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A INFLUÊNCIA DO PROCESSO INFLAMATÓRIO NAS CONVULSÕES E NO DÉFICIT COGNITIVO INDUZIDOS PELO ÁCIDO GLUTÁRICO EM RATOS JOVENS

TESE DE DOUTORADO

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Santa Maria, RS, Brasil

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por

Danieli Valnes Magni

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do grau de **Doutor em Ciências Biológicas: Bioquímica Toxicológica.**

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Universidade Federal de Santa Maria
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A Comissão Examinadora, abaixo assinada,
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Como requisito parcial para a obtenção do grau de
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Essa tese é dedicada a minha mãe Neuza.

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“O saber é que nada se sabe.
Esta é a definição do verdadeiro conhecimento.”
Confúcio (551- 479 a.c.), filósofo chinês

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

A INFLUÊNCIA DO PROCESSO INFLAMATÓRIO NAS CONVULSÕES E NO DÉFICIT COGNITIVO INDUZIDOS PELO ÁCIDO GLUTÁRICO EM RATOS JOVENS

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A acidemia glutárica tipo I (GA-I) é um erro inato do metabolismo (EIM) caracterizada bioquimicamente pelo acúmulo principal de ácido glutárico (GA) e patologicamente por uma característica degeneração estriatal. As manifestações clínicas são predominantemente neurológicas, e desenvolvem-se principalmente na infância (até os 5 anos de idade). Entre estas alterações, destacam-se as convulsões e os déficits cognitivos, os quais podem ser precipitados por processos infecciosos. A partir disso, a primeira hipótese a ser testada neste estudo foi investigar se o lipopolissacarídeo sorotipo E. coli 055 B5 (LPS; 2 mg/Kg; i.p.), um agente inflamatório, facilitaria as convulsões induzidas pelo GA em ratos jovens. Para isso, primeiramente determinou-se a dose intraestriatal aguda de GA (1.3 µmol/estriado) que causa convulsões comportamentais e eletroencefalográficas (EEG) em ratos jovens (21 dias). Em seguida foi verificado que a administração de LPS 3 horas antes da injeção intraestriatal de GA não alterou as convulsões, mas quando o LPS foi administrado 6 horas antes do GA, ele reduziu a latência e aumentou a duração das convulsões comportamentais e EEG induzidas pelo GA em ratos jovens. Observou-se também que injeção de LPS causou uma queda inicial na temperatura retal dos ratos jovens (até 2 horas), seguida de uma elevação na temperatura que iniciou em 3 horas e permaneceu alta até 6 horas após a injeção de LPS. Além disso, foi verificado que injeção de LPS 3 e 6 horas antes da injeção intraestriatal de GA causou um aumento nos níveis estriatais de IL-1β nos ratos jovens, sendo esse aumento estatisticamente maior em 6 do que em 3 horas. Também foi observado que o aumento nos níveis estriatais de IL-1β, causado pela administração de LPS, correlacionou-se positivamente com o tempo total de convulsões. Por fim, verificou-se que uso prévio do anticorpo da IL-1β preveniu a redução da latência e o aumento da duração das convulsões causadas pela administração de LPS 6 horas antes da injeção intraestriatal de GA nos ratos jovens. Assim, estes achados sugerem que a sinalização da IL-1β presente no processo inflamatório produzido pelo LPS contribui decisivamente para a hiperexcitabilidade neuronal e, consequentemente, para a redução da latência e o aumento da duração das convulsões induzidas pelo GA. Dessa maneira, tratamentos farmacológicos específicos que bloqueiam a superprodução ou as funções da IL-1β na GA-I, podem representar uma estratégia

não convencional para o tratamento dessa patologia. Entretanto, estudos clínicos devem ser realizados a fim de avaliar a eficácia desse tratamento nos pacientes glutaricoacidêmicos que apresentam convulsões. Desde que os pacientes com GA-I apresentam outras alterações neurológicas importantes além das convulsões, como prejuízos cognitivos, a segunda hipótese a ser testada neste estudo foi verificar se o tratamento crônico com GA (5 µmol/g; s.c.; duas vezes por dia; do 5° ao 28° dia de vida) causaria déficit de memória espacial em ratos jovens, bem como se a inflamação produzida pelo LPS (2 mg/Kg; i.p.; uma vez por dia; do 25° ao 28° dia de vida) facilitaria o déficit cognitivo induzido pelo GA. Além disso, também foi objetivo avaliar o impacto desses tratamentos sobre possíveis alterações funcionais e estruturais no hipocampo desses animais. Inicialmente verificou-se que o tratamento crônico com GA, assim como os tratamentos com LPS e GA-LPS, causaram um déficit no aprendizado espacial dos ratos jovens. No entanto, foi observado que o tratamento com GA-LPS produziu um maior prejuízo na memória espacial comparado com os outros tratamentos. Em seguida foi observado que nenhum dos tratamentos alterou o peso ou a atividade locomotora/exploratória dos animais. Verificou-se também que o tratamento crônico com GA, assim como os tratamentos com LPS e GA-LPS, aumentaram os níveis hipocampais de IL-1β e TNF-α nos ratos jovens. Além disso, foi observado que tratamentos com GA, LPS e GA-LPS causaram uma redução no volume hipocampal total dos ratos jovens. Finalmente verificou-se que os tratamentos com GA, LPS e GA-LPS causaram uma redução na atividade da subunidade α1 da enzima Na^+,K^+-ATPase. Por outro lado, foi observado que os tratamentos com GA e LPS causaram um aumento na atividade das subunidades α2/3 da enzima. Assim, somente o tratamento com GA-LPS apresentou uma redução na atividade total da enzima Na^+,K^+-ATPase no hipocampo dos ratos jovens. Estes dados indicam que o prejuízo no aprendizado espacial observado nos ratos tratados com GA, LPS e GA-LPS parece estar relacionado a um aumento nos níveis de citocinas inflamatórias, a uma redução no volume hipocampal e a uma inibição na atividade da subunidade α1 da enzima Na^+,K^+-ATPase. No entanto, o maior prejuízo na memória espacial observado nos ratos tratados com GA-LPS ocorreu devido a inibição na atividade total da enzima Na^+,K^+-ATPase, que foi específica das isoformas α2/3, já que somente este grupo não apresentou resposta compensatória na atividade destas subunidades. Portanto, esta segunda parte do estudo demonstrou que o tratamento crônico com GA causou um déficit no aprendizado espacial de ratos jovens, e que a presença de um processo inflamatório potencializou o prejuízo na memória espacial induzida pelo GA sozinho. Assim, o entendimento dos mecanismos envolvidos nas convulsões e no déficit cognitivo observados nos paciente com GA-I frente a um processo inflamatório é importante para o desenvolvimento de novas terapias para o tratamento dessa patologia, bem como de outras doenças associadas à presença de mediadores inflamatórios.

Palavras-chave: ácido glutárico; LPS; IL-1β; TNF-α; convulsões; estriado; memória espacial; hipocampo.

