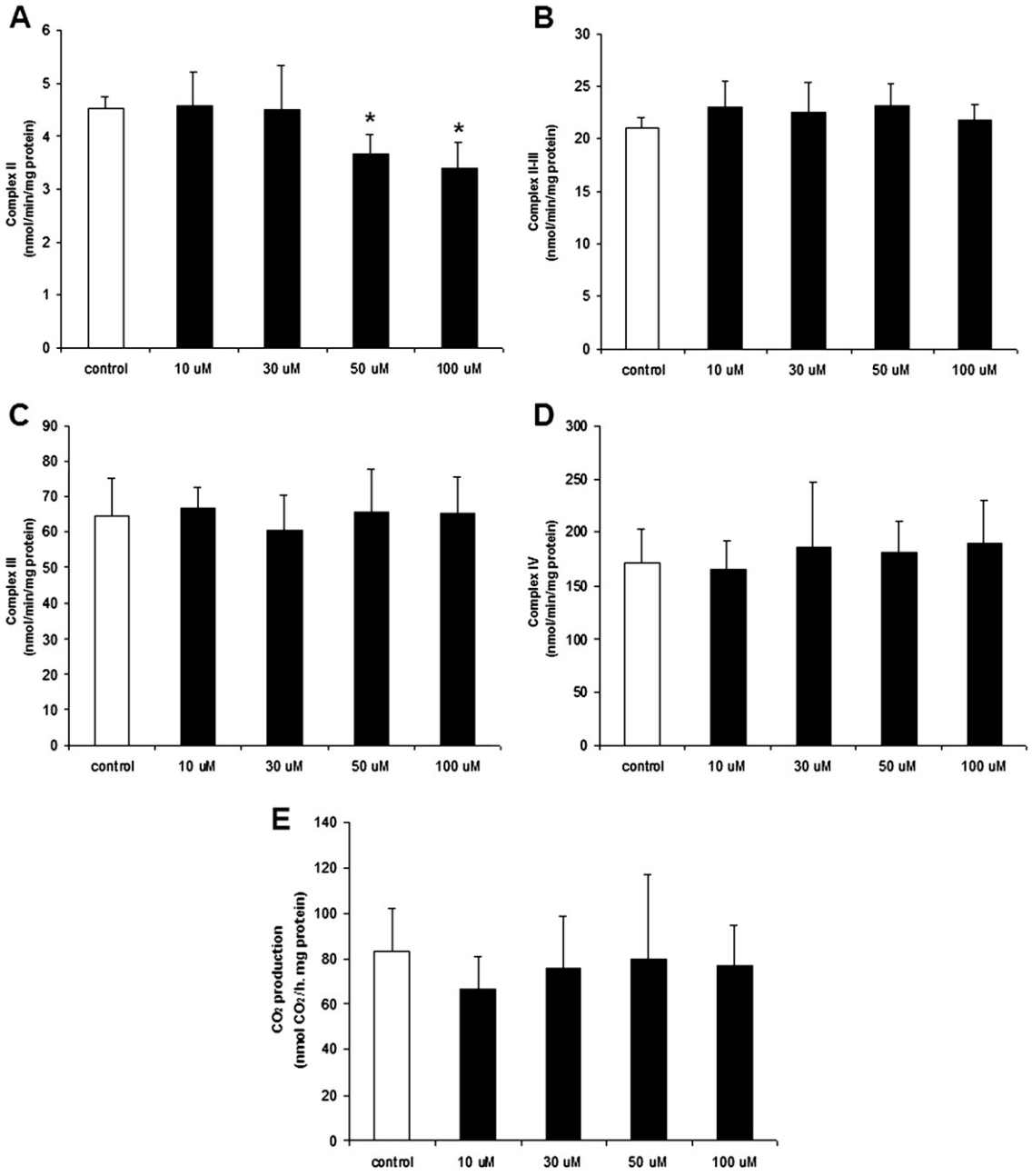

Fig. 2. Effect of guanidinoacetate addition on the assays of complex II (A), complex II–III (B), complex III (C) and complex IV (D) activities and CO_2 production (E) in striatum from 60-day-old rats. Values are mean±standard deviation for five to six independent experiments per group. *Different from control, $p<0.01$ (Duncan's multiple range test).

dose-dependent effects. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. A value of $p<0.05$ was considered to be significant.

3. Results

First, we tested the effect of intrastriatal administration of GAA on CO_2 production from acetate (citric acid cycle activity) and on complexes II, II–III, III and IV activities of the respiratory chain in striatum of 60-day-old rats. Fig. 1 shows that GAA significantly inhibited (20–25%) complex II [$t(8)=6.74$, $p<0.01$] (A) and complex II–III [$t(8)=3.22$, $p<0.01$] (B), but did not alter complexes III (C) and IV activities (D), neither CO_2 production (E). We also investigated the *in vitro* effects of GAA on the same parameters studied *in vivo*. Fig. 2 shows that 50–100 μM of GAA significantly inhibited complex II (around 25%) [$F(4,20)=4.73$, $p<0.01$] (A), but did not alter complexes II–III (B), III (C) and IV activities (D), neither CO_2 production (E).

A previous work from our group has shown that GAA inhibits Na^+,K^+-ATPase and CK activities in rat striatum [14]. Therefore, since GAA induces oxidative stress [38,39], we also examined the influence of taurine and vitamins E and C, which have antioxidant properties, on the inhibitory effects caused by intrastriatal administration of GAA on complexes II and complex II–III activities, as well as on Na^+,K^+-ATPase and CK activities. Results showed that taurine and vitamins E and C administration did not alter *per se* the parameters studied, but only taurine prevented the inhibition of the activities of complexes II

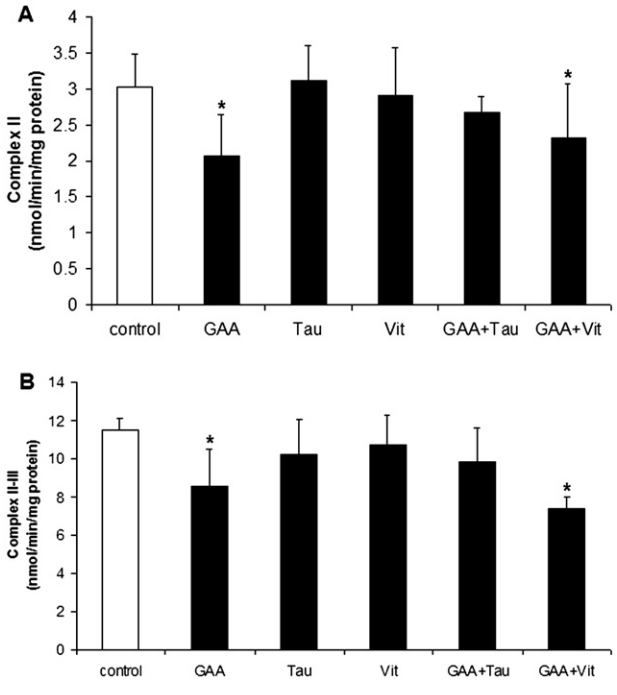

Fig. 3. Effect of pretreatment with taurine and vitamins E and C on the inhibition caused by intrastriatal administration of guanidinoacetate on complex II (A) and complex II–III (B) activities in striatum from 60-day-old rats. Values are mean ± standard deviation for five to seven independent experiments per group. *Different from control, $p<0.01$ (Duncan's multiple range test). GAA = guanidinoacetate; Tau = taurine; Vits = vitamins E and C; GAA + Tau = guanidinoacetate plus Taurine; GAA + Vits = guanidinoacetate plus vitamins E and C.

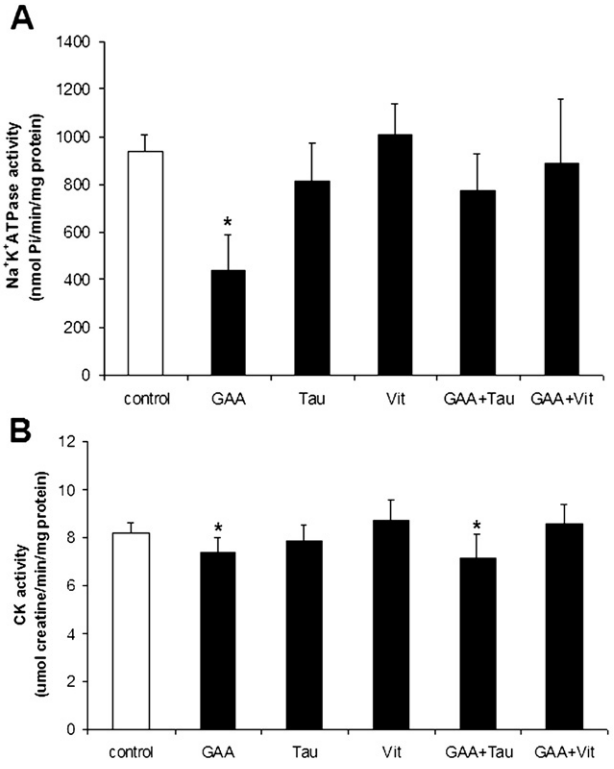

Fig. 4. Effect of pretreatment of administration with taurine and vitamins E and C on the inhibition caused by intrastriatal administration of guanidinoacetate on Na^+,K^+-ATPase (A) and creatine kinase (B) activities in striatum from 60-day-old rats. Values are mean ± standard deviation for five to seven independent experiments per group. *Different from control, $p<0.01$ (Duncan's multiple range test). GAA = guanidinoacetate; Tau = taurine; Vits = vitamins E and C; GAA + Tau = guanidinoacetate plus Taurine; GAA + Vits = guanidinoacetate plus vitamins E and C.

[$F(5,36)=3.98$, $p<0.01$] (A) and II–III [$F(5,30)=6.04$, $p<0.01$] (B) caused by GAA (Fig. 3). Furthermore, taurine and vitamins E plus C prevented the inhibitory effects caused by GAA on Na^+,K^+-ATPase activity [$F(5,24)=7.30$, $p<0.01$] (Fig. 4A), whereas vitamins E plus C prevented the GAA-induced inhibition on CK [$F(5,30)=3.97$, $p<0.01$] (Fig. 4B) activity.

Next, we evaluated the effect of taurine or vitamins E and C administration on GAA-induced *in vivo* increase of the thiobarbituric acid reactive substances (TBARS) measurement, an index of lipid peroxidation in rat striatum. Fig. 5 shows that GAA significantly increased TBARS and vitamins E and C, but not taurine, prevented this effect [$F(5,22)=7.821$, $p<0.001$].

4. Discussion

GAMT-deficiency is an inherited metabolic disease biochemically characterized by tissue accumulation of GAA. Affected patients present neurological damage whose underlying mechanisms are poorly known [1]. In a previous work we demonstrated that GAA inhibits Na^+,K^+-ATPase and CK activities in rat striatum. In the present study we investigated the *in vivo and in vitro* effects of GAA on some parameters of energy metabolism, including CO_2 production from [U-^{14}C] acetate (citric acid cycle activity) and the activities of complexes

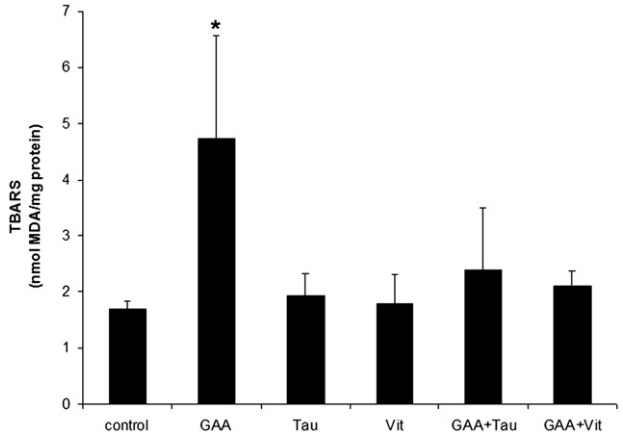

Fig. 5. Effect of pretreatment with administration of taurine and vitamins E and C on the increase of TBARS caused by intrastriatal administration of guanidinoacetate in striatum from 60-day-old rats. Values are mean±standard deviation for four to six independent experiments per group. *Different from control, $p<0.01$ (Duncan's multiple range test). GAA=guanidinoacetate; Tau=taurine; Vits=vitamins E and C; GAA+Tau=guanidinoacetate plus Taurine; GAA+Vits=guanidinoacetate plus vitamins E and C.

II, II–III, III and IV of the respiratory chain in rat striatum. We verified that intrastriatal administration of GAA significantly reduced complexes II and II–III and did not affect complexes III and IV. We also observed that complex II, but not the other complexes analyzed, was inhibited when exposed to 50 and 100 μM of GAA *in vitro*. These results indicate that GAA is a selective inhibitor of complex II of the respiratory chain. Furthermore, CO_2 production was also not affected *in vivo and in vitro* by GAA, indicating that the activity of the citric acid cycle was not disturbed by this organic acid.

Considering that GAA is a compound that induces oxidative stress [20,39] and that complex II, Na^+,K^+-ATPase and CK activities are vulnerable to free radical attack [40–42], it is possible that the reduction of the enzymatic activities caused by GAA administration, as demonstrated in a previous work for Na^+,K^+-ATPase and CK [14] and in the present study for complex II, could occur through oxidative damage. To test this hypothesis, we pretreated the animals during a week with taurine or vitamins E and C and injected GAA afterwards. Taurine was previously demonstrated to possess antioxidant activities [18,19] and to be a neuroprotector [43]. In this context, we have recently demonstrated that taurine prevents the inhibition of Na^+,K^+-ATPase activity caused by GAA *in vitro* [20]. Furthermore, vitamins E and C are classic antioxidants [18]. Vitamin E is a lipid-soluble vitamin that interacts with cell membranes, traps free radicals and interrupts the oxidative chain that damages cells [44,45], preventing the uncontrolled propagation of lipid peroxidation by free radicals [45]. The resultant tocopheroxil radical requires ascorbate (vitamin C) for its regeneration back to reduced tocopherol [46,47].

Our results showed that taurine prevented the inhibitory effect caused by intrastriatal administration of GAA on the activities of complexes II and II–III. Furthermore, the inhibition of Na^+,K^+ATPase activity caused by intrastriatal administration of GAA was prevented by both taurine and vitamins E and C. Finally, we found that the inhibition of the activity of CK as well as the increase of TBARS caused by GAA was prevented by pre-treatment with vitamins E and C, but not by taurine. The exact mechanisms underlying taurine effects are not clearly understood. However, strong evidences show that taurine acts as a reactive oxygen species and nitrogen radical scavenger [48] and also as a membrane stabilizer [49]. Vitamins E and C are known to prevent the propagation of lipid peroxidation by free radicals [45].