ABSTRACT

Thesis of Doctor's Degree
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Federal University of Santa Maria, RS, Brazil

THE INFLUENCE OF THE INFLAMMATORY PROCESS IN SEIZURES AND COGNITIVE DEFICIT INDUCED BY GLUTARIC ACID IN YOUNG RATS

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Glutaric acidemia type I (GA-I) is an inborn error of metabolism (EIM), characterized biochemically by major accumulation of glutaric acid (GA) and pathologically by a characteristic striatal degeneration. The clinical manifestations are mainly neurological and develop during childhood (up to 5 years old). Among these changes, there are the seizures and cognitive deficits, which may be precipitated by infectious processes. From this, the first hypothesis to be tested in this study was to investigate whether lipopolysaccharide E. coli 055 B5 serotype (LPS; 2 mg/Kg; i.p.), an inflammatory agent, could facilitate seizures induced by GA in young rats (21 days of life). For this, firstly it was determined the acute dose of intrastriatal GA (1.3 µmol/striatum) that cause behavioral and electroencephalographic (EEG) seizures in young rats. Moreover, it was shown that LPS administration 3 hours before GA intrastriatal injection did not change the seizures, but when LPS was administered 6 hours before the GA, it reduced the latency and increased the duration of behavioral and EEG seizures induced by GA in young rats. It also was observed that LPS injection caused an initial drop in rectal temperature of young rats (up to 2 hours), followed by a rise in temperature that started at 3 hours and remained high until 6 hours after LPS injection. Furthermore, it was shown that LPS injection 3 and 6 hours before intrastriatal injection of GA caused an increase in striatal levels of IL-1β in young rats, and this increase was statistically higher in 6 than in 3 hours. In addition, it was observed that the increase in IL-1β striatal levels, caused by LPS administration, positively correlated with total time of seizures. Finally, it was observed that previous use of IL-1β antibody prevented the latency reduction and the increased duration of seizures caused by LPS administration 6 h before intrastriatal injection of GA in young rats. Thus, these findings suggest that the signaling of IL-1β present in inflammation produced by LPS contributes significantly to neuronal hyperexcitability, and thus to reduce latency and increase the duration of seizures induced by GA. Therefore, pharmacological treatments that block the specific functions or overproduction of IL-1β in GA-I, may represent an unconventional strategy to treat this condition. However, clinical studies should be conducted to evaluate the effectiveness of treatment in glutaricoacidemic patients with convulsions. Since patients with GA-I have other important neurological changes addition to the seizures, as

cognitive impairments, the second hypothesis to be tested in this study was to determine whether chronic treatment with GA (5 µmol/g; s.c.; twice per day; from the 5th to the 28th day of life) could cause spatial memory impairment in young rats, and verify whether the inflammation produced by LPS (2 mg/Kg; i.p.; one per day; from the 25th to the 28th day of life) could facilitate the cognitive deficit induced by GA. In addition, it also was evaluated the possible impact of these treatments on functional and structural changes in the hippocampus of these animals. Initially it was shown that chronic treatment with GA, as well as the treatments with LPS and GA-LPS, caused a deficit in spatial learning of young rats. However, it was demonstrated that the treatment with GA-LPS produced a greater impairment in spatial memory compared to other treatments. In addition, it was observed that none of the treatments affected weight or locomotor activity/exploratory of animals. It also was shown that chronic treatment with GA, as well as treatments with LPS and GA-LPS, increased the hippocampal levels of IL-1β and TNF-α in young rats. Furthermore, it was demonstrated that treatments with GA, LPS and GA-LPS caused a reduction in total hippocampal volume of young rats. Finally it was observed that treatments with GA, LPS and GA-LPS caused a reduction of α1 subunit activity of Na^+,K^+-ATPase enzyme. On the other hand, it was shown that treatments with GA and LPS caused an increase in activity of α2/3 subunits of the enzyme. Thus, only treatment with GA-LPS showed a reduction in total activity of Na^+,K^+-ATPase in the hippocampus of young rats. These data indicate that the impairment in spatial learning observed in rats treated with GA, LPS and GA-LPS was due to increased levels of inflammatory cytokines, the reduction in hippocampal volume and the inhibition of α1 subunit activity of Na^+,K^+-ATPase enzyme. However, the worsening in spatial memory observed in rats treated with GA-LPS was due to inhibition of total activity of Na^+,K^+-ATPase, which was specific α2/3 isoforms, since only this group showed no compensatory response the activity of these subunits. Therefore, this second part of the study showed that chronic treatment with GA caused a deficit in spatial learning in young rats, and that the presence of an inflammatory process increased the impairment in spatial memory induced by GA alone. Thus, understanding the mechanisms involved in seizures and cognitive deficits observed in patients with GA-I in the presence of an inflammatory process is important for the development of new therapies to treat this condition, as well as other diseases associated with the presence of inflammatory mediators.

Keywords: glutaric acid; LPS; IL-1β; TNF-α; seizures; striatum; spatial memory; hippocampus.

LISTA DE FIGURAS E TABELAS

3. REVISÃO BIBLIOGRÁFICA

4. RESULTADOS

4.1. Capítulo I

4.2. Capítulo II

LISTA DE ABREVIATURAS

3-OH-GA – ácido 3-hidroxiglutárico
AIDS – síndrome da imunodeficiência adquirida
AMPA – α-amino-3-hidróxi-5-metil-4-isoxazol propiônico
ATP – trifosfato de adenosina
CNQX – 6-ciano-7-nitroquinoxalina-2,3-diona
CO_2 – dióxido de carbono
CREB – proteína ligante do elemento responsivo ao AMPc
DNQX – 6,7-dinitroquinoxalina-2,3-diona
EEG – eletroencefalográficos
EIM – erros inato do metabolismo
GA – ácido glutárico
GA-I – Acidemia Glutárica tipo I
GABA – ácido γ-aminobutírico
GCDH – glutaril-CoA desidrogenase
HMBG1 – grupo caixa-1 de alta mobilidade
ICE – enzima conversora de interleucina-1
i.c.v. – intracerebroventricular
IL-1β – interleucina 1β
IL-6 – interleucina 6
IL-1Ra – antagonista do receptor da IL-1
IL-1R1 – receptor da interleucina-1
i.p. – intraperitoneal
KA – ácido caínico
LPS – lipopolissacarídeo
LTP – potenciação de longa duração
NMDA –*N*-metil-D-aspartato
PTZ – pentilenotetrazol
SNC – sistema nervoso central
TLR-4 – receptores toll-like do tipo 4
TNF-α – fator de Necrose tumoral α
TNFR1 – receptor 1 do TNF

SUMÁRIO

APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos, os quais se encontram no item RESULTADOS. Esse item, por sua vez, está subdividido em Capítulo I e Capítulo II. No capítulo I encontra-se o primeiro artigo científico publicado. No capitulo II está o manuscrito do segundo artigo científico. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos capítulos I e II que representam a íntegra deste estudo. Os itens, DISCUSSÃO E CONCLUSÕES, encontram-se no final desta tese e apresentam interpretações e comentários gerais sobre o artigo e o manuscrito contidos neste trabalho. As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem nos itens INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA e DISCUSSÃO desta tese.

1. INTRODUÇÃO

1. INTRODUÇÃO

A Acidemia Glutárica tipo 1 (GA-I) é uma doença hereditária autossômica recessiva causada pela deficiência na atividade da enzima mitocondrial glutaril-CoA desidrogenase (GCDH; EC 1.3.99.7) envolvida no catabolismo dos aminoácidos L-lisina, L-hidroxilisina e L-triptofano. Essa deficiência enzimática leva ao acúmulo de ácido glutárico (GA; 500-5000 µM), ácido 3-hidroxiglutárico (3-OH-GA; 40-200 µM) e ácido glutacônico nos fluídos corporais e tecidos dos pacientes afetados (Goodman et al., 1977; Goodman e Freman, 2001; Strauss e Morton, 2003; Strauss et al., 2003; Sauer et al., 2006). Aproximadamente 500 pacientes foram identificados, mundialmente distribuídos, desde o primeiro relato desta acidemia (Goodman et al., 1975; Kölker et al., 2006), e mais de 150 mutações patogênicas já foram descritas para o gene da GCDH (Zschocke et al., 2000; Christensen et al., 2004).

Após um período de desenvolvimento normal, as crianças glutaricoacidêmicas podem apresentar crises encefalopáticas, as quais são precipitadas por processos infecciosos entre os 3 meses e 5 anos de idade (Strauss et al., 2003; Kölker et al., 2006). Essas crises induzem o aparecimento das manifestações clínicas da GA-I que são predominantemente neurológicas, incluindo convulsões e atraso cognitivo, acompanhadas pela degeneração estriatal (Morton et al., 1991; Hoffmann e Zschocke, 1999). Como se sabe que as crises encefalopáticas que levam ao aparecimento das manifestações clínicas nos pacientes glutaricoacidêmicos são precipitadas por infecções comuns, doenças febris ou imunizações rotineiras, tem-se sugerido um possível envolvimento de citocinas inflamatórias na neuropatogênese da GA-I (Hoffmann et al., 1996; Hoffmann e Zschocke, 1999).

De acordo, alguns estudos mostraram que uma inflamação sistêmica durante os períodos críticos do desenvolvimento cerebral pode resultar em aumento da vulnerabilidade do sistema nervoso central (SNC) e periférico (Hagberg e Mallard, 2005; Godbout e Johnson, 2006). Nesse contexto, estudos observaram que a administração de lipopolissacarídeo (LPS) no período perinatal piora a memória de ratos (Graciarena et al., 2010; Hao et al., 2010), e que a sua injeção pós-natal (P14) aumenta a susceptibilidade às convulsões induzidas por lítio-pilocarpina, ácido caínico (KA) e pentilenotetrazol (PTZ) em ratos (Galic et al., 2008).

Nesse sentido, utiliza-se a administração de LPS como um modelo para produzir neuroinflamação, pois a resposta inflamatória induzida pelo LPS é caracterizada por ativação do sistema imune inato e pela produção de mediadores proinflamatórios, como o fator de necrose tumoral α (TNF-α) e interleucina 1β (IL-1β), concomitantemente no SNC e periférico (Miyake, 2004). Estas citocinas inflamatórias são consideradas moduladoras da transmissão tanto normal como anormal dos neurônios (Merrill, 1992).

Nesse contexto, tem sido demonstrado que a administração de LPS no período pré e pós-natal resulta em uma forma crônica de astrogliose, uma característica comumente encontrada em modelos experimentais de convulsões (Somera-Molina et al., 2007; Oberheim et al., 2008), déficit cognitivo (Hao et al., 2010) e em pacientes com epilepsia (Eid et al., 2008). Nesta linha de visão, um estudo prévio demonstrou que os metabólitos da GA-I induzem a proliferação de astrócitos, estando essa associada à disfunção mitocondrial e ao estresse oxidativo (Olivera et al., 2008). Dessa maneira, o aparecimento de novos astrócitos pode estar relacionado ao desenvolvimento de gliose, interrompendo o desenvolvimento cerebral e, talvez contribuindo para o estabelecimento dos déficits neurológicos encontrados nos pacientes com GA-I (Goodman e Frerman, 2001).

Embora as evidências clínicas e experimentais sugiram que a infecção ou a inflamação facilita a predisposição às crises convulsivas (Vezzani e Granata, 2005; Vezzani e Baram, 2007; Auvin et al, 2010) e piora o desempenho em testes de memória (Casadesus et al., 2007; Hein et al., 2007), a patogênese das convulsões e do déficit cognitivo induzidos pelo GA é ainda desconhecida. Portanto, decidiu-se investigar o envolvimento de citocinas pró-inflamatórias durante os períodos críticos do desenvolvimento (Galic et al., 2008) no déficit cognitivo e nas alterações eletroencefalográficas (EEG) e neuroquímicas induzidas pelo GA. Dessa maneira, é de especial interesse determinar como a presença de um processo inflamatório pode contribuir para o desenvolvimento das alterações funcionais e estruturais em ratos jovens submetidos ao tratamento com GA.

Assim, dado o elevado grau de limitação que a GA-I traz para a criança portadora desta patologia, o entendimento dos mecanismos envolvidos nas alterações neurológicas induzidas pelo acúmulo de GA após um processo infeccioso é importante para o desenvolvimento de novas estratégicas terapêuticas para essa patologia.

2. OBJETIVOS

2. OBJETIVOS

2.1. Capítulo I

Objetivo geral

Investigar se a presença de um processo inflamatório, induzido pelo LPS, pode contribuir para o desenvolvimento de convulsões desencadeadas a partir de uma injeção intraestriatal de GA em ratos jovens (21 dias de vida).

Objetivos específicos

1. Determinar a dose de GA que causa convulsões em ratos jovens.
2. Avaliar se a administração de LPS potencializa as convulsões induzidas pelo GA em ratos jovens.
3. Determinar os níveis de IL-1β no estriado dos ratos jovens.
4. Verificar se existe correlação entre os níveis estriatais de IL-1β e as convulsões apresentadas pelos ratos jovens.
5. Avaliar se a potencialização das convulsões causada pelo LPS deve-se a alterações na temperatura dos ratos jovens.
6. Avaliar se a administração prévia do anticorpo da IL-1β previne a potencialização das convulsões causadas pela administração de LPS 6 horas antes do GA.

2.2. Capítulo II

Objetivo geral

Investigar se o tratamento crônico com GA causa déficit na memória espacial de ratos jovens, e verificar se a presença de um processo inflamatório produzido pelo LPS potencializa o déficit cognitivo induzido pelo GA.

Objetivos específicos

7. Avaliar a memória espacial de ratos jovens injetados cronicamente com GA na presença de um processo inflamatório no labirinto de Barnes.