Taken together, the present data indicate that disturbances of bioenergetics and oxidative stress are interrelated mechanisms underlying the neurological dysfunction characteristic of GAMT-deficient patients. However, it should be emphasized that since we did not measure brain GAA concentrations after GAA administration, we cannot establish precisely whether our present results of altered oxidative metabolism are of relevance for the GAMT deficient patients.

In conclusion, we demonstrated here that the respiratory chain activity is compromised in vivo and in vitro by GAA probably via oxidative attack. Considering previous reports demonstrating that other important enzyme activities of the CNS, i.e. Na^+,K^+-ATPase and CK, were also inhibited by GAA, it is conceivable that brain accumulation of GAA may disturb CNS functioning. However, it should be emphasized that creatine deficiency is neurotoxic *per se*, since patients with AGAT deficiency and creatine transporter deficiency in which there is no accumulation of GAA present serious neurological dysfunction [5,6]. The present findings may explain, at least in part the neurotoxicity caused by GAA in patients affected by GAMT-deficiency. Our results also suggest that the administration of antioxidants should be considered as an adjuvant therapy to specific diets or to other pharmacological interventions. However, more studies must be conducted before administration of antioxidants is given to GAMT-deficient patients.

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Artigo 3

Guanidinoacetate decreases antioxidant defenses and total protein sulfhydryl content in striatum of rats.

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Guanidinoacetate decreases antioxidant defenses and total protein sulfhydryl content in striatum of rats

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Abstract

Guanidinoacetate methyltransferase (GAMT) deficiency is an inherited neurometabolic disorder biochemically characterized by tissue accumulation of guanidinoacetate (GAA) and depletion of creatine. Affected patients present epilepsy and mental retardation whose pathogeny is unclear. In the present study we investigated the *in vitro* and *in vivo* (intrastriatal administration) effect of GAA on oxidative stress parameters in rat striatum. Sixty day-old rats were used for intrastriatal infusion of GAA. For *in vitro* studies, sixty day-old Wistar rats were killed by decapitation and the striatum was pre-incubated for 1 h at 37°C in the presence of GAA at final concentrations ranging from 10 to 100 uM. Parameters of oxidative stress such as total radical-trapping antioxidant potential (TRAP), antioxidant enzymes (SOD, GPx and CAT), protein carbonyl and sulfhydryl contents were measured. DNA damage was also evaluated. Results showed that GAA administration (*in vivo* studies) or the addition of 100 μM GAA to assays (*in vitro* studies) significantly decreased TRAP, SOD activity and total sulfhydryl content in rat striatum. In contrast, protein carbonyl content and the activities of CAT and GPx were not altered by this guanidino compound. DNA damage was not found after intrastriatal administration of GAA. These findings suggest that GAA decreases total antioxidant capacity and total sulfhydril content, suggesting an induction of oxidative stress in brain. It is tempting to speculate that these alterations may contribute, at least in part, to the neurological dysfunction found in GAMT-deficiency patients.

Key words: GAMT-deficiency, metabolic disease, guanidinoacetate, total antioxidant defense, DNA damage, comet assay.

INTRODUCTION

Guanidinoacetate (GAA) accumulates in patients affected by the inborn error of creatine biosynthesis known as guanidinoacetate methyltransferase (GAMT) deficiency. The disorder is clinically characterized by severe neurological symptoms, including muscular hypotonia, involuntary extrapiramidal movements, mental retardation and epilepsy. Biochemically, tissue accumulation of GAA and deficiency of creatine/phosphocreatine, occurs, especially in brain (Von Figura et al., 2001).

The exact mechanisms underlying the brain dysfunction, particularly epilepsy and mental retardation, are poorly understood in this disease. However, the neurological dysfunction, commonly found in human GAMT-deficiency, has been mainly attributed to reduction of creatine or/and increase of GAA levels (Leuzzi et al., 2000). It has been suggested that GAA alters neurotransmission (Neu et al., 2002), decreases membrane fluidity (Hiramatsu et al., 1992) and can provokes epilepsy in man (Schulze et al., 2001). It should be however emphasized that creatine deprivation is highly neurotoxic *per se*, as evidenced by the serious neurological dysfunction affecting patients with AGAT deficiency and creatine transporter deficiency in which there is no accumulation of GAA (Schulze, 2003; Sykut-Cegielska et al., 2004).

Oxidative stress is an important event that has been related to the pathogenesis of diseases affecting the central nervous system (CNS), such as neurodegenerative disorders, epilepsy, demyelination (multiple sclerosis), and dementia (Halliwell, 2006; Reznick and Packer, 1993). This is understandable

since the CNS is highly sensitive to oxidative stress due to its high oxygen consumption, its high iron and lipid contents, especially polyunsaturated fatty acids, and the low activity of antioxidant defenses (Halliwell, 1996). In this context, we have shown that GAA administration increases TBARS in rat striatum, which suggest an increase of lipid peroxidation (Zugno et al., 2006). These data are in agreement with the other studies, showing that GAA increases free radical production (Mori et al., 1996).

In the present study we investigated the effect of intrastriatal administration (*in vivo* study) of GAA on non-enzymatic (total radical-trapping antioxidant potential (TRAP) and enzymatic antioxidant defenses (catalase-CAT, glutathione peroxidase-GPx and superoxide dismutase-SOD activities), as well as on protein oxidation markers such as carbonyl and sulfhydryl (thiol) content in rat striatum. Besides, we also verified the effect of this compound on DNA damage. The *in vitro* effect of GAA on the same parameters also was studied. Striatum was studied because it has been demonstrated that basal ganglia abnormalities are common in patients with GAMT-deficiency.

MATERIAL AND METHODS

Animals and reagents

Wistar rats obtained from the Central Animal House of Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil, were used in the experiments. Animals were maintained on a 12/12 h light/dark cycle (lights on from 7 a.m. to 7 p.m.) in an air-conditioned constant temperature (22°C) colony room, and had free access to a 20% (w/w) protein

commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Society for Experimental Biology and was approved by the Ethics Committee of the Federal Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical, St Louis, MO, USA.

In vivo studies

For the *in vivo* studies, surgery and intrastriatal infusion were performed as described by Zugno and colleagues (2004). Sixty day-old rats were anesthetized with a mixture intraperitoneal injection of ketamine and xylazine (100 mg/kg and 14 mg/kg, respectively). The head of the animals were fixed in a stereotaxic apparatus, the skin of the skull was removed and a 27-gauge 9-mm guide cannula was then placed above the striatum (AP: -0.5mm; L: -0.5mm; DV: -2.5mm). The cannula was fixed with jeweler acrylic cement. Experiments were performed 48 h after surgery. A 30-gauge cannula was fitted into the guide cannula and connected by polyethylene tube to a 5 μL Hamilton microsyringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula towards the striatum. The animals were divided into two groups: group 1 (control group), rats that suffered surgery and received saline; and group 2 (GAA-treated), rats that received 10 μM of GAA solution (0.02 nmol/striatum). The volume administered intrastrially (saline or GAA solution) was 2 μL. The animals were killed by decapitation without anesthesia 30 min after insertion.

Tissue Preparation

The animals were killed by decapitation and the brains were removed and kept on an ice-plate. Striatum was weighed and kept chilled until homogenization in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 750 *g* for 10 min at 4ºC. The pellet was discarded and the supernatant was immediately separated and used for the measurements.

For measure of TRAP, sulfhydryl and carbonyl contents, the striatum was homogenized with 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. For test the antioxidant enzymes CAT and GPx, striatum was homogenized (1:10 w/v) in 10 mM potassium phosphate buffer, pH 7.6 and for SOD activity, striatum was homogenized (1:10 w/v) in 50 mM Tris-HCl buffer with 1.0 mM EDTA, pH 8.2. For alkaline comet assay the striatum was removed and washed in cold RPMI.

In vitro studies

For the *in vitro* studies, striatum from 60-day-old non-treated rats was dissected and homogenized in a similar manner as that for the *in vivo* experiments to obtain supernatants. The supernatants were then incubated for one hour in the presence of 10, 30, 50 or 100 μM GAA

Total Radical-Trapping Antioxidant Potential (TRAP)

TRAP represents the total antioxidant capacity of the tissue and was determined by measuring the luminol chemiluminescence intensity induced by 2,20-azo-bis (2-amidinopropane) (ABAP) by the method of Lissi and

colleagues (Lissi et al., 1992). Briefly, 4 mL of 10 mM ABAP was added to the vial and background chemiluminescence measured. Ten microliter of 4 mM luminol was then added and the chemiluminescence measured. This was considered the initial value. Ten microliter of 0.2 mM trolox or homogenates (1:10 w/v in 0.1M glycine buffer, pH 8.6) was added and chemiluminescence measured until it reached the initial levels. The addition of trolox or tissue homogenate to the incubation medium reduces the chemiluminescence. The time necessary to return to the levels present before the addition was considered to be the induction time (IT). IT is directly proportional to the antioxidant capacity of the tissue and was compared to the IT of Trolox. The results are reported as nmol of trolox per mg protein.

Superoxide Dismutase assay

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on O_2^-, which is substrate for SOD (Marklung, 1985). The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm, using a double-beam spectrophotometer with temperature control. A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results were reported as units of SOD/mg protein.

Catalase assay

CAT activity was assayed using a double-beam spectrophotometer with temperature control. This method is based on the disappearance of H_2O_2 at

240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 0.1-0.3 mg protein/mL (Aebi, 1984). One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute and the specific activity is reported as units per mg protein.

Glutathione peroxidase assay

GPx activity was measured using *tert*-butyl-hydroperoxide as substrate (Wendel, 1981). NADPH disappearance was monitored at 340 nm using a double-beam spectrophotometer with temperature control. The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl-hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as one μmol of NADPH consumed per minute and the specific activity is represented as units per mg protein.

Protein carbonyl content

Oxidatively modified proteins present an enhancement of carbonyl content (Stadtman, 1990). In this study, carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm. Briefly, 100 μL of homogenate were added to plastic tubes containing 400 μL of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This was kept in the dark for 1 hour and vortexed each 15 minutes. After that, 500 μL of 20% trichloroacetic acid were added to each tube. The moisture was vortexed and centrifuged at 14,000 rpm for 3 minutes. The supernatant obtained was discarded. The pellet was washed with 1 mL

ethanol:ethyl acetate (1:1, v/v), vortexed and centrifuged at 14,000 rpm for 3 minutes. This washing procedure was repeated once again and, after centrifugation, the supernatant was discarded and the pellet re-suspended in 600 μL of 6 M guanidine (prepared in a 20 mM potassium phosphate solution pH 2.3). The sample was vortexed and incubated at 60^{o}C for 15 minutes. After that, it was centrifuged at 14,000 rpm for 3 minutes and the absorbance was measured at 370 nm (UV) in a quartz cuvette. Results were reported as carbonyl content (nmol/mg protein).

Protein sulfhydryl content

This assay is based on the reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) by thyols, which in turn become oxidized (disulfide), generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). Briefly, 50 μL of homogenate were added to 1 mL of PBS buffer pH 7.4 containing 1 mM EDTA. Then 30 μL of 10 mM DTNB, prepared in a 0.2 M potassium phosphate solution pH 8.0, were added. Subsequently, 30 min incubation at room temperature in a dark room was performed. Absorption was measured at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein.

Alkaline comet assay

This analysis included an investigation of DNA damage in striatum of adult rats. For such analysis, the alkaline comet assay was performed as described by Singh and colleagues (1988). Briefly, striatum cells (20 μL) were

embedded in 80 μL of 0.75% low-melting-point agarose. After solidification, the slides were placed in lysis buffer (2.5 M NaCl, 100 mM Tris, pH 10.0-10.5, with freshly added 1% Triton X-100 and 10% dimethyl sulfoxide) for a minimum 1 hour and a maximum of 2 weeks. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 20 minutes. The DNA was subjected to electrophoresis for 15 minutes at 25 V (0.90 V/cm) and 300 mA, and the buffer was then neutralized with 0.4 M Tris (pH 7.5). Finally, the DNA was stained with ethidium bromide (2 μg/mL). The slides were coded for blind analysis. To demonstrate the electrophoresis conditions and efficiency of this methodology, negative and positive controls of human blood cells were used for each electrophoresis treatment. For a positive control, 50 μL of whole blood was mixed with 13 μL of methyl methanesulfonate at $8x10^{-5}$ M. This mixture was then incubated for 2 hours at $37^{O}C$. The result of each electrophoresis was considered yielded negative and positive results, respectively. Images of 100 randomly selected cells (50 cells from each of two replicate slices) were analyzed from each rat, by using a fluorescence microscope equipped with an excitation filter of BP546/12 nm and barrier filter of 590 nm. Cells were also scored visually according to tail size, into five classes, from n tails (0) to maximally long tails (4); this visual scoring resulted in a single DNA damage score for each study group. Therefore, the group damage index was calculated and ranged from 0 (all tails, 100 cells x 4) (Collins et al.,1995; Collis et al., 1997). Damage frequency (%) was calculated by dividing the number of cells with tails by the number of cells without tails.

Protein determination

Protein was measured by the method of Lowry (1951) using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by the Student's t-test or by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the F-test was significant. Pearson linear regression coupled to ANOVA. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. A value of $p<0.05$ was considered to be significant.

RESULTS

Initially, we evaluated the effect of intrastriatal administration of GAA on antioxidant defenses in striatum of rats. Our results showed that GAA administration significantly decreased (45%) TRAP [$t(8)=8.596$, $p<0.01$] (Figure 1). In contrast, GAA did not alter the activities of antioxidant enzymes: CAT [$t(10)=1.255$, $p>0.05$], GPx [$t(12)=0.979$, $p>0.05$] and SOD [$t(8)=0.366$, $p>0.05$] (Table 1).

We also evaluated the effect of intrastriatal administration of GAA on protein oxidation (sulfhydryl and carbonyl contents) and DNA damage (comet assays) in striatum of rats. As can be seen in figure 2(A-B), GAA significantly reduced (20%) total sulfhydryl content (B) [$t(12)=5.142$, $p<0.01$] and did not

alter carbonyl levels (A) [$t(8)=1.172$, $p>0.05$] as compared to control group. Figure 3 shows that GAA infusion did not cause DNA damage in rat striatum [$t(13)=1.155$, $p>0.27$].

Next, we verified the *in vitro* effect of different concentrations of GAA on same parameters of oxidative stress in rat striatum evaluated after GAA administration. Figure 4 shows that 100 μM GAA significantly reduced (40%) TRAP [$F(4,15)=3.12$, $p<0.01$] as compared to control. As can be seen in table 2, GAA significantly inhibited the activity of SOD, at 50 and 100 μM, [$F(4,20)=5.63$, $p<0.01$], but did not alter the activities of CAT [$F(4,25)=0.49$, $p>0.05$] and GPx [$F(4,20)=0.46$, $p>0.05$].