8. Determinar se o prejuízo na memória espacial se deve a uma desnutrição dos ratos jovens.

9. Avaliar se o déficit na memória espacial ocorre devido a um prejuízo na atividade locomotora dos animais.

10. Determinar os níveis das citocinas, IL-1β e TNF-α, no hipocampo dos animais.

11. Analisar histologicamente o volume hipocampal total dos animais.

12. Determinar a atividade total e das subunidades α1 e α2/3 da enzima Na^+, K^+-ATPase no hipocampo dos ratos jovens.

3. REVISÃO BIBLIOGRÁFICA

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3.1. Erros Inatos do Metabolismo

Erros inatos do metabolismo (EIM) são distúrbios hereditários, resultado de deficiências em atividades enzimáticas, o que ocasiona um bloqueio de diversas rotas metabólicas (Goodman e Frerman, 2001). Em consequência deste bloqueio metabólico, pode ocorrer o acúmulo de precursores tóxicos da reação catalisada pela enzima envolvida, com a formação de rotas metabólicas alternativas e a deficiência de produtos essenciais ao organismo (Bickel, 1987), provocando assim, distúrbios no desenvolvimento físico e mental (Oberholzer et al., 1967).

Archibald E. Garrod, em 1908, foi o primeiro a empregar o termo EIM para designar doenças como a alcaptonúria, em que os indivíduos afetados excretam grandes quantidades de ácido homogentísico na urina. Garrod observou uma maior frequência desta doença em indivíduos de uma mesma família e com maior incidência de consanguinidade entre os pais dos pacientes. Baseando-se nas leis de Mendel e no fato de que os pais dos indivíduos afetados não apresentavam a doença, Garrod propôs um modelo de herança autossômica recessiva para este distúrbio. Através da observação de que o ácido homogentísico presente em excesso na urina dos pacientes era um metabólito normal da degradação protéica, ele relacionou este acúmulo a um bloqueio na rota de catabolismo da tirosina (Scriver, 2008).

Com o surgimento de novos distúrbios relacionados a alterações genéticas que envolviam o acúmulo de substâncias nos líquidos biológicos dos pacientes, utilizou-se o termo EIM para designar esse grupo de doenças geneticamente determinadas que resultam da síntese qualitativa ou quantitativamente anormal de uma proteína, enzimática ou não, pertencente ao metabolismo. Até o momento, foram descritos mais de 500 EIM, a maioria deles envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas (Scriver et al., 2001).

Os EIM afetam aproximadamente afetam 1 a cada 500 a 2.000 recém nascidos vivos (Baric et al., 2001), e apesar de serem eventos individualmente raros, esse grupo de doenças representa um importante problema de saúde e seu diagnóstico, frequentemente, se constitui em desafio para o clínico.

3.2. Acidemias Orgânicas

As acidemias ou acidurias orgânicas são um grupo de EIM caracterizadas pelo acúmulo de um ou mais ácidos orgânicos nos líquidos biológicos e tecidos dos pacientes afetados, devido à deficiência na atividade de enzimas do metabolismo de aminoácidos, lipídeos ou carboidratos (Chalmers e Lawson, 1982). Um subgrupo de acidemias orgânicas tem sido classificado como doenças de ácidos orgânicos "cerebrais" (Hoffmann et al., 1994) devido às manifestações neurológicas serem predominantes ou mesmo exclusivas, sendo que neste grupo encontra-se a Acidemia Glutárica tipo I (GA-I).

Os sintomas neurológicos nas acidemias orgânicas são frequentemente manifestados durante crises agudas precipitadas por estresse catabólico. Durante esses episódios existe um déficit de energia, com a consequente mobilização dos estoques de carboidratos, ácidos graxos e proteínas. Estes são catabolisados, mas devido ao bloqueio metabólico, os ácidos orgânicos e outros compostos são acumulados. Neste contexto, tem sido sugerido que alguns desses metabólitos podem agir como toxinas endógenas e podem tornar-se neurotóxicos (McLaughlin et al., 1998; Kölker et al., 2002a; 2004).

Clinicamente, os pacientes afetados apresentam predominantemente disfunções neurológicas em suas mais diversas formas de expressão: regressão neurológica, convulsões, coma, ataxia, hipotonia, hipertonia, irritabilidade, tremores, movimentos coreatetóticos, tetraparesia espástica, atraso no desenvolvimento psicomotor, retardo mental, entre outros (Scriver et al., 2001; Saudubray et al., 2006).

Bioquimicamente, as mais frequentes manisfestações laboratoriais apresentadas pelos pacientes são cetose, cetonúria, neutropenia, trombocitopenia, acidose metabólica, baixos níveis de bicarbonato, hiperglicinemia, hiperamonemia, hipo/hiperglicemia, acidose láctica, aumento dos níveis séricos de ácidos graxos livres e outros (Scriver et al., 2001; Saudubray et al., 2006).

Além disso, o uso da tomografia computadorizada revelou na maioria dos pacientes afetados por essas acidemias alterações na substância branca (hipomielização e/ou desmielização), atrofia cerebral generalizada ou dos gânglios da base (necrose ou calcificação), atrofia frontotemporal e cerebelar, e macrocefalia (Mayatepek et al., 1996).

A incidência dos EIM na população é pouco conhecida, o que pode ser creditado à falta de laboratórios especializados para o seu diagnóstico que requer equipamentos de alto custo e/ou ao desconhecimento médico dessas patologias. Entretanto, na Holanda, país referência para o diagnóstico de EIM, a incidência destes é estimada em 1: 2.200 recém-nascidos, enquanto que, na Alemanha, Israel e Inglaterra é de aproximadamente 1: 6.000 a 9.000 nascimentos (Hoffmann et al., 2004). Em países onde a taxa de consanguinidade é elevada, como na Arábia Saudita, a frequência é de 1: 740 nascidos vivos (Rashed et al., 1994).

Frequência estimada de alguns EIM	
Fenilcetonúria (caucasianos)	1/15.000
Acidemia Glutárica tipo I	1/30.000
Homocistinúria	1/100.000
Acidemia Metilmalônica	1/100.000

Tabela 1. Frequência estimada de alguns EIM (Goodman e Frerman, 2001).

Em 1980, Chalmers e colaboradores demonstraram que as acidemias orgânicas eram os EIM mais frequentes em crianças hospitalizadas, incentivando assim, diversos estudos clínicos, laboratoriais e epidemiológicos nos anos seguintes a respeito dessas doenças. De fato, em função dos progressos feitos no campo do diagnóstico dos EIM, evidenciou-se que as acidemias orgânicas são, realmente, os erros inatos mais frequentes na população (Scriver et al., 2001; Wajner et al., 2001).

3.3. Acidemia Glutárica tipo I

A GA-I (OMIM # 231670) é um EIM que foi descrito pela primeira vez por Goodman e colaboradores em 1975. É uma desordem neurometabólica autossômica recessiva, caracterizada por uma deficiência parcial ou total na atividade da enzima da matriz mitocondrial glutaril-CoA desidrogenase (GCDH; E.C. 1.3.99.7). A GCDH está envolvida na via de degradação dos aminoácidos L-lisina, L-hidroxilisina e L-

triptofano (Goodman e Frerman, 2001), catalisando a descarboxilação oxidativa do glutaril-CoA a crotonil-CoA e CO_2, e transferindo os elétrons para a cadeia respiratória via flavoproteína transferidora de elétrons (Lenich e Goodman, 1986). Essa reação possui duas diferentes etapas: a desidrogenação de glutaril-CoA a glutaconil-CoA e a descarboxilação de glutaconil-CoA a crotonil-CoA (Härtel et al., 1993).

O gene da GCDH localiza-se no cromossomo 19p 13.2 e codifica um polipeptídeo de 438 aminoácidos que sofre uma clivagem na porção *N*-terminal na qual são retirados 44 aminoácidos formando uma proteína madura dentro da matriz mitocondrial (Goodman et al., 1998). A maioria das mutações conhecidas está relacionada com simples mudanças de bases como no caso da R402W, mutação mais frequente em caucasianos (Goodman et al., 1998; Zschocke et al., 2000). Existe uma grande heterogeneidade de mutações na deficiência da GCDH, porém, dentro de comunidades específicas o padrão pode ser mais homogêneo (Busquets et al., 2000). Apesar do conhecimento de diferentes mutações, não há correlação entre o genótipo, a atividade enzimática e o prognóstico dos pacientes (Goodman et al., 1998; Hoffmann e Zschocke et al., 1999; Kölker et al., 2006).

Com o bloqueio da atividade da enzima GCDH, formam-se rotas metabólicas alternativas que culminam na presença de concentrações elevadas de ácido glutárico (GA), 3-hidroxiglutárico (3-OH-GA) e, algumas vezes, ácido glutacônico nos fluidos biológicos (plasma, urina e líquor) e tecidos corporais dos indivíduos afetados (Goodman et al., 1975; Goodman e Frerman, 2001; Figura 1).

Tem sido proposto que o acúmulo desses ácidos orgânicos pode apresentar um efeito neurotóxico que seria o responsável pelas mudanças neuropatológicas na doença. As concentrações plasmáticas destes ácidos variam entre 5 e 400 µmol/L (Hoffmann et al., 1991, 1996; Merinero et al., 1995), entretanto, as concentrações cerebrais podem atingir 500-5000 µmol/L para o GA e 40-200 µmol/L para o 3-OH-GA (Sauer et al., 2006). Tais diferenças podem ser explicadas pelo fato de que o GA e o 3-OH-GA são produzidos nas células neurais e que a barreira hematoencefálica é pouco permeável a esses ácidos orgânicos, ocasionando assim o acúmulo dessas substâncias no sistema nervoso central (SNC) o que se constitui em um fator de risco para a neurodegeneração característica dos pacientes afetados (Sauer et al., 2006; Kölker et al., 2006).

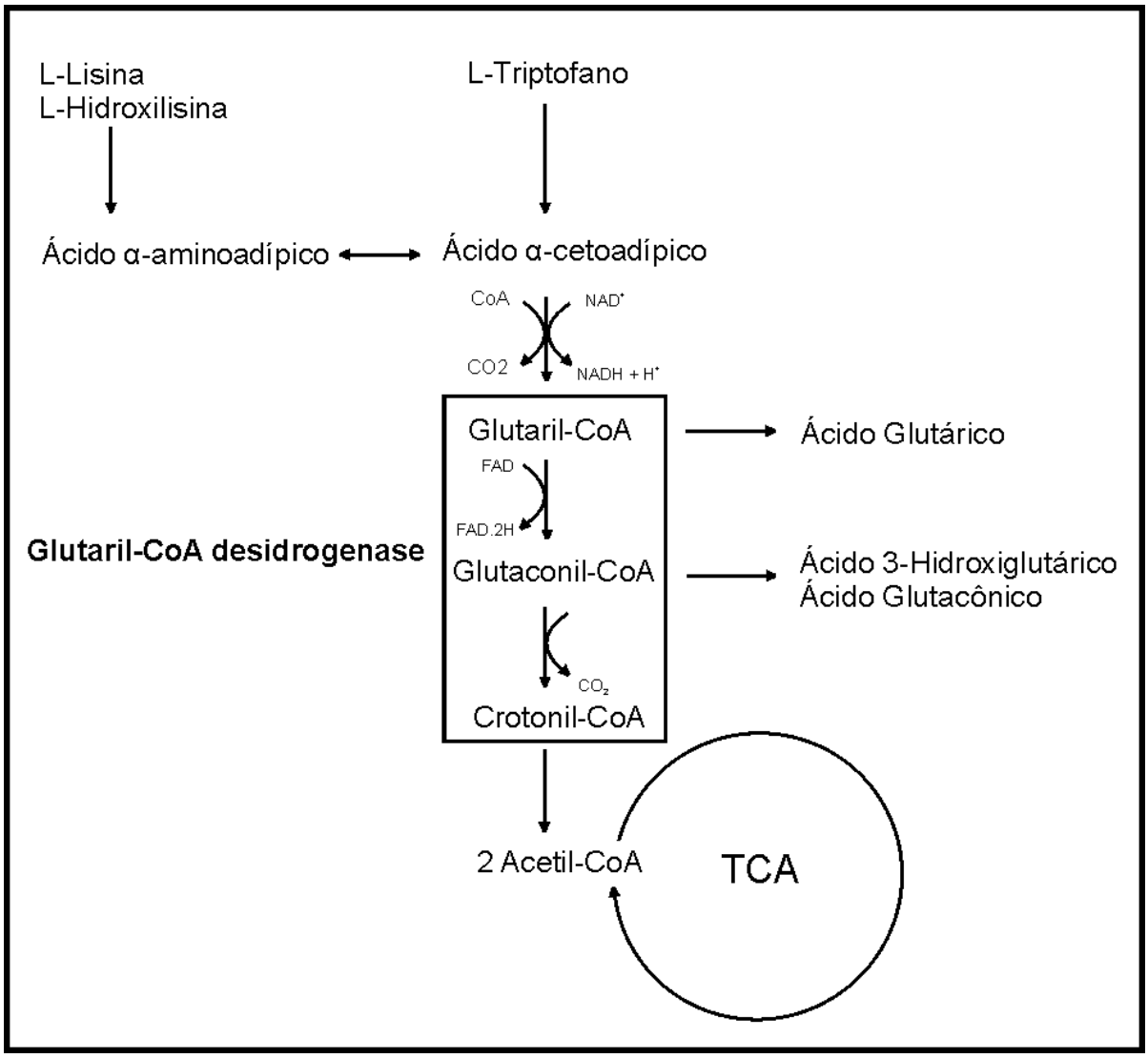

Figura 1. Deficiência da enzima glutaril-CoA desidrogenase (Adaptado de Goodman e Frerman, 2001).