Figure 5 shows GAA significantly reduced that sulfhydryl content (B) at 100 μM [$F(4,28)=3.69$, $p<0.01$], but carbonyl levels (A) [$F(4.25)=0.80$, $p=0.98$] was not altered by this compound when added to assays. Finally, as can be observed in figure 6, the addition of GAA to comet assay did not induce DNA damage. However, we observed a significant reduction of viable cells at 50 and 100 μM after 1h of incubation [$F(4,17)=3.75$, $p<0.05$].

DISCUSSION

GAMT-deficiency is an inherited metabolic disease biochemically characterized by tissue accumulation of GAA. Affected patients present neurological damage whose underlying mechanisms are poorly known (von Figura et al., 2001). In a previous work we demonstrated that GAA infusion increases TBARS (an index of lipid peroxidation) and inhibits Na^+,K^+-ATPase

and creatine kinase activities in striatum of rats, probably by oxidative stress (Zugno et al., 2006). In the present study we investigated the *in vivo and in vitro* effects of GAA on some parameters of brain oxidative stress. We initially investigated the effect of intrastriatal administration of GAA on antioxidant defenses in striatum of rats. Our results showed that GAA significantly reduced TRAP and did not alter enzymatic antioxidant enzymes, suggesting a decrease of non-enzymatic antioxidant capacity (Lissi et al., 1995).

We also investigated the effect of infusion of GAA into striatum on protein carbonyl and sulfhydryl levels in rat striatum. Results showed that GAA significantly decreased protein sulfhydryl (decreased content of total thiol) and did not carbonyl content. These findings suggest that GAA may induce the oxidation of sulfhydryl groups, but not the carbonylation of amino acids residues in proteins. On the other hand, previous studies from our group have showed the administration of intrastriatal of GAA reduces the activities of Na^+, K^+-ATPase and creatine kinase, which are enzymes that contain thiol groups in their structure (Zugno et al., 2006). More studies are necessary to elucidate such mechanisms.

The role of free radicals in cell death is associated to accumulation of cellular oxidatively damaged DNA (Badisa et al., 2007; Tsutsui et al., 2006). It may represent the direct effect of some damaging agent on this molecule. On the other hand, alkaline comet assay allows to detection the DNA damage (Frenzilli et al., 2006). In our study, we verified that the administration of GAA did not induce damage on DNA in striatum of adult rats.

In order to verify whether GAA act directly on the parameters of oxidative stress evaluated *in vivo*, we also verified the *in vitro* effect of

different concentrations of GAA on the same parameters studied after aministration. Results showed that 100 μM GAA significantly decreased TRAP and sulfhydryl content in striatum of rats. On the other hand, 50 and 100 μM GAA decreased SOD activity, but not CAT and GPx. These results suggest that GAA increases superoxide radical *in vitro,* but not *in vivo*. Our results showing that *in vivo* exposure of striatum to GAA provoked no alteration on all antioxidant enzymes activities suggest that the *in vitro* alteration in SOD may occur via distinct mechanisms. It is possible that GAA *in vitro* has a direct effect on SOD and *in vivo* this inhibition may not be detected. Besides, GAA may be in contact with these enzymes for a shorter time than that needed to alter their activities.

The *in vitro* evaluation of GAA in alkaline comet assay showed no effect of this compound on this DNA damage index. However, after 1 h of incubation, there was a significant reduction of viable cells at 50 and 100 μM. This result suggests a citotoxic effect of GAA at these concentrations. However, we did not discard that GAA when added to incubation medium for 1 h (in vitro studies) caused a citotoxic effect since there are poor available cells in samples, explaining the decrease of cells values, this results are similar with data from the literature that suggests no available cells by toxicity effects (Andrade et al., 2004; Rank and Jensen, 2003).

Considering that TRAP measures the content of non-enzymatic antioxidant defenses (Lissi et al., 1995) and GAA was also able to cause oxidative damage on thiol groups in proteins as verified by the significant decrease of sulfhydryl contents, taken together our results indicate that GAA reduces the non-enzymatic antioxidant capacity in rat brain. It is possible that

this mechanism may be involved in the neuropathological findings and motor delayed development observed in the patients affected by GAMT-deficiency. Besides as can be observed in our study, GAA when added to enzymatic assay (*in vitro study)* inhibited SOD activity. This enzyme catalyzes the dismutation of superoxide anion to hydrogen peroxide and water (Lawer and Song, 2000). The generation of superoxide anion induces membrane lipid peroxidation and causes damage to protein and nucleic acids, resulting in tissue injury (Freeman and Crapo, 1982; Ogawa et al., 1982). In agreement with this evidence, we have shown that GAA administraion increases lipid peroxidation in striatum of rats (Zugno et al., 2006).

Considering that oxidative stress can be elicited by the imbalance between free radical production and antioxidant defenses (Schulz et al., 2000), that previous evidences shows that GAA increases free radical formation (Mori et al., 1996; Halliwell, 2001; Hiramatsu 2003) and our present results showing that GAA decreases the brain antioxidant defenses and increases protein damage, we suggest that GAA, metabolite accumulated in GAMT-deficiency, induces oxidative stress in brain. Taken together, it is tempting to speculate that oxidative stress may contribute, at least in part, to the neurological dysfunction found in GAMT-deficiency.

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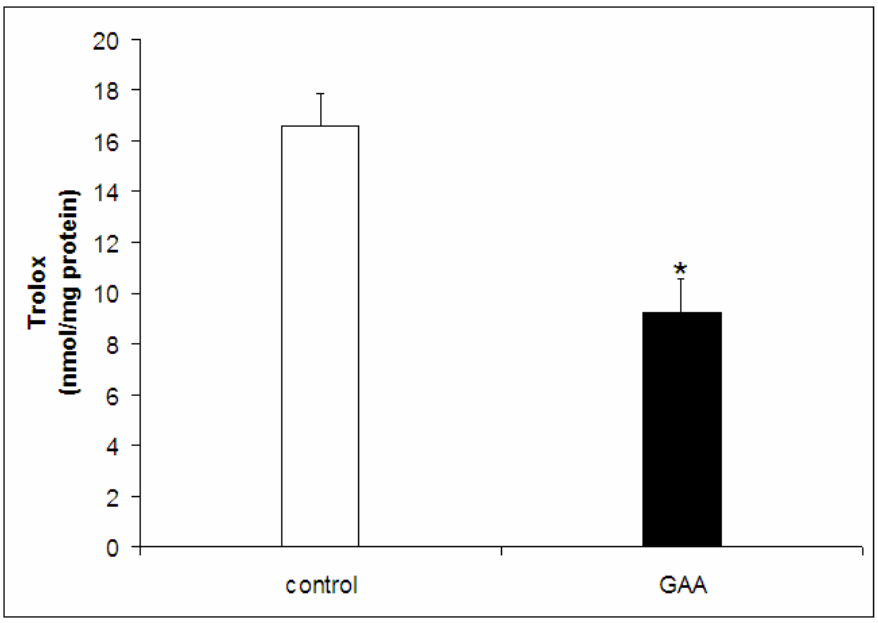

Figure 1. Effect of intrastriatal administration of guanidinoacetate on TRAP in striatum of rats. Data are expressed as mean ± SD of five animals performed in duplicate. *Different from control, $p<0.01$ (Student's *t* test for unpaired samples).

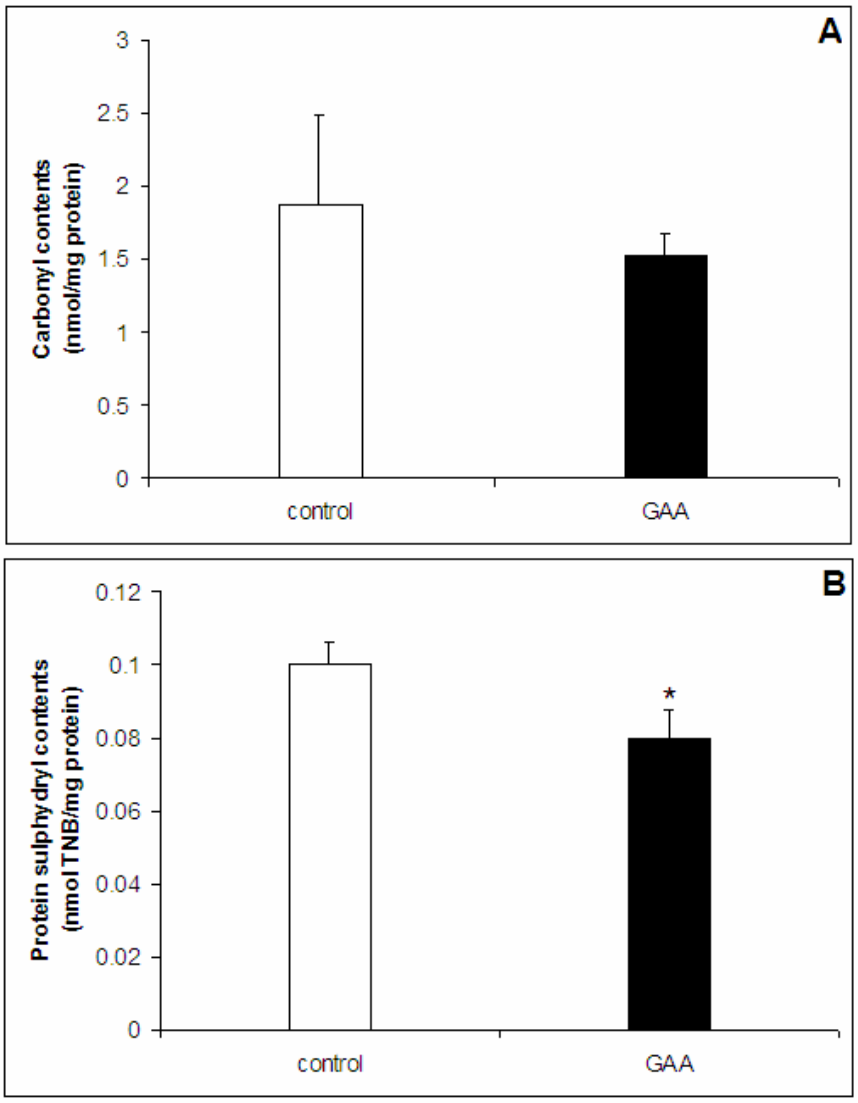

Figure 2. Effect of intrastriatal administration of guanidinoacetate on carbonyl (A) and sulfhydryl (B) contents in striatum of rats. Data are expressed as mean ± SD of five animals performed in duplicate. *Different from control, $p<0.01$ (Student's *t* test for unpaired samples).

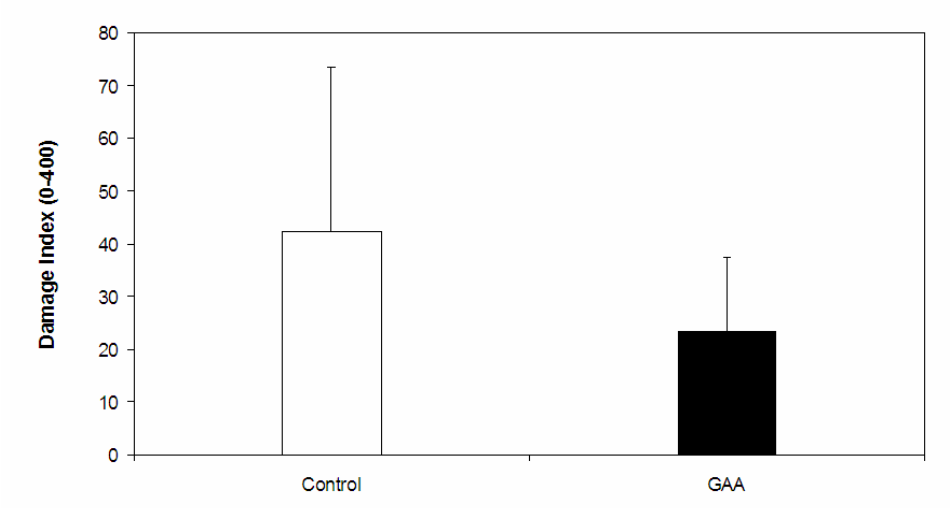

Figure 3. Effect of intrastriatal administration of guanidinoacetate on comet assay in striatum of rats. Data are expressed as mean ± SD of five animals performed in duplicate. Damage index represents mean values obtained from an average of 100 cells per sample.

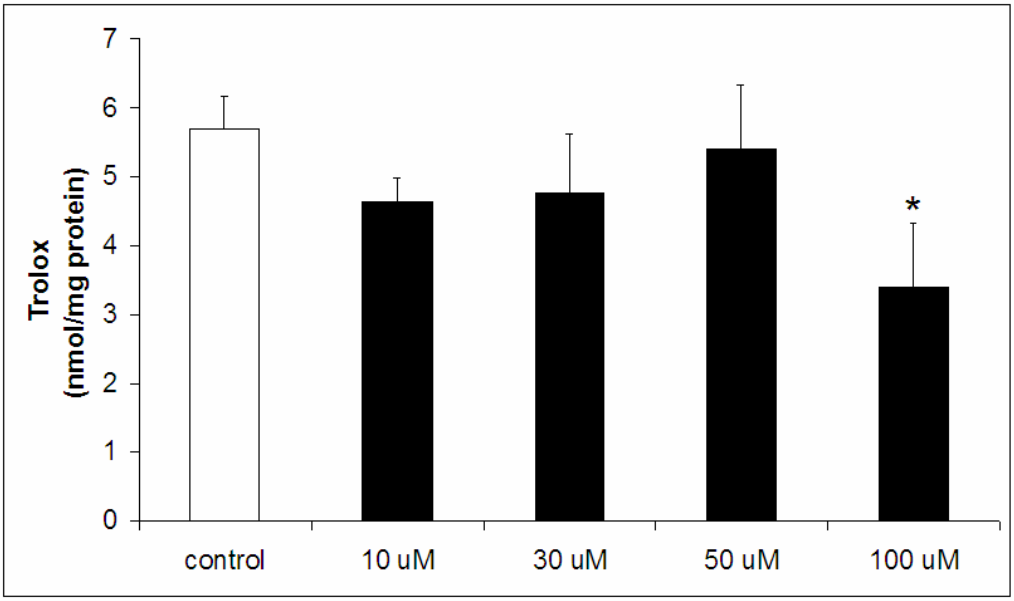

Figure 4. *In vitro* effect of guanidinoacetate on TRAP in striatum of rats. Data are expressed as mean ± SD of five animals performed in duplicate. *Different from control, $p<0.01$ (Duncan´s multiple range test).