3.3.1. Prevalência

A prevalência da GA-I é estimada em 1: 30.000 a 80.000 (Goodman e Frerman, 2001) ou 1: 100.000 nascimentos (Lindner et al., 2004). Porém, pode ter uma prevalência aumentada em até 1: 300 nascidos vivos (Kölker et al., 2004; 2006) em comunidades geneticamente homogêneas, como na Ordem Amish da Pensilvânia (Biery et al., 1996) e nos índios Salteaux/Ojibway do Canadá (Greenberg et al., 1995).

3.3.2. Diagnóstico

Apesar do desenvolvimento de diversas estratégias terapêuticas para o tratamento da GA-I, o diagnóstico precoce continua sendo determinante para um melhor prognóstico dos pacientes afetados, uma vez que medidas preventivas podem ser tomadas. No entanto, a identificação desta acidemia apresenta grandes dificuldades, pois o grau de deficiência da enzima não está correlacionado com a intensidade dos sintomas apresentados pelos pacientes, e a excreção de GA pode ser normal em pacientes que apresentam sintomas graves (Goodman e Frerman, 2001).

Entretanto, a presença de quantidades elevadas de GA e 3-OH-GA nos líquidos biológicos (especialmente urina) dos pacientes afetados (Goodman et al., 1977; Funk et al., 2005; Kölker et al., 2006) constitui o marcador bioquímico da GA-I. O diagnóstico é geralmente realizado através da detecção desses compostos e seus ésteres de glicina e carnitina na urina por cromatografia gasosa acoplada à espectrometria de massa (Hoffmann, 1994; Kölker et al., 2006). O perfil de acilcarnitinas e a diminuição de carnitinas livres nos líquidos biológicos, também determinados por espectrometria de massa, podem ser utilizados como métodos auxiliares no diagnóstico dessas doenças (Ziadeh et al., 1995).

A análise mutacional não é muito utilizada para fins de diagnóstico devido ao grande número de mutações conhecidas, aproximadamente 150 mutações já foram descritas para o gene da GCDH, e também pelo fato de não existir correlação entre o genótipo e fenótipo clínico (Pineda et al., 1998; Christensen et al., 2004). Portanto, a análise mutacional apresenta maior valor em estudos de comunidades onde a cosanguinidade é elevada e para fins de pesquisa (Busquets et al., 2000; Kölker et al., 2006).

Nos pacientes que apresentam excreção pouco elevada, ausente ou normal de GA (Merinero et al., 1995; Hoffmann et al., 1996), a determinação da atividade da GCDH em fibroblastos ou leucócitos deve ser realizada sempre que houver fortes suspeitas clínicas e neuro-radiológicas da doença (Goodman e Frerman, 2001).

3.3.3. Achados Neuropatológicos

Patologicamente, a GA-I é caracterizada principalmente por atrofia frontotemporal cortical ao nascimento, formação espongiforme, diminuição da substância branca (leucoencefalopatia progressiva) e uma característica degeneração estriatal (caudado e putâmen) bilateral (Kyllerman et al., 1994). Essa degeneração estriatal ocorre após crises encefalopáticas que são precipitadas por infecções, doenças febris ou mesmo vacinações rotineiras (situações de estresse catabólico) entre os primeiros 3 meses a 5 anos de vida (Amir et al., 1987; Chow et al., 1988; Hoffmann e Zschocke, 1999). Frequentemente, os pacientes apresentam um alargamento dos espaços subaracnóideos que, devido à alta irrigação sanguínea, os tornam susceptíveis a hemorragias agudas (Drigo et al., 1996; Hoffmann e Zschocke, 1999).

De acordo, exames neuroradiológicos em pacientes afetados demonstram atrofia frontotemporal cortical, degeneração estriatal, dilatação dos ventrículos laterais e atraso na mielinização (Hoffmann et al., 1991; Twomey et al., 2003; Neumaier-Probst et al., 2004; Figura 2).

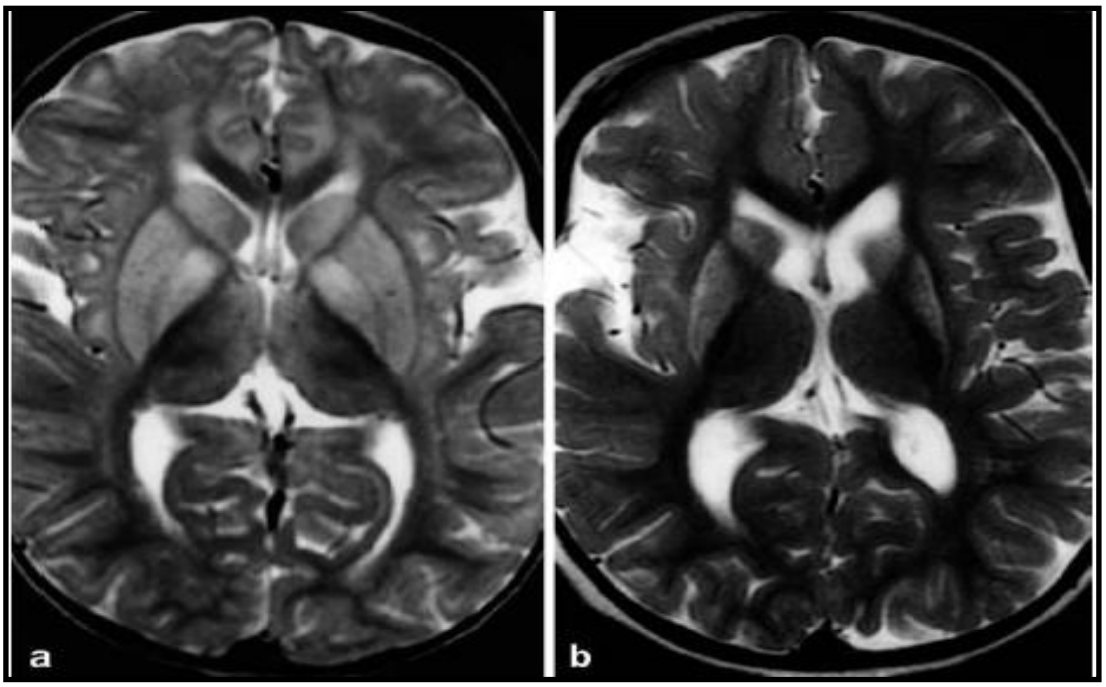

Figura 2. Ressonância magnética nuclear de crânio de uma criança com GA-I. a) Aumento anormal da intensidade do sinal nos núcleos caudado e putâmen; b) Dois anos mais tarde, o estriado ainda apresenta uma intensidade de sinal anormal, além de uma acentuada redução. Também observa-se uma dilatação dos ventrículos cerebrais neste intervalo (Twomey et al., 2003).

3.3.4. Manifestações Clínicas

Um dos achados clínicos mais frequente é a macrocefalia, que geralmente já está presente ao nascimento. A sintomatologia inicial é branda com alguns pacientes desenvolvendo-se normalmente até o aparecimento das crises encefalopáticas (quadro de estresse catabólico precipitado por infecções rotineiras; Hoffmann et al., 1995).