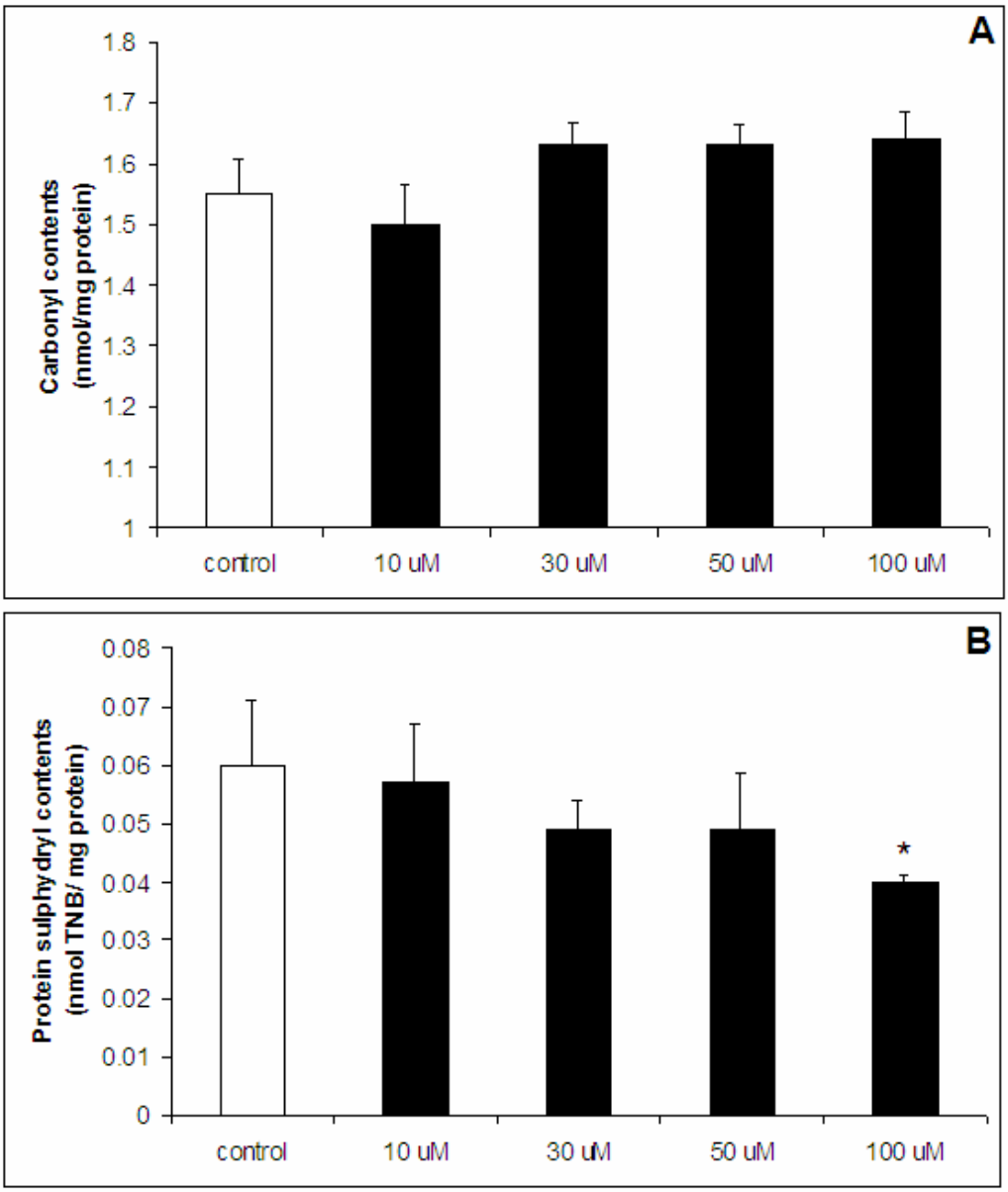

Figure 5. *In vitro* effect of guanidinoacetate on carbonyl (A) and sulfhydryl (B) contents in striatum of rats. Data are expressed as mean ± SD of five animals performed in duplicate. *Different from control, $p<0.01$ (Duncan´s multiple range test).

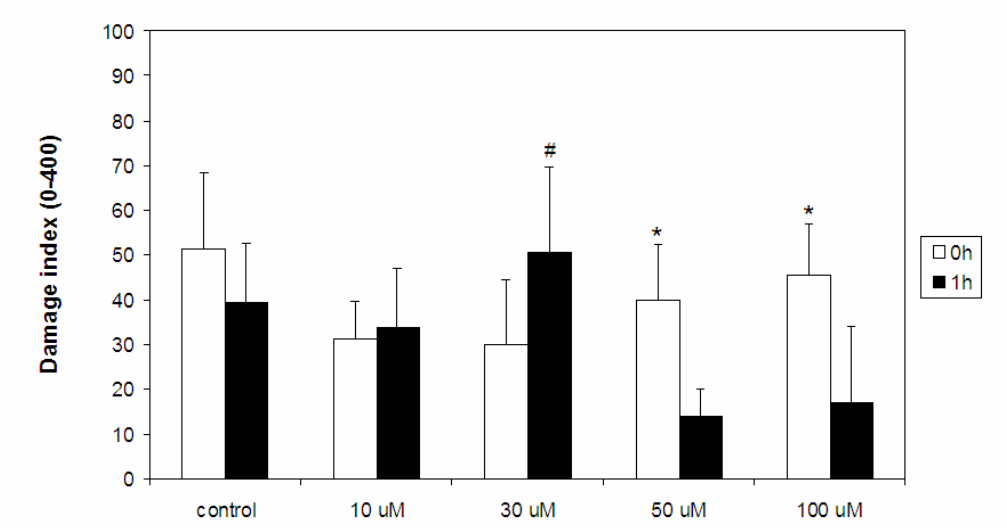

Figure 6. *In vitro* effect of guanidinoacetate on comet assay in striatum of rats. Data are expressed as mean ± SD of five animals performed in duplicate and damage index represents mean values obtained from an average of 100 cells per sample. *Significant in relation to 1 hour, $p<0.05$. #Significant in relation to 50 uM. (*t* test).

Table 1. Effect of intrastriatal administration of guanidinoacetate on catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) in striatum from 60-day-old rats.

Antioxidants enzymes (U/mg protein)	control	GAA
Catalase	0.48 ± 0.03	0.45 ± 0.06
GPx	19.61 ± 6.87	22.77 ± 5.05
SOD	6.58 ± 0.85	6.81 ± 1.15

Values are means ± SD for five to six independent experiments (animals) per group.
*Different from control, $p<0.01$ (Duncan´s multiple range test).

Table 2. In vitro effect of guanidinoacetate on catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) in striatum from 60-day-old rats.

Antioxidants enzymes (U/mg protein) GAA	control	10 uM	30 uM	50 uM	100 uM
Catalase	0.38 ± 0.04	0.40 ± 0.04	0.38 ± 0.05	0.38 ± 0.03	0.37 ± 0.02
GPx	1.68 ± 0.14	1.64 ± 0.38	1.46 ± 0.28	1.61 ± 0.24	1.65 ± 0.33
SOD	11.87 ± 2.74	9.05 ± 3.61	9.17 ± 2.81	5.35 ± 0.57*	5.39 ± 2.41*

Values are means ± SD for five to six independent experiments (animals) per group.
*Different from control, $p<0.01$ (Duncan´s multiple range test).

Artigo 4

Guanidinoacetate *in vitro* inhibits glutamate uptake in rat striatum of rats at different ages

Alexandra I. Zugno, Diogo L. Oliveira, Emilene B.S. Scherer, Moacir Wajner, Susana Wofchuk and Angela T.S. Wyse

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ORIGINAL PAPER

Guanidinoacetate Inhibits Glutamate Uptake in Rat Striatum of Rats at Different Ages

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Abstract Glutamate plays a central role in the excitatory synaptic transmission and is important for brain development and functioning. Increased glutamate levels in the synaptic cleft are related to neuronal damage associated with excitotoxicity. Guanidinoacetate methyltransferase (GAMT) deficiency is an inherited neurometabolic disorder biochemically characterized by tissue accumulation of guanidinoacetate (GAA) and depletion of creatine. Affected patients present epilepsy and mental retardation whose pathogeny is unclear. In the present study we investigated the in vitro and in vivo (intrastriatal administration) effect of GAA on glutamate uptake by striatum slices of developing and adult rats. Results showed that GAA significantly inhibited in vitro glutamate uptake at 50 μM and 100 μM in all ages tested. We also tested the effect of taurine on the inhibition of glutamate uptake caused by GAA. Taurine significantly attenuated the inhibitory effect caused by 50 μM GAA, but did not alter that provoked by 100 μM GAA. Furthermore, intrastriatal administration of a solution of 30 μM GAA (0.06 nmol/striatum) significantly inhibited glutamate uptake by rat striatum slices. Our results suggest that the inhibition of striatal glutamate uptake caused by GAA might be involved in the neuropathology and especially in the acute neurological features present in patients with GAMT-deficiency.

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Keywords GAMT-deficiency · Metabolic disease · Guanidinoacetate · Glutamate uptake · Taurine

Introduction

Guanidinoacetate methyltransferase (GAMT) deficiency is an inborn error of creatine biosynthesis characterized by severe neurological symptoms, including muscular hypotonia, involuntary extrapiramidal movements, mental retardation and generalized convulsions. It is biochemically characterized by brain accumulation of guanidinoacetate (GAA) and deficiency of creatine/phosphocreatine [1].

Although neurological dysfunction commonly found in human GAMT-deficiency has been mainly attributed to reduction of creatine or/and increase of GAA levels [2, 3], the exact mechanisms of brain dysfunction, particularly epilepsy and mental retardation, are poorly understood in this disease. In this context, Schulze and colleagues have suggested that GAA may be epileptogenic in man [3].

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS), being fundamental for normal brain development and function [4, 5]. However, glutamate at high concentrations behaves as a potent neurotoxin. Its toxicity (excitotoxicity) has been related to the neuronal death and neurodegeneration observed in brain ischemia, epilepsy, Huntington's disease and Alzheimer's disease [4, 6, 7]. Extracellular glutamate levels are maintained below neurotoxic levels in the synaptic cleft primarily through its uptake by Na^+-dependent high affinity glutamate transporters present mainly in astrocytes [4, 5, 8].

Taurine in the CNS exerts various functions, including modulation of intracellular calcium, membrane stabilization, osmoregulation and neurotransmition [9]. Beneficial effects of taurine have been described in epilepsy, hypertension and diabettes [10]. It has been also demonstrated that treatment with taurine inhibits ischemia/reperfusion-induced compartment syndrome attenuating oxidative stress injury in rabbits [11]. Besides, we have recently shown that taurine prevents the inhibition of Na^+,K^+-ATPase caused by GAA [12].

In the present study we investigated the in vitro and in vivo (intrastriatal injection) effects of GAA on glutamate uptake by striatum slices of developing and young adult rats in order to clarify the neurological manifestations, particularly seizures, in GAMT-deficients patients. Striatum was used because basal ganglia abnormalities are commonly found in these patients [1].

Material and methods

Animals and reagents

Wistar rats obtained from the Central Animal House of Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil, were used in the experiments. Animals were maintained on a 12/12 h light/dark cycle (lights on from 7 a.m. to 7 p.m.) in an air-conditioned constant temperature (22°C) colony room, and had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Society for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical, St Louis, MO, USA.

In vitro studies

For the in vitro studies, 6-, 14- and 60-day-old Wistar rats were used. The animals were killed by decapitation and the brains were removed and humidified with Hank's balanced salt solution (HBSS). Striatum was rapidly dissected and cut into slices (0.4 mm) by using a McIlwain tissue chopper. Slices were transferred to 24-well culture plates and washed with 300 µl HBSS followed by 280 µl HBSS. Slices were then preincubated at 37°C for 30 min in the absence (control group) or presence of GAA (10–100 µM). Glutamate uptake assays were then performed. In some experiments, slices from striatum of 60-day-old rats were preincubated at 37°C for 30 min with 50 or 100 µM GAA alone or combined with 1.0 mM taurine. The concentration of taurine was chosen according to Qi et al. [13].

In vivo studies

For the in vivo studies, surgery and intrastriatal infusion were performed according to Folbergovà [14] and Zugno et al. [15]. Sixty day-old rats were anesthetized with an intraperitoneal injection of ketamine plus xylazine (100 mg/kg and 14 mg/kg, respectively). The head of the animals were fixed in a stereotaxic apparatus, the skin of the skull was removed and a 27-gauge 9-mm guide cannula was then placed above the striatum (AP: –0.5 mm; L: –2.5 mm; DV: –2.5 mm). The cannula was fixed with jeweler acrylic cement. Experiments were performed 48 h after surgery. A 30-gauge cannula was fitted into the guide cannula and connected by polyethylene tube to a 5 µl Hamilton microsyringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula towards the striatum. The animals were divided into two groups: group 1 (sham group), rats that suffered surgery and received saline solution; group 2 (GAA-treated), rats that received a solution of 10 µM (0.02 nmol/striatum) or 30 µM (0.06 nmol/striatum) GAA. The volume administered intrastrially (saline or GAA solution) was 2 µl. The animals were killed by decapitation without anesthesia at 30 min after injection. Striatal slices were prepared afterwards as described above and glutamate uptake assays were then performed.

Glutamate uptake

Glutamate uptake was performed according to a previous report [16]. The uptake assay was carried out by adding 0.66 μCi ml^{-1} L- [^{3}H] glutamate and 100 µM unlabeled glutamate (final concentration) in 20 µl HBSS at 37°C. Incubation proceeded at 35°C and was stopped after 3 min by two ice-cold washes with 1 ml HBSS, immediately followed by addition of 0.5 N NaOH, which was kept overnight. Incorporated radioactivity was measured using a scintillation counter (Wallac 1400). Sodium-independent uptake was determined in parallel assays using *N*-methyl-D-glucamine instead of sodium chloride. This uptake was substracted from the total uptake to obtain the sodium-dependent uptake. All experiments were performed in triplicate.

Protein determination

Protein was measured by the method of Lowry [17] using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by the Student's *t*-test for unpaired samples or by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the *F* was significant. Pearson linear regression coupled to ANOVA was also used to verify dose-dependent effects. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. A value of $P < 0.05$ was considered to be significant.

Results

First, we studied the in vitro effect of GAA on glutamate uptake into slices of striatum of 60-day-old rats. Figure 1 shows that GAA significantly inhibited this parameter at 50 μM and 100 μM by around 30%, when compared to the control group ($F(4,24) = 4.89$, $P < 0.01$).

We also investigated the in vitro effect of GAA on glutamate uptake by striatum slices from 6- and 14-day-old rats in order to investigate GAA action on glutamate uptake throughout development. Figure 2 shows that a significant decrease (25–40% inhibition) of glutamate uptake caused by 50 and 100 μM GAA occurred in both ages tested (14-day-old rats: ($F(2,28) = 8.16$, $P < 0.01$); 6-day-old animals; ($F(2,29) = 5.87$, $P < 0.01$)).

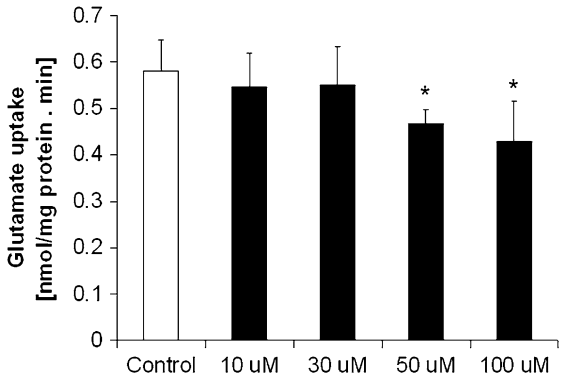

Fig. 1 In vitro effect of guanidinoacetate on glutamate uptake in slices of striatum at 60-day-old rats. Slices were maintained for 30 min in HBSS solution in the presence or absence (control) of the acid followed by 3 min of [^{3}H] glutamate uptake. Values are means ± SEM of six independent experiments carried out in triplicate. $^*P < 0.01$, different from control (Duncan multiple range test). (□) control; (■) guanidinoacetate

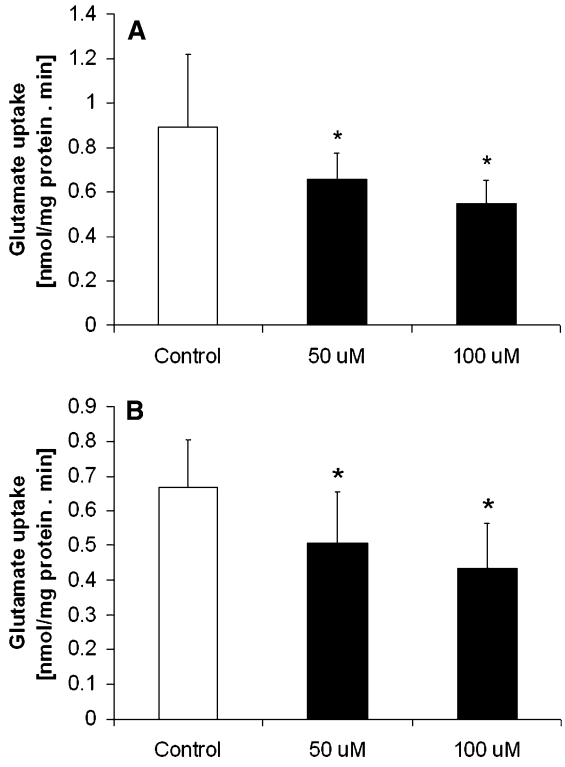

Fig. 2 In vitro effect of guanidinoacetate on glutamate uptake in slices of striatum at 6- (**A**) and 14- (**B**) day-old rats. Slices were maintained for 30 min in HBSS solution in the presence or absence (control) of the acid followed by 3 min of [^{3}H] glutamate uptake. Values are means ± SEM of eight independent multiple range test). (□) control; (■) guanidinoacetate

Next, we evaluated the effect of taurine, a known neuroprotector [18], on the inhibitory effect of GAA on glutamate uptake by striatum slices. Figure 3 shows that taurine fully prefented the inhibitory effect caused by 50 μM GAA on glutamate uptake but not of that provoked by 100 μM GAA ($F(5,34) = 12.57$, $P < 0.01$).

Finally, we tested the in vivo effect of intrastriatal administration of GAA on glutamate uptake into rat striatum slices. Figure 4 shows that infusion of a solution of 10 μM (A) GAA did not alter glutamate uptake (A) [$t(9) = 1.248$, $P = 0.244$], whereas a higher dose (30 μM GAA solution) inhibited glutamate uptake by striatum slices (B) [$t(7) = 3.162$, $P < 0.05$], as compared to control.

Discussion

Tissue accumulation of GAA is the biochemical hallmark of GAMT-deficiency and, besides the low brain levels of creatine, may be involved in the clinical manifestations, particularly the generalized convulsions that occur in patients affected by this disorder.

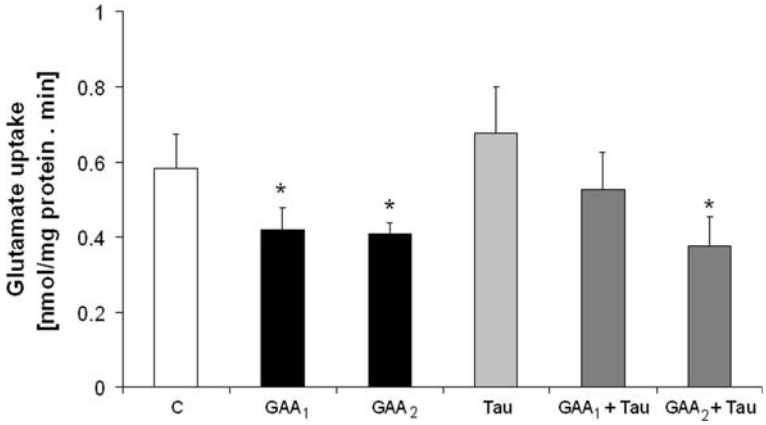

Fig. 3 In vitro effect of guanidinoacetate and taurine on glutamate uptake in slices of striatum at 60-day-old rats. Slices were maintained for 30 min in HBSS solution in the presence or absence (control) of guanidinoacetate or taurine alone or combined followed by 3 min of [^{3}H] glutamate uptake. Values are means ± SEM of seven independent experiments carried out in triplicate. $^*P < 0.01$, different from control (Duncan multiple range test). C = control; GAA_1 = 50 μM guanidinoacetate; GAA_2 = 100 μM guanidinoacetate; Tau = taurine; GAA_1 + -Tau = 50 μM guanidinoacetate plus Taurine; GAA_2 + -Tau = 100 μM guanidinoacetate plus Taurine. (□) control; (■) GAA; (▒) Tau; (▓) GAA + Tau

However, the exact mechanisms underlying the brain dysfunction encountered in GAMT-deficiency are poorly understood [1].

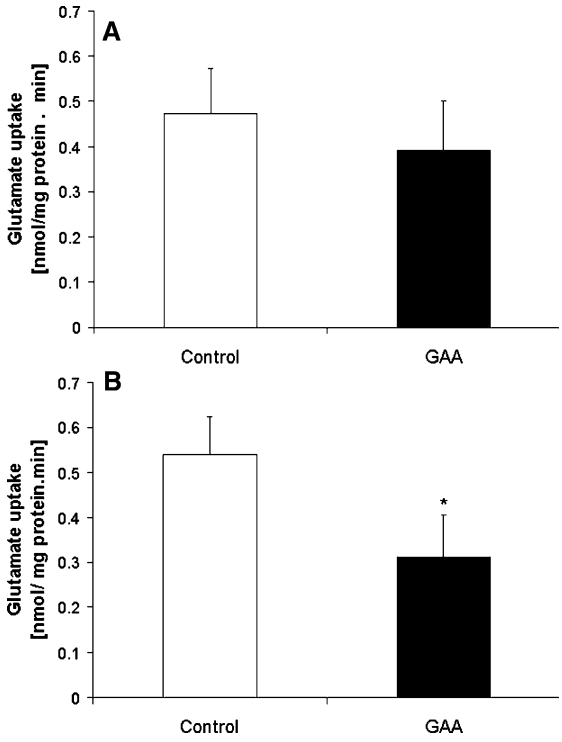

Fig. 4 Effect of intrastriatal administration of guanidinoacetate (GAA) on glutamate uptake by slices of striatum of 60-day-old rats. Two doses of GAA were administered to the animals. (**A**) A solution of 10 μM GAA (0.02 nmol/striatum); (**B**) 30 μM GAA (0.06 nmol/striatum). Values are means ± SEM of six independent experiments carried out in triplicate. $^*P < 0.05$, different from the control group (Student́s *t* test for unpaired samples). (□) control; (■) guanidinoacetate

Under normal conditions glutamate is released into the synaptic cleft and binds to glutamate receptors resulting in the propagation of an action potencial [19]. However, overstimulation of glutamate receptors and alterations in glutamate transporters may occur in neurodegenerative conditions, including epilepsy [4, 5, 19]. Thus, it is not surprising that extracellular glutamate levels must be tightly regulated by removing glutamate from the synaptic cleft, which is mainly performed by astrocytic uptake through high affinity sodium-dependent glutamate transporters [4, 8].

In the present study we tested the in vitro effect of GAA on glutamate uptake into striatum slices from rats of 6, 14 and 60 days of life. Our results showed that GAA markedly inhibited glutamate uptake by striatum at all ages tested.

Although we cannot presently explain the exact mechanism(s) by which glutamate uptake was inhibited by GAA, it may have occurred due to oxidation of astrocytic glutamate transporters since it has been established that GAA induces free radical formation [20, 21] and that reactive species are able to oxidize the high affinity sodium-dependent glutamate transporters localized in the astrocytes, reducing therefore glutamate transport into the astrocytes [22, 23]. An alternative explanation could be energy deprivation caused by GAA, since it has been previously shown that GAA strongly inhibits creatine kinase activity [15], which is crucial for cellular energy transfer. In that case, less ATP would be available for the active transport of glutamate. Furthermore, during energy-limiting conditions, the Na^+ gradient collapses leading to the deficient functioning or even the inverse activation of

glutamate transporters, extruding glutamate to the extracellular space [24–26].

We also investigated whether taurine could reverse the inhibition of glutamate uptake caused by GAA since it is well established that taurine, the most abundant free amino acid in the CNS, protects neural cells against glutamate-induced excitotoxicity [27–29]. It has been also demonstrated that taurine has antioxidant properties, stabilizes membrane and regulates Ca^{2+} influx [30]. In addition, elevated extracelular levels of taurine have been shown to contribute to the maintenance of homeostasis [18] and to ameliorate epileptic symptoms in experimental animals and human [31]. We observed that exposure of striatum slices to 1.0 mM taurine per se did not alter glutamate uptake, but was able to totally prevent the inhibitory effect of 50 μM GAA on glutamate uptake into striatum of 60-day-old rats. However, taurine did not modify the inhibition of glutamate uptake provoked by 100 μM GAA, that could be possibly explained by a single taurine concentration used in our study. The apparent neuroprotective effect of taurine observed in the present study preventing the inhibitory effect of GAA on glutamate uptake may have occurred through a competition between GAA and taurine for the same transporters since guanidino compounds are competitors of taurine uptake [32]. If that is the case, this may be another neuroprotective effect of taurine, especially in situations in which the guanidino compounds are increased in the body. In addition, the antioxidant properties of taurine may have potentially protected oxidative damage caused by GAA to the astrocytic transporters reducing glutamate entrance into these cells.

We cannot also exclude the possibility that taurine could stabilize Na^+,K^+-ATPase activity, an enzyme embedded in plasma membrane and necessary for glutamate transport, as previously shown in retina of diabetic rats [33] and in erythrocyte membrane exposed to ozone [13] and in striatum of rats caused by GAA [15].

We also evaluated the in vivo effect of intrastriatal administration of GAA on glutamate uptake by striatum slices. We observed that the injection of 0.06 nmol/striatum (2 μl of 30 μM GAA) provoked a significant inhibition of this uptake, in contrast with the administration of a 3-fold lower concentration of GAA, which failed to inhibit glutamate uptake. These data suggest a dose-dependent effect for GAA. These data allied to the findings of the in vitro experiments strongly indicate that GAA indeed inhibit glutamate uptake into rat striatum.

Considering that maintenance of homeostasis is crucial for normal brain function and that reduction of glutamate uptake is related to many neurological disorders [4], our findings showing that GAA inhibits glutamate uptake may contribute to understanding of the neurological dysfunction characteristic of GAMT-deficient patients, especially the generalized convulsions (epilepsy) occuring during crises following catabolic states, in which metabolism is accelerated and GAA concentrations tend to increase. Our system of short exposition of striatum to GAA may therefore better represent an acute model of GAMT-deficiency. We also observed that taurine was able to prevent the inhibition of glutamate uptake caused by GAA. However, more studies are necessary to investigate additional mechanisms involved in GAMT-deficiency. It will be also desirable to perform more studies to investigate the in vivo effect of taurine on the clinical manifestations of GAMT-deficient patients as an attempt to evaluate whether this therapy is beneficial to these patients.

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Artigo 5

Guanidinoacetate administration increases acetylcholinesterase activity in striatum of rats and impairs retention of an inhibitory avoidance task

Alexandra I. Zugno, Lenir O. Pereira, Cristiane Mattos, Emilene B. S. Scherer, Moacir Wajner, Carlos A. Netto, Angela T. S. Wyse.

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Abstract

Guanidinoacetate methyltransferase deficiency (GAMT-deficiency) is an inborn error of metabolism characterized biochemically by accumulation of guanidinoacetate (GAA) and depletion of creatine. The pathogenesis of the brain dysfunction in this disorder is not yet established. In the present study we investigated the effect of intrastriatal administration of GAA on acetylcholinesterase (AChE) activity and on acquisition, consolidation and retrieval of memory step-down inhibitory in rat. Results showed that GAA significantly increased AChE activity in rat striatum at 30 min (50%) and 3 h (25%), but not 6 h, after GAA administration. GAA impaired test session performance; indicate that 30 minutes after GAA administration induced impairment of memory acquisition, consolidation and retrieval and, at 6 hours after GAA administration test session shown that GAA administration impairment of retrieval memory. The mechanisms of action of GAA on AChE activity and on memory are unclear and further studies will be necessary to evaluate it but we suggest that the increased of GAA found in GAMT-deficiency patients may be one of the mechanisms involved in the neuronal dysfunction observed.

Key words: Guanidinoacetate; Acetylcholinesterase; Inhibitory avoidance task

1. Introduction

Guanidinoacetate methyltransferase (GAMT) deficiency is an inherited neurometabolic disorder biochemically characterized by tissue accumulation of guanidinoacetate (GAA) and depletion of creatine. Affected patients present severe neurological symptoms, including muscular hypotonia, involuntary extrapiramidal movements, mental retardation and generalized convulsions [22]. Some symptoms such as autistic or self-aggressive behavior have also been related in patients suffering from GAMT-deficiency [20, 21]. However, the exactly pathogeny of this disease is still unclear.