Aproximadamente 90% das crianças apresentam severos sintomas clínicos antes dos 36 meses de idade, e nenhuma crise encefalopática aguda tem sido descrita após os 5 anos de vida, sugerindo que a vulnerabilidade é restrita a um período limitado de desenvolvimento do cérebro (Hoffmann et al., 1996; Bjugstad et al., 2000).

Após as crises encefalopáticas surgem sintomas relacionados à destruição estriatal, como hipotonia, rigidez muscular, espasticidade, distonia e discinesia facial (Hoffmann e Zschocke, 1999; Strauss et al., 2003; Kölker et al., 2004; Figura 3). As convulsões, o déficit cognitivo, a ataxia, a irritabilidade, o retardo mental e a demência também estão entre os achados clínicos da GA-I (Külkens et al., 2005; Boneh et al., 2008; Beauchamp et al., 2009).

Além dessas manifestações clínicas, os pacientes apresentam um quadro laboratorial que caracteriza-se, principalmente, por acidose metabólica, hipercetonemia, hiperamonemia, hipoglicemia, neutropenia e trombocitopenia (Goodman e Frerman, 2001).

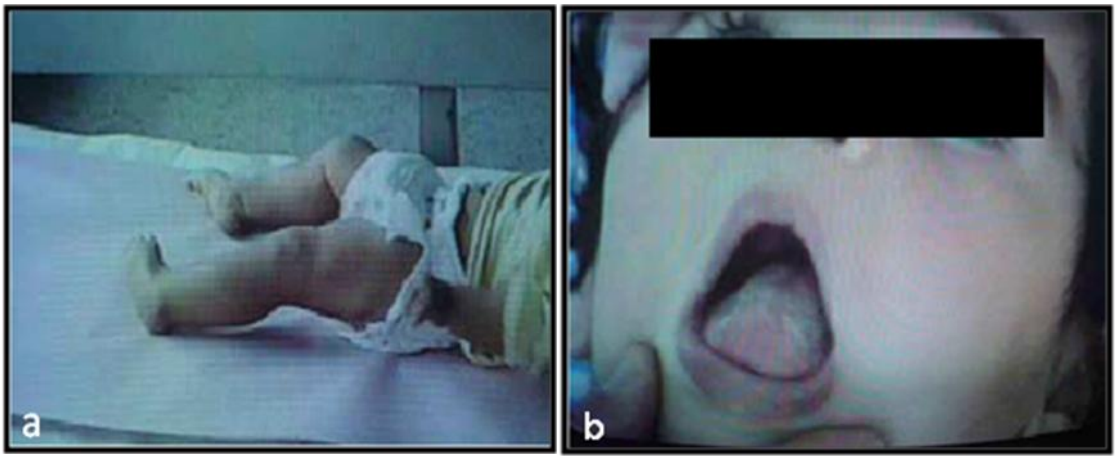

Figura 3. Manisfestações clínicas de crianças com GA-I. a) Movimentos distônicos dos membros inferiores; b) Discinesia Facial (Barreiro et al., 2004).

3.3.5. Tratamento

Uma ampla variedade de medicamentos tem sido usada na terapêutica da GA-I com alguns resultados satisfatórios, entre eles destacam-se os anticonvulsivantes (Yamaguchi et al., 1987; Hoffmann et al., 1996), os anticolinérgicos e a toxina botulínica (Burlina et al., 2004). Mais recentemente, Zinnanti e colaboradores (2007) propuseram a utilização da suplementação com glicose e homoarginina como terapia adjuvante para reduzir o acúmulo cerebral dos metabólitos tóxicos gerados pela deficiência da GCDH, baseado em estudo de um modelo animal de GA-I (Zinnanti el al., 2006).

Entretanto, o diagnóstico precoce continua sendo determinante para um bom prognóstico dos pacientes glutaricoacidêmicos, pois a restrição dietética dos aminoácidos hidroxilisina, triptofano e principalmente a lisina é essencial para um adequado desenvolvimento destes pacientes (Goodman e Frerman, 2001; Kölker et al., 2006; Sauer et al., 2011). Além disso, a suplementação com dieta hipercalórica, L-carnitina e riboflavina têm demonstrado bons resultados na prevenção das crises encefalopáticas agudas e no subsequente dano estriatal na maioria dos pacientes (Hoffmann et al., 1996; Strauss et al., 2003; Kyllerman et al., 2004). No entanto, a avaliação e a intervenção neurocirúrgica também podem ser necessárias em pacientes com lesões estruturais associadas a esta patologia (Hou et al., 2007).

Convém destacar que novas estratégias terapêuticas estão sendo pesquisadas, porém a utilização dessas terapias requer muito estudo e cautela quando de seu uso para os pacientes. Por outro lado, na medida em que se conheçam os mecanismos exatos do dano cerebral nos pacientes com GA-I, melhores terapias se tornarão disponíveis.

3.3.6. Modelos animais de Acidemia Glutárica tipo I

Tem sido investigado o desenvolvimento de modelos animais que mimetizem as características metabólicas e neuropatológicas apresentadas pelos pacientes com GA-I. Neste sentido, Koeller e colaboradores (2002) desenvolveram um modelo de camundongos geneticamente modificados (nocaute) para o gene da GCDH. Apesar dos animais apresentarem um fenótipo bioquímico similar ao dos pacientes,

com elevados níveis de GA, 3-OH-GA e conjugados de glicina e carnitina, esse modelo não reproduz o fenótipo neurológico e a degeneração estriatal característica dos pacientes afetados. Um aperfeiçoamento deste modelo foi proposto por Zinnanti e colaboradores (2006) com a administração via oral de uma sobrecarga de lisina aos animais. Neste em particular, foi verificado que as concentrações de GA no cérebro dos camundongos nocaute do gene da GCDH aumentaram significativamente e que os mesmos apresentaram um padrão de neurodegeneração dependente do estágio de desenvolvimento semelhante ao apresentado pelos pacientes afetados pela GA-I (lesão estriatal), além de provocar a perda de seletividade da barreira hematoencefálica.

Por outro lado, Strauss e Morton (2003) propuseram um modelo de degeneração estriatal aguda com o uso de ácido 3-nitropropiônico, um inibidor clássico do complexo II da cadeia respiratória utilizado em modelos de doença de Huntington, que apresenta características neuro-radiológicas idênticas às observadas em pacientes com GA-I.

Além destes, tem sido utilizada a administração intraestriatal dos ácidos orgânicos acumulados na GA-I como um modelo para reproduzir às crises convulsivas apresentadas pelos pacientes. Nesse sentido, trabalhos têm demonstrado que a injeção intraestriatal de GA (Lima et al., 1998; Fighera et al., 2006; Magni et al., 2007) e 3-OH-GA (De Mello et al., 2001) causam o aparecimento de episódios convulsivos em ratos.