Neurological dysfunction commonly found in human GAMT-deficiency has been mainly attributed to reduction of creatine or/and increase of GAA levels [13,20]. In this context, data from literature suggests that GAA may be epileptogenic in man [19]. Inborn metabolic disorders lead a considerable portion of patients for the morbidity, affecting the central nervous system (CNS) and causing cognitive impairment. Many authors have been use compartmental tasks to clarify the cognitive functions in animal models [3]. Besides, previous studies from our group shows that intrastriatal administration of GAA can decrease important enzymes such as Na^+,K^+-ATPase and creatine kinase in CNS, inhibit glutamate uptake and parameters from energetic metabolism [24,25,26].

It has been described that impaired cholinergic neurotransmission contributes to cognitive deficit and behavioral disturbances, especially in Alzheimer's disease. This disease is characterized by a loss of cholinergic neurons and their projections to the cortex [7,8,11]. Acetylcholinesterase

(AChE) is a acetylcholine-hydrolyzing enzyme, is implicated in cognitive functions and may play important roles in neurodegenerative diseases [1, 12]. In this context, it has been reported that AChE inhibitors are important therapeutic keys to achieve cognitive improvement in Alzheimer's disease patients [12,2]. These agents prevent the hydrolysis of acetylcholine after its release from the terminals of cholinergic neurons [11].

Considering that high levels of GAA are associated with neurological dysfunction, that GAMT-deficiency patients present cognitive impairment, and that AChE activity is related to cognitive processes, in the present study we investigated the acute effect of intrastriatal administration of GAA on AChE activity in rat and on acquisition, consolidation and retrieval of memory step-down inhibitory avoidance task. Striatum was used because patients with GAMT-deficiency present basal ganglia abnormalities [22]

2. Material and Methods

2.1. Animals and reagents

Wistar rats obtained from the Central Animal House of Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil, were used in the experiments. Animals were maintained on a 12/12 h light/dark cycle (lights on from 7 a.m. to 7 p.m.) in an air-conditioned constant temperature (22°C) colony room, and had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Society for Experimental Biology and was approved by the Ethics Committee of the Federal

University of Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical, St Louis, MO, USA.

2.2. In vivo studies

For the *in vivo* studies, surgery and intrastriatal infusion were performed according to Folbergovà and colleagues [6] and Zugno and colleagues [24]. Sixty day-old rats were anesthetized with an intraperitoneal injection of ketamine plus xylazine (100 mg/kg and 14 mg/kg, respectively). The head of the animals were fixed in a stereotaxic apparatus, the skin of the skull was removed and a 27-gauge 9-mm guide cannula was then placed above the striatum (AP: -0.5mm; L: -2.5mm; DV: -2.5mm). The cannula was fixed with jeweler acrylic cement. Experiments were performed 48 h after surgery. A 30-gauge cannula was fitted into the guide cannula and connected by polyethylene tube to a 5 µL Hamilton microsyringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula towards the striatum. The animals were divided into two groups: group 1 (sham group), rats that suffered surgery and received saline solution; group 2 (GAA-treated), rats that received 10 µM GAA solution (0.02 nmol/striatum). The volume administered intrastrially (saline or GAA solution) was 2 µL. For determination of AChE activity, the animals were killed 30 minutes, 3 hours or 6 hours after intrastriatal administration. For behavioral studies, the animals were subjected to training and test session 30 minutes or 6 hours before training, after training or before testing sessions.

2.3. AChE activity assay

AChE activity was determined by the method of Ellman *et al.* [4].

Hydrolysis rate *v* was measured at acetylthiocholine (S) concentration of 0.8 mM in 1-mL assay solutions with 100 mM phosphate buffer (pH 7.5) and 1.0 mM DTNB. Fifty microliters of rat serum was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) at 25±C. All samples were run in duplicate.

2.4. Protein determination

Protein was measured by the method of Lowry [14] using bovine serum albumin as standard.

2.5. Behavioral procedures

2.5.1. Step-down inhibitory avoidance (aversive task): animals were subjected to training and test sessions on a step-down avoidance task, with an interval of 24 h in between [10, 23], carried out in an automatically operated, brightly illuminated box. The left extreme of the grid was covered by a 7.0 cm wide, 2.5 cm high formic platform. Animals were placed on the platform and their latency to step down placing their four paws on the grid (42.0 X 25.0 cm grid of parallel 0.1 cm caliber stainless steel bars spaced 1.0 am apart) was measured.

2.5.2. Open-field habituation (non aversive task): the task was run in a wooden box measuring 60X40X50 cm with a frontal glass wall, whose floor was divided by white lines into 12 squares. The animals were gently placed facing the rear left corner of the arena and observed for 2 minutes. The latency to leave the first square (timidity) and the

defecation (number of stools) were taken as measures of rats emotionality. The number of crossings from one square to another is indicative of motor activity and the number of rearing responses in a measure of habituation [16]. The number of squares crossed with the fours paws, number of rearing responses and number of fecal boli were manually recorded by an observer who was not aware of the subject's condition.

2.6. Statistical analysis

Data from AChE activity were analyzed by the Student's *t*-test for unpaired samples or by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the F was significant. Training-test session latency differences were assessed by the Wilcoxon test followed by individual Mann-Whitney *U*-test. Open field behaviour was analysed by unpaired Student *t*-test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. A value of $p<0.05$ was considered to be significant.

3. Results

First, we studied the effect of intrastriatal administration of GAA on AChE activity. Figure 1 shows that GAA significantly increased AChE activity at 30 min (A) [$t(8)=7.58$, $p<0.05$] and 3 hours (B) [$t(8)=7.76$, $p<0.05$], but not at 6 hours (C) after the administration of this guanidine compound [$t(8)=7.84$, $p=0.841$].

For behavioral studies, GAA was administered into striatum 30 minutes and 6 hours before training, after training and before test session. This study

aimed to investigate memory acquisition, consolidation and retrieval [10].

Figure 2 shows that there were no differences among groups in training session, and saline-treated rats showed improvement of test session latency, when compared to training session, indicating memory for the task (Wilcoxon test, $p<0.05$). GAA impaired test session performance, as showed by Mann-Whitney *U*-test, $p<0.05$. The test session indicates that 30 minutes after GAA administration there was an impairment of memory acquisition (Figure 3A) (Mann-Whitney = -3.261; $p<0.01$) and consolidation (Figure 3B) (Mann-Whitney = -3.059, $p<0.01$), and retrieval memory(C) (Mann-Whitney = -2.607, $p<0.01$). We also tested the effect of GAA administration 6 hours before training, after training and before the test. In the test session we verified that GAA administration impaired retrieval memory (Figure 3C) (Mann-Whitney = -3.027, $p<0.05$).

Finally, we tested the open field behavior task. Rats that received GAA administration did not display changes in any measure taken in the arena, suggesting that the effect in the inhibitory avoidance task is nor due to any impairment in locomotor activity (Table1).

4. Discussion

GAMT-deficiency is an inherited metabolic disease biochemically characterized by tissue accumulation of GAA. Patients present neurological damage whose underlying mechanisms are poorly known [22]. However, many

symptoms such as epilepsy and cognitive impairment are found in patients with GAA-deficiency. We have recently demonstrated that GAA provoked an inhibition of Na^+,K^+-ATPase and creatine kinase activities in rat striatum [24]. Recent studies from our group also showed that glutamate uptake is affected in the presence of GAA in rats at different ages [25].

In the present study we investigated the effect of intrastriatal administration of GAA on AChE activity and on memory of step-down inhibitory avoidance task. We verified that AChE activity was significantly increased 30 min or 3 h after administration of GAA. The cholinergic system plays a crucial role in cognitive function [5]. It has been shown that AChE activity is increased around amyloid plaques in Alzheimer disease and it is suggested that this increase could play a pathogenic role in this disease by influencing the process leading to amyloidal toxicity [15]. In this context, it has also been showed that acetylcholine has a potential neuroprotective role as a scavenger of superoxide anions and is able to reduce lipid peroxidation, which suggests that a decrease of acetylcholine could result in the decrease of neuroprotection that can lead to neurodegeneration [18]. Data from the literature also support the fact that reduced cholinergic activity leads to memory impairment. In this context, the inhibition of AChE, the main enzyme that hydrolyses acetylcholine, increases the neurotransmitter levels in the synaptic cleft and ameliorates cognitive processes [11].

We also observed that GAA impaired acquisition, consolidation and retrieval memory 30 min after GAA administration. We also demonstrated that administration of GAA 6 hours before training caused an impairment of retrieval memory. These results seem to indicate that GAA decrease kinds of different

memory in all times tested. These effects may possible mediated by oxidative stress, since it is known that GAA is able to produce free radicals [9] or by a neurotoxic effect since it was demonstrated that GAA alters neurotransmission [17].Taking together our present findings, it is possible that AChE activity increased by GAA may decrease acetylcholine brain levels. This fact may be related to memory impairment, since this neurotransmitter is important for the processes of memory acquisition and consolidation.

In conclusion, we demonstrated that AChE activity and memory are compromised 30 min or 6 h after GAA injection in striatum. These findings suggest that brain accumulation of GAA may disturb CNS functioning. It may explain, at least in part, the neurotoxicity caused by GAA in patients affected by GAMT-deficiency.

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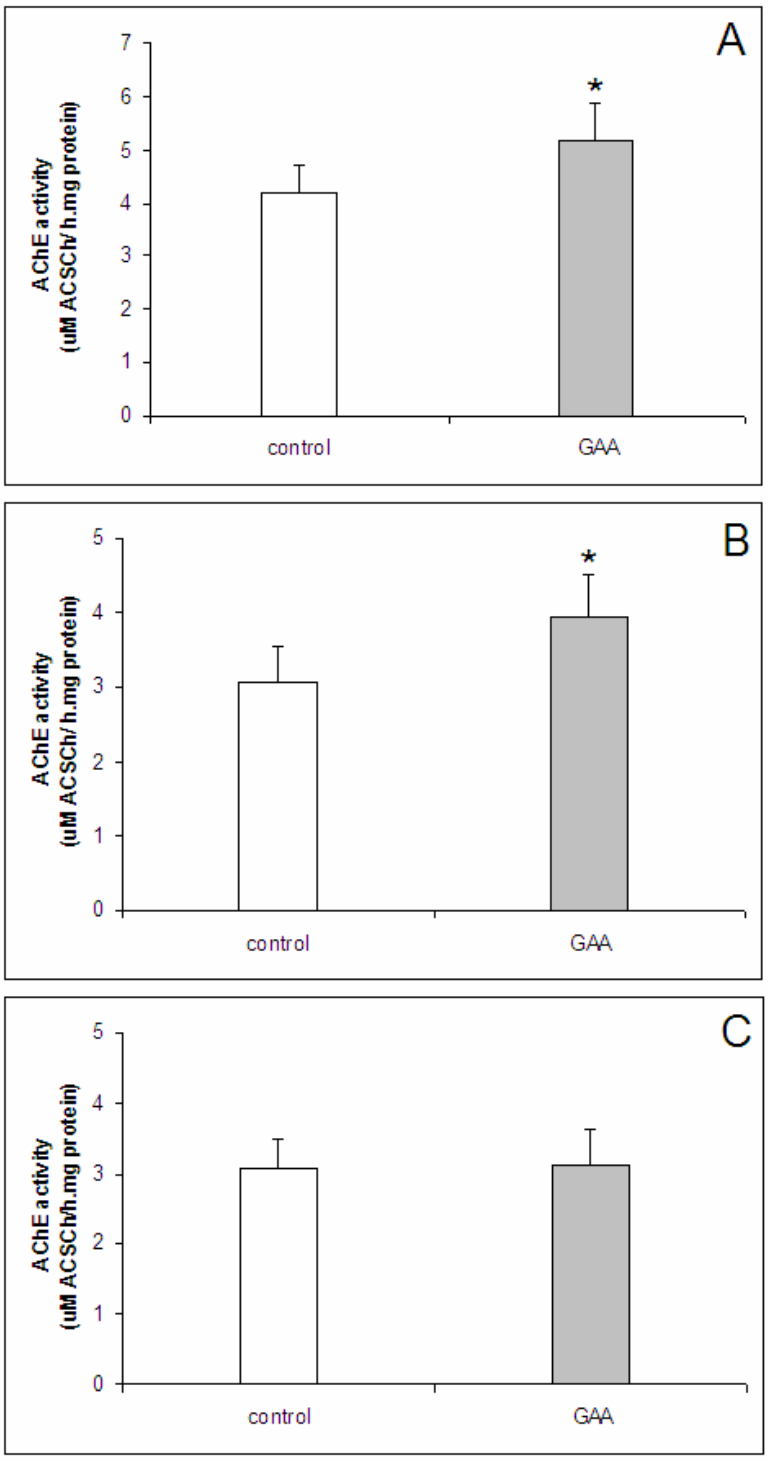

Figure 1. Effect of intrastriatal administration of GAA on acetylcholinesterase activity in striatum of adult rats. Results show the effect at 30 minutes (A), 3 hours (B) and 6 hours (C) after GAA administration. Data are expressed as mean ± SD for five animals in each group. *$p < 0.05$ compared to control group (Student's *t* test for unpaired samples).

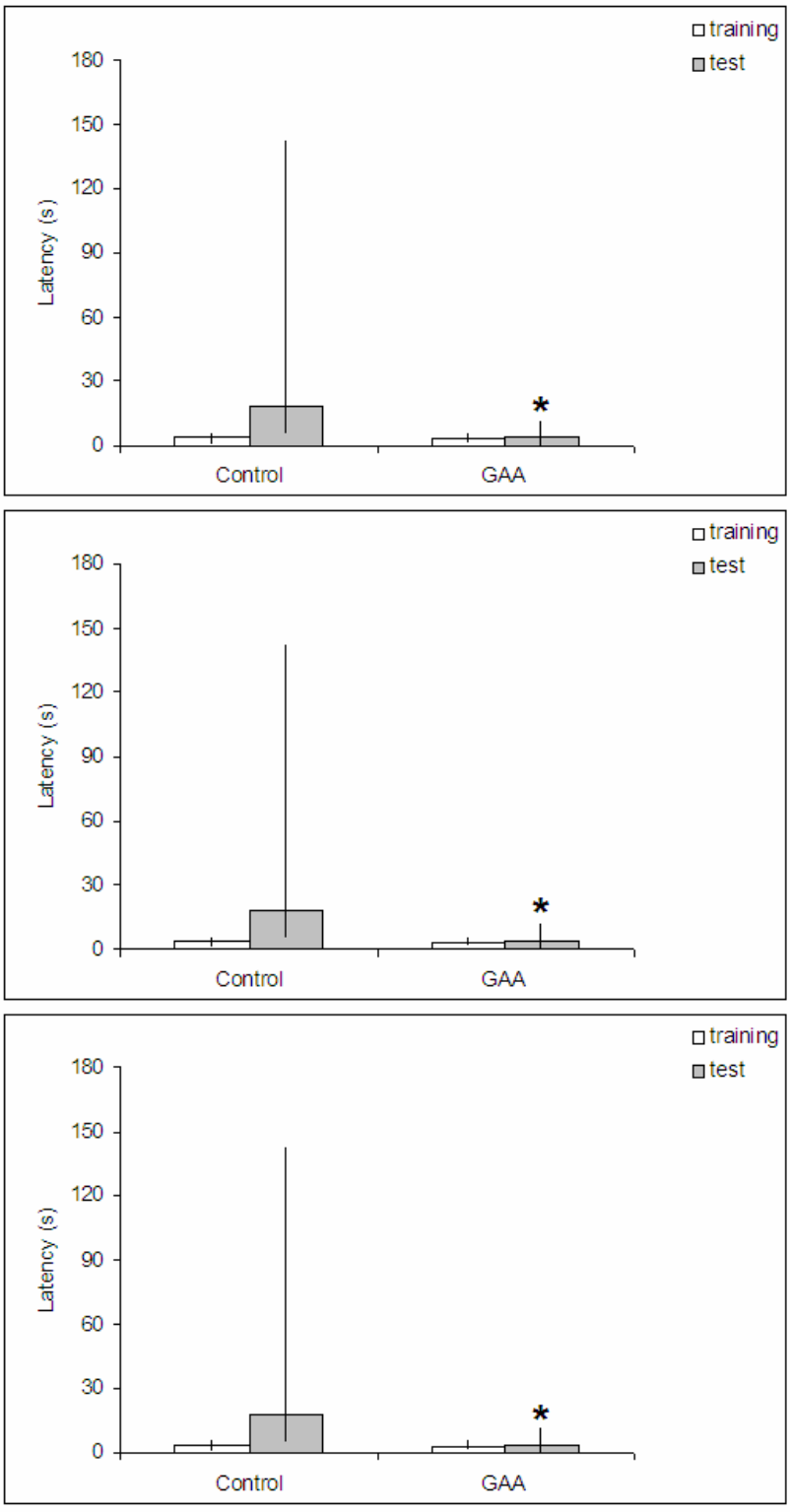

Figure 2. Effect of intrastriatal administration of GAA on test session performance of step-down inhibitory avoidance task. Results show the effect at 30 minutes after GAA administration: before training (A), after training (B) and before test (C) sessions. Data are median (interquartile range) of at least 10 animals each group. Different from the all groups, * $p < 0.05$.

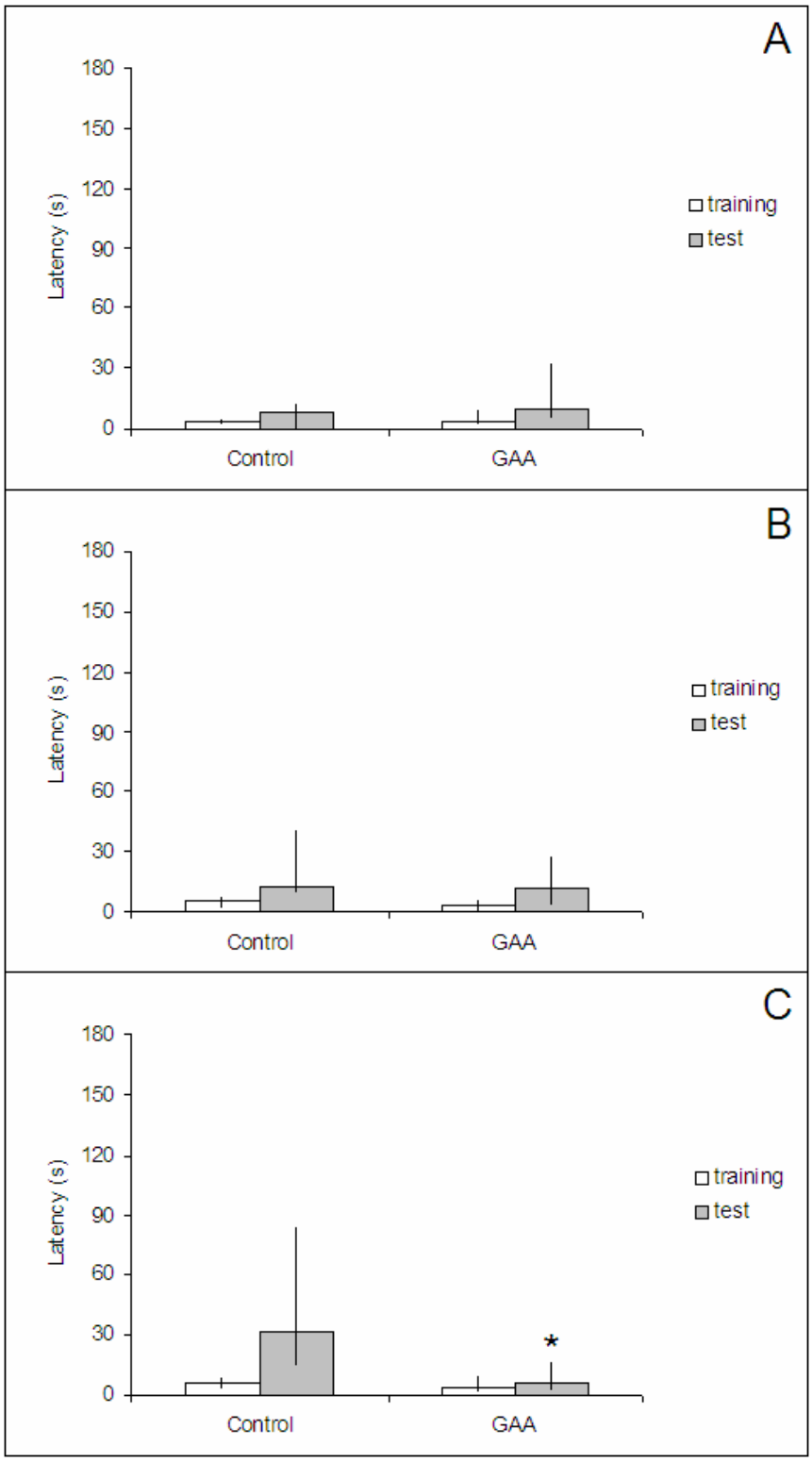

Figure 3. Effect of intrastriatal administration of GAA on test session performance of step-down inhibitory avoidance task. Results show the effect at 6 hours after GAA administration: before training (A), after training (B) and before test (C) sessions. Data are median (interquartile range) of at least 10 animals each group. Different from the all groups, * $p < 0.05$.

Table 1 – Effect of intrastriatal administration of GAA 30 min before training on open field behavior task in adult rats (n=10).

	Treatment	
	Saline	GAA
Latency to leave the first aquare	7.13 ± 5.48	4.4 ± 2.77
Number of rearings	13.2 ± 4.47	14.20 ± 4.6
Number of crossings	32.53 ± 11.24	31.73 ± 5.53
Number of fecal boli	1.86 ± 1.95	1.73 ± 1.53

5. *Discussão*

A deficiência de GAMT é uma doença autossômica recessiva do metabolismo da creatina descrita pela primeira vez por Stöckler e colaboradores em 1994. Junto com a deficiência de AGAT e deficiência de CRTR, formam um conjunto de doenças conhecidas como síndromes de creatina (SCHULZE, 2003). No entanto, dentre as três, a deficiência de GAMT é a única em que se observa acúmulo de GAA nos tecidos dos pacientes afetados (SCHULZE *et al.*, 1997). Essa doença tem como sintomas principais o retardo mental e a epilepsia. Os pacientes também apresentam movimentos involuntários extrapiramidais, provavelmente causados pelas lesões nos gânglios da base (VON FIGURA *et al.*, 2001).

Embora a redução nos níveis cerebrais de creatina contribua para disfunções encontradas em doenças neurodegenerativas (MALCON *et al.*, 2000), atribui-se ao acúmulo de GAA os danos neurológicos encontrados nesses pacientes. Os mecanismos de ação do GAA são pouco conhecidos, mas estudos da literatura mostram que o seu acúmulo provoca danos ao SNC (SCHULZE *et al*., 2001), além de ser capaz de produzir ERO (MORI *et al.*, 1996). Sabe-se que a suplementação oral de creatina em pacientes portadores da deficiência de GAMT aumenta os níveis de creatina e fosfocreatina no cérebro, apresentando efeitos benéficos sobre as manifestações clínicas como, por exemplo, atraso no desenvolvimento, fraqueza muscular e hipotonia. Por outro lado, a suplementação de creatina não provoca melhora das crises epiléticas apresentadas pelos pacientes, sugerindo assim, um papel

fundamental de GAA na patologia dos pacientes afetados com a deficiência de GAMT (STÖCKLER *et al.*, 1996).

O presente trabalho teve por objetivo estudar o efeito da administração intra-estriatal do GAA sobre parâmetros bioquímicos e comportamentais em ratos, bem como avaliar o efeito *in vitro* do GAA sobre os mesmos parâmetros bioquímicos estudados *in vivo*.

Primeiramente, investigamos o efeito da administração intra-estriatal de GAA sobre as atividades da Na^+,K^+-ATPase e CK em estriado de ratos adultos. As frações citosólica e mitocrondrial da CK foram isoladas e testadas para avaliar mais especificamente a inibição. O resultado desse estudo mostra que somente a fração mitocondrial da CK foi inibida pela administração intra-estriatal de GAA. Considerando que a atividade dessas enzimas é crucial para a manutenção da atividade normal do cérebro e a redução das mesmas pode levar a dano neuronal (LEES 1993; TOMIMOTO *et al.*, 1993), nossos resultados mostraram que o GAA provocou uma inibição significativa sobre as atividades da Na^+,K^+-ATPase e da CK após 30 minutos d administração desse composto, mas não 3 horas depois.

A Na^+,K^+-ATPase é uma enzima transmembrana responsável, dentre outras funções, por manter o gradiente iônico necessário para a atividade normal do SNC. Já foi demonstrado que a diminuição na atividade dessa enzima é relacionada a transtornos neurodegenerativos (LIGURI *et al.*, 1990; RENKAWEK *et al.*, 1992; GRISAR *et al.*, 1992; HATTORI *et al.*, 1998; WYSE *et al.*, 2000; YU, 2003) e que ela é inibida por ERO (YOUSEF *et al.*, 2002).

A CK, assim como a Na^+,K^+-ATPase, também é uma enzima fundamental para o funcionamento normal do SNC e a sua relação com

doenças neurodegenerativas vem sendo estudada (TOMIMOTO *et al.*, 1993). Dados encontrados em nosso grupo já mostraram que ácidos orgânicos acumulados em alguns EIM, tais como o ácido metilmalônico e o ácido etilmalônico inibem as atividades da Na^+,K^+-ATPase e CK (WYSE *et al.*, 2000; SCHUCK *et al.*, 2002; LEIPNITZ *et al.*, 2003).

Dados de literatura indicam que o GAA aumenta a produção de ERO (MORI *et al.*, 1996; HIRAMATSU, 2003) e diminui a fluidez de membrana (HIRAMATSU *et al.*, 1992). Considerando que a Na^+,K^+-ATPase é uma enzima de membrana, a atividade dessa enzima pode ser inibida por ERO (LEES, 1993) e que a manutenção da fluidez é uma propriedade importante para a integridade celular (SCHREIER *et al.*, 1978), podemos sugerir que os efeitos causados pelo GAA podem estar envolvidos na inibição da atividade da Na^+,K^+-ATPase. Da mesma forma, é possível presumir que a inibição da atividade da CK pode ser mediada por oxidação de grupos sulfidrila já que esta enzima é composta por resíduos de cisteína em sua estrutura, os quais podem reagir com óxido nítrico ou radicais livres (WOLOSKER *et al.*, 1996).

Para testar essa hipótese e avaliar os possíveis mecanismos pelos quais o GAA é capaz de inibir as atividades da Na^+,K^+-ATPase e a CK, nós investigamos o efeito da administração intraestriatal de GAA sobre o TBARS, que é uma medida de lipoperoxidação. Em trabalhos anteriores, mostramos que a inibição da atividade da Na^+,K^+-ATPase causada por GAA *in vitro* foi prevenida por L-NAME e taurina (ZUGNO *et al.*, 2004). No presente estudo, nós mostramos um aumento na medida de TBARS, 30 minutos após a injeção intraestriatal de GAA, indicando que esse composto guanidínico é capaz de induzir a lipoperoxidação em estriado de ratos.

A inibição das enzimas Na^+,K^+-ATPase e CK observada nesse primeiro estudo, sugere uma diminuição da atividade energética cerebral. Dessa forma, achamos interessante avaliar o efeito *in vivo* e *in vitro* do GAA sobre parâmetros do metabolismo energético, incluindo a produção de CO_2 a partir de acetato [U-^{14}C] e as atividades dos complexos II, II-III, III e IV da cadeia respiratória em estriado de ratos.

Neste trabalho, nós verificamos que a administração intra-estriatal de GAA reduz significativamente a atividade dos complexos II e II-III, mas não altera os complexos III e IV. Também observamos que somente o complexo II foi inibido quando exposto a 50 e 100 µM de GAA *in vitro.* Esses resultados indicam que o GAA é um inibidor seletivo do complexo II. A produção de CO_2 não foi alterada quando testada *in vivo* ou *in vitro* pelo GAA, indicando que a atividade do ciclo do ácido cítrico não é afetada por esse ácido orgânico.

Considerando que o GAA induz o estresse oxidativo (ZUGNO *et al.,* 2004; HIRAMATSU, 2003) e que o complexo II, a Na^+,K^+-ATPase e a CK são susceptíveis ao ataque de radicais livres (WOLOSKER *et al.,* 1996; RUSTIN e RÖTIG, 2002; YOUSEF *et al.,* 2002), é possível dizer que a redução nas atividades dessas enzimas causada por GAA ocorra por indução de estresse oxidativo.

Para testar essa hipótese, foi realizado um pré-tratamento em ratos de 60 dias, durante uma semana com taurina ou com vitaminas E e C. Após o tratamento, foram injetados GAA ou salina por via intra-estriatal. A taurina tem propriedades antioxidante (SENER *et al.,* 2005; ORIYANHAN *et al.,* 2005) e neuroprotetora (SARANSAARI e OJA, 2000). Em nosso grupo já foi demonstrado que a taurina previne a inibição da atividade da Na^+,K^+-ATPase

causada pelo GAA *in vitro* (ZUGNO *et al.,* 2004). As vitaminas E e C são antioxidantes clássicos. A vitamina E é lipossolúvel e interage com membranas, interrompendo o processo oxidativo causado por radicais livres (BURTON *et al.,* 1990; AMES *et al.,* 1993), prevenindo a propagação descontrolada da lipoperoxidação (BURTON *et al.,* 1990). Além disso, a vitamina C é necessária para a regeneração do tocoferol reduzido (FREI *et al.*, 1990; CARR e FREI, 1999).