Mais recentemente, tem sido reportado um modelo de tratamento crônico com GA através da injeção subcutânea deste ácido em ratos jovens (Da Costa Ferreira et al., 2008). Embora, este modelo não mimetiza exatamente a GA-I em humanos, na qual, além do GA outros metabólitos são acumulados em menores quantidades, ele reproduz a principal característica bioquímica dessa doença, que são altos níveis teciduais de GA (~0.72 mM) no cérebro de ratos jovens, em concentração semelhante à encontrada em pacientes com GA-I (Ferreira et al., 2005).

3.3.7. Fisiopatologia

Tem sido proposto que os ácidos orgânicos acumulados na GA-I podem ser a causa das alterações neuropatológicas apresentadas pelos pacientes (Flott-Rahmel

et al., 1997; Lima et al., 1998; Hoffmann e Zschocke, 1999; Goodman, 2004; Wajner et al., 2004). No entanto, apesar dos sintomas neurológicos serem predominantes nessa acidemia, pouco se sabe sobre a causa da degeneração estriatal e o mecanismo pelo qual o GA e o 3-OH-GA são neurotóxicos (Goodman e Frerman, 2001).

O primeiro trabalho proposto com o intuito de explicar a fisiopatogenia da GA-I estava relacionado à neurotransmissão GABAérgica, e foi realizado por Stokke e colaboradores (1976). Este trabalho demonstrou a inibição competitiva da glutamato descarboxilase, enzima que catalisa a conversão do glutamato a ácido γ-aminobutírico (GABA), na presença do GA, 3-OH-GA e ácido glutacônico, sugerindo assim que o desequilíbrio na produção de GABA poderia estar envolvido na patogênese da GA-I (Wajner et al., 2004). Nesse contexto, estudos mostraram que as convulsões induzidas pela injeção intraestriatal de GA (Lima et al., 1998; Fighera et al., 2006) e 3-OH-GA (De Mello et al., 2001) são prevenidas pelo muscimol, um agonista de receptores $GABA_A$, sugerindo a participação dos receptores GABAérgicos na neurotoxicidade induzida por estes ácidos orgânicos em ratos. Contudo, concentrações reduzidas de GABA no estriado foram encontradas somente em um paciente com GA-I (Leibel et al., 1980). Além disso, Ullrich e colaboradores (1999) não encontraram efeitos do 3-OH-GA sobre receptores GABA em oócitos de *Xenopus laevis*.

Nas últimas décadas, distintos mecanismos têm sido propostos para explicar a fisiopatogenia do dano cerebral na GA-I. Entre esses mecanismos pode-se citar as alterações no metabolismo energético (Ullrich et al., 1999; Silva et al., 2000; Das et al., 2003; Ferreira et al., 2005; Latini et al., 2005a), a produção de espécies reativas (Latini et al., 2002; 2005b; De Oliveira Marques et al., 2003; Fighera et al., 2006) e a excitotoxicidade (Kölker et al.,1999; 2000; 2002a,b; De Mello et al., 2001; Porciúncula et al., 2004; Rosa et al., 2004; 2007).

Em relação ao metabolismo energético, estudos mostraram que o 3-OH-GA provoca uma inibição moderada dos complexos II e V da cadeia respiratória e reduz significantemente os níveis de fosfocreatina em culturas de neurônios de ratos (Ullrich et al., 1999; Das et al., 2003). Da mesma forma, Latini e colaboradores (2005a) também evidenciaram uma inibição do complexo II da cadeia transportadora de elétrons pelo 3-OH-GA em homogeneizados de córtex cerebral e células C6 de glioma de ratos. Além disso, o mesmo estudo demonstrou que o 3-OH-GA pode

interferir com o consumo de oxigênio em preparações mitocondriais, funcionando talvez, como um desacoplador da fosforilação oxidativa em situações onde a mitocôndria esteja sob condições de estresse. Entretanto, Kölker e colaboradores (2002a) encontraram uma pequena inibição do complexo V somente em altas concentrações de 3-OHGA (10 mM) sem nenhuma alteração dos outros complexos da cadeia respiratória em culturas neuronais de telencéfalos de embriões de pinto, concordando em parte com um estudo realizado em partículas submitocondriais de coração bovino que não mostrou nenhum efeito do 3-OH-GA sobre os complexos enzimáticos da cadeia transportadora de elétrons (Sauer et al., 2005). Em relação ao GA, foi demonstrado que este metabólito inibe os complexos I-III e II-III da cadeia respiratória, diminui a produção de CO_2 e os níveis de ATP em córtex cerebral de ratos (Silva et al., 2000). Além disso, foi demonstrado uma inibição dos complexos I-III, II, II-III em músculos esqueléticos e cérebros de ratos tratados de forma aguda (Ferreira et al., 2005) e crônica (Ferreira et al., 2007) com GA. Resultados semelhantes foram descritos também *in vitro*, além da demonstração da inibição da enzima creatina quinase em cérebros de ratos (Da C. Ferreira et al., 2005). A partir destes achados, tem sido postulado que o GA pode causar interferência no metabolismo aeróbico celular, levando provavelmente a uma redução na produção de ATP.

Por outro lado, vários trabalhos relacionam o aumento na produção de radicais livres e a redução das defesas antioxidantes no cérebro de ratos com a neurotoxicidade induzida pelo GA e 3-OH-GA (Kölker et al., 2001b; Latini et al., 2002; 2005b; 2007; De Oliveira Marques et al., 2003; Fighera et al., 2006). De acordo, Latini e colaboradores (2002, 2005b) mostraram que o 3-OH-GA induz um aumento na lipoperoxidação, na produção de óxido nítrico e peróxido de hidrogênio, além de diminuir as defesas antioxidantes no córtex cerebral e no estriado de ratos. Uma produção aumentada de espécies reativas na presença de 3-OH-GA também foi demonstrada em culturas neuronais de telencéfalos de embriões de pinto, sendo que esse aumento foi reduzido após incubação com antioxidantes (Kölker et al., 2001b). Quanto ao GA, foi evidenciado que este causa um aumento no conteúdo de proteínas carboniladas e na peroxidação lipídica no estriado de ratos (Fighera et al., 2006). Também foi demonstrado que o GA aumenta a produção de espécies reativas e reduz as defesas antioxidantes em cérebro de ratos (De Oliveira Marques et al., 2003). De acordo com os demais trabalhos, Latini e colaboradores (2007)

verificaram que tanto a administração aguda quanto crônica de GA aumenta a peroxidação lipídica e diminui as defesas antioxidantes em diferentes estruturas cerebrais, no fígado e nos eritrócitos de ratos. Além disso, foi demonstrada uma redução nas concentrações de glutationa cerebral e hepática em camundongos deficientes da enzima GCDH (Sauer et al., 2005). A partir destes achados, tem sido postulado que o GA pode causar interferência no metabolismo oxidativo celular, levando provavelmente a uma redução nas defesas antioxidantes e/ou um aumento na produção de espécies reativas, e a consequente excitotoxicidade.

É conhecido que o padrão de lesões no SNC causadas pela administração