Nossos resultados mostraram que a taurina preveniu o efeito inibitório causado pela administração intra-estriatal de GAA sobre as atividades dos complexos II e II-III. A inibição da atividade da Na^+,K^+ATPase, bem como o aumento do TBARS, foram prevenidos por ambos antioxidantes, taurina e vitaminas E e C. Finalmente, a inibição da atividade da CK causada pela administração de GAA foi prevenida pelo pré-tratamento das vitaminas E e C, mas não pela taurina. Os mecanismos pelos quais a taurina age ainda não estão totalmente esclarecidos, no entanto, evidências mostram que a taurina age um seqüestrador de EROS (GUPTA *et al.,* 2005) além de estabilizador de membrana (QI *et al.,* 1995). As vitaminas E e C são conhecidas por prevenir a propagação da lipoperoxidação induzida por radicais livres (BURTON *et al.,* 1990). Considerando a prevenção observada na administração desses agentes protetores, esses resultados indicam que o GAA é capaz de causar distúrbios bioenergéticos e induzir o estresse oxidativo.

Devido a forte evidência do GAA causar insultos oxidativos, como foi visto em trabalhos prévios apresentados e descritos na literatura (MORI *et al.*, 1996, ZUGNO *et al.,* 2004) considerou-se necessário investigar o efeito *in vivo* e *in vitro* do GAA sobre alguns parâmetros de estresse oxidativo cerebral.

Inicialmente, nós investigamos o efeito da administração intra-estriatal de GAA sobre as defesas antioxidantes em estriado de ratos (LISSI *et al*., 1995). Nossos resultados mostraram que o GAA reduziu o TRAP, mas não alterou as enzimas antioxidantes, sugerindo uma diminuição da capacidade antioxidante não-enzimática do tecido.

Nós também investigamos o efeito da administração de GAA sobre os níveis de carbonilas e sulfidrilas, parâmetros avaliados para medir a oxidação de em proteínas em estriado de ratos. Os resultados mostraram uma diminuição significativa nos níveis de sulfidrilas, mas não de carbonilas. Esses resultados indicam que o GAA inibe a quantidade de tióis totais das proteínas. Esses achados sugerem que o GAA induz a oxidação de grupos sulfidrila e estão de acordo com estudos prévios de nosso grupo que mostram que o GAA inibe a atividade da Na^+, K^+-ATPase, a qual contém grandes quantidades de grupos sulfidrila em sua estrutura (ZUGNO *et al.*, 2003).

O papel dos radicais livres na morte neuronal vem sendo associado ao acúmulo de DNA de células oxidadas. O teste do cometa nos permite detectar o dano ao DNA (FRENZILLI *et al*., 2006). Nessa parte do trabalho, nós verificamos que a administração de GAA não induz dano ao DNA em estriado de ratos.

Com o objetivo de verificar se o GAA tem um efeito direto sobre os parâmetros de estresse oxidativo estudados *in vivo*, nós testamos os mesmos parâmetros *in vitro,* em diferentes concentrações de GAA. Os resultados mostraram que a concentração de 100 μM de GAA diminui significativamente o TRAP e a quantidade de grupos sulfidrila em estriado de ratos. Por outro lado, as concentrações de 50 e 100 μM de GAA inibiram significativamente a

atividade da SOD, mas não das outras enzimas antioxidantes (catalase e GPx). Esses resultados sugerem que o GAA produz o radical superóxido *in vitro*. Nossos resultados mostraram que a exposição do GAA *in vivo* não provoca alterações em nenhuma das enzimas antioxidantes, sugerindo que a alteração da SOD observada *in vitro* pode ter ocorrido por mecanismos distintos. É possível que o efeito de GAA *in vivo* não tenha sido detectado ou que o GAA tenha ficado pouco tempo em contato com as enzimas.

A avaliação do GAA sobre o ensaio cometa mostrou que não houve dano ao DNA. No entanto, após uma hora de incubação, houve uma redução significativa na quantidade de células viáveis quando a concentração de GAA estava em 50 e 100 μM. Da mesma forma que os estudos *in vivo,* esses resultados sugerem que o GAA tem um efeito citotóxico nessas concentrações. Esses resultados estão de acordo com resultados prévios do nosso grupo sugerindo tal efeito desse ácido (ZUGNO *et al*., 2003; ZUGNO *et al.*, 2004).

Considerando que o estresse oxidativo é o desequilíbrio entre a produção de radicais livres e a capacidade de defesa antioxidantes do organismo (SCHULZ *et al.*, 2000), que relatos na literatura mostram que o GAA é capaz de induzir a formação de radicais livres (MORI *et al.*, 1996; HIRAMATSU, 2003), que este trabalho mostra que o GAA diminui a capacidade de defesas antioxidantes e aumenta o dano a proteínas, nós sugerimos que o GAA induz o estresse oxidativo no cérebro. Dessa forma, outros estudos são necessários para avaliar se o dano oxidativo é capaz de contribuir para a disfunção observada em pacientes com a deficiência de GAMT. Esses mecanismos podem estar envolvidos na neuropatologia e deficiência motora encontrada nos pacientes com a deficiência de GAMT. Além

disso, as alterações *in vitro*, que mostram a inibição na atividade da SOD, reforçam a hipótese de formação de radicais livres tais como o anion superóxido que induz lipoperoxidação e causa dano a proteínas e ao DNA (FREEMAN E CRAPO, 1982; OGAWA *et al.*, 1982).

Muitas hipóteses têm sido estudadas para explicar a fisiopatologia de doenças neurodegenerativas, tais como Alzheimer, Huntington e Parkinson, sem que haja uma explicação satisfatória para o dano cerebral causado por essas doenças. Acredita-se que possíveis mecanismos envolvam deficiência no metabolismo energético, estresse oxidativo e neurotoxicidade mediada por receptores glutamatérgicos do tipo NMDA, ou, possivelmente, um somatório desses fatores (ROSE e HENNEBERRY, 1994).

Com o objetivo de buscar possíveis mecanismos de ação da deficiência de GAMT, estudamos o efeito *in vitro* bem como o efeito da administração intra-estriatal do GAA sobre a captação de glutamato em fatias de estriado de ratos.

O glutamato é o neurotransmissor excitatório mais importante do SNC, é fundamental para o desenvolvimento normal de cérebro e tem diversas funções como excitabilidade, memória e aprendizado (DANBOLT, 2001; SEGOVIA *et al.*, 2001). Sob condições normais, o glutamato é liberado na fenda sináptica, ligando-se a receptores e resultando na propagação do potencial de ação (MARAGAKIS e ROTHSTEIN, 2001). No entanto, a hiperestimulação dos receptores de glutamato bem como alterações nos transportadores podem ocorrer em transtornos neurodegenerativos, tais como a epilepsia (DANBOLT, 2001; SEGOVIA *et al.,* 2001; MARAGAKIS e ROTHSTEIN, 2001). Dessa forma, os níveis de glutamato extracelular devem ser altamente regulados pela

remoção do glutamato da fenda sináptica, que é controlado pelos astrócitos através da recaptação por transportadores de glutamato sódio-dependentes (DANBOLT, 2001; CHEN e SWNASON, 2003).

Inicialmente, nós testamos o efeito *in vitro* do GAA sobre a captação de glutamato em fatias de ratos de 6, 14 e 60 dias. Nossos resultados mostram que o GAA inibiu a captação de glutamato em todas as idades testadas nas concentrações de 50 e 100 μM. Embora o mecanismo pelo qual a captação de glutamato foi inibida não possa ser totalmente explicado, e considerando que o GAA capaz de formar ERO (MORI *et al.,* 1996), essa alteração pode ter ocorrido devido à oxidação dos transportadores de glutamato, já que estes são susceptíveis ao ataque de radicais livres (BEGNI, *et al*., 2004; SALA *et al*., 2005). Uma outra possível explicação pode ser a diminuição de energia causada pelo GAA já que esse composto inibe a atividade da CK. Além disso, com uma diminuição nos níveis de ATP, acarreta em diminuição da atividade da Na^+,K^+-ATPase e o gradiente de sódio pode entrar em colapso ativando o transporte reverso, liberando ainda mais glutamato na fenda sináptica (SZATKOWSKI *et al*., 1990; ATTWELL *et al.*, 1993; ROSSI, *et al*., 2000).

Com o intuito de observar uma possível prevenção da inibição da captação de glutamato causada pelo GAA, nós investigamos o efeito da taurina sobre esse parâmetro. Como foi descrito anteriormente, a taurina é um aminoácido abundante no SNC que tem propriedades antioxidantes, estabilizando membranas e regulando o influxo de cálcio (PETROSIAN e HAROUTOUNIAN, 2000). Além disso, há dados mostrando que esse amino ácido protege os neurônios da excitotoxicidade induzida por glutamato (FARIELLO *et al*., 1982; FRENCH *et al.*, 1986; TRENKNER, 1990), ajuda a

manter a homeostase (SARANSAARI e OJA, 2000) e melhora crises epiléticas em modelos animais e em humanos (KONTRO e OJA, 1987). No presente estudo, nós observamos que a exposição de fatias de estriado a 1,0 mM de taurina não altera a captação de glutamato, mas foi capaz de prevenir o efeito inibitório causado por 50 μM de GAA em ratos de 60 dias. No entanto, a taurina não foi capaz de prevenir a inibição provocada por 100 μM de GAA, o que pode ser explicado pela grande quantidade de GAA utilizada nesse estudo.

O efeito protetor da taurina no nosso trabalho pode ter ocorrido por competição entre o GAA e a taurina pelo mesmo receptor, já que os compostos guanidínicos são competidores da recaptação de taurina (FROSINI *et al.,* 2003). A taurina também pode ter atuado como estabilizador da Na^+,K^+-ATPase (QI *et al.,* 1995; DI LEO *et al.,* 2002), enzima necessária para o transporte de glutamato, protegendo assim, a recaptação do mesmo para os astrócitos.

Nós também testamos o efeito da administração intra-estriatal de GAA sobre a captação de glutamato. Inicialmente, uma dose de 10 μM de GAA foi testada, mas não foi capaz de inibir a captação de glutamato. Dessa forma, decidimos utilizar uma dose maior (30 μM) e assim verificamos uma diminuição na captação de glutamato.

Pacientes com a deficiência de GAMT apresentam dano neurológico e distúrbios cognitivos (VON FIGURA *et al.*, 2001). Por outro lado, sabe-se que o sistema colinérgico desempenha um papel fundamental na função cognitiva (EVERITT *et al*, 1997) e que o aumento da atividade da AChE nas placas amilóides na doença de Alzheimer já foi descrito e parece ter um papel importante na patogenia desta doença (MELO *et al.,* 2002). Além disso, dados

mostram que a ACh tem um efeito protetor atuando como seqüestrador de ânios superóxido e reduzindo a lipoperoxidação (PILLAY *et al.,* 2003).

Com o objetivo de investigar possíveis alterações na memória dos animais submetidos ao modelo GAMT deficiência, investigamos o efeito da administração intra-estriatal de GAA sobre a atividade da AChE em estriado de ratos e sobre a tarefa aversiva de esquiva inibitória em ratos adultos. Resultados mostraram que a atividade da AChE aumentou significativamente 30 minutos e 3 horas após a administração de GAA.

Em relação à tarefa comportamental (esquiva inibitória), testamos as memórias de aquisição, consolidação e evocação e, observamos que a administração intra-estriatal de GAA foi capaz de alterar essas memórias 30 minutos após sua administração. Também observamos que 6 horas após a administração de GAA houve alteração na memória de evocação. Esses resultados indicam que o GAA diminui tipos de diferentes memórias em todos os tempos testados. Esses efeitos podem ser mediados por produção de radicais livres (HIRAMATSU, 2003) ou por possíveis efeitos neurotóxicos causados pelo GAA (NEU *et al.*, 2002).

Considerando os resultados encontrados no nosso trabalho, é possível que o aumento da atividade da AChE causado pelo GAA diminua os níveis de ACh cerebrais. Esse fato pode estar relacionado com as alterações de memória encontradas já que a ACh é um importante neurotransmissor nos processos de memória.

Os resultados apresentados sugerem que a inibição da atividade da Na^+,K^+-ATPase, da CK, do complexo II, além das alterações na recaptação de glutamato e do estresse oxidativo causados pelo acúmulo de GAA podem

mediar o funcionamento normal de SNC. Foi mostrado também que as vitaminas E e C, bem como a taurina podem prevenir essas modificações. Os dados apresentados podem explicar, ao menos em parte, as alterações encontradas nos pacientes com a deficiência de GAMT.

6. Conclusões

1. A administração intraestriatal de GAA inibiu a atividade da Na^+,K^+-ATPase, e da CK bem como aumentou o TBARS após 30 minutos de administração em estriado de ratos adultos.
2. O GAA alterou parâmetros de metabolismo energético tais como complexo II e complexo II-III tanto *in vitro* quanto 30 minutos após a administração intraestriatal de GAA.
3. O tratamento prévio de uma semana de taurina ou vitaminas E e C foi capaz de prevenir os efeitos causados pela administração intraestriatal de GAA sobre as atividade da Na^+,K^+-ATPase, da CK, do complexo II e do TBARS.
4. A administração intra-estriatal de GAA foi capaz de produzir radicais livres mostrados na alteração de parâmetros como o TRAP, TBARS e sulfidrilas e enzimas antioxidantes.
5. O GAA *in vitro* inibiu a recaptação de glutamato em fatias de estriado de ratos de idades diferentes (6, 15 e 60 dias). Também foi capaz de alterar a recaptação de glutamato após 30 minutos de administração intra-estriatal de GAA na concentração de 30 µM.
6. A atividade da AChE 30 minutos e 3 horas após a administração de GAA em estriado de ratos de 60 dias.
7. As memórias de aquisição, consolidação e evocação foram alteadas após 30 minutos de administração de GAA.

8. A memória de evocação foi alterada após 6 horas de administração de GAA.

Conclusão Geral

A inibição de parâmetros de metabolismo energético (Na^+,K^+-ATPase, CK, complexo II), a produção de radicais livres e a diminuição de defesas antioxidantes, o aumento da toxicidade glutamatérgica e a alteração de memória induzidas pelo GAA parecem ser mecanismos envolvidos na disfunção neuronal observada nos pacientes com a deficiência de GAMT.

7. Perspectivas

1. Estudar o efeito da administração intra-estriatal de GAA sobre outros parâmetros comportamentais, tais como a tarefa do labirinto aquático de Morris.
2. Investigar o efeito de antioxidantes e taurina sobre as alterações comportamentais em ratos submetidos à administração de GAA.
3. Verificar se a administração de creatina é capaz de prevenir as alterações comportamentais e bioquímicas em animais submetidos à administração de GAA.

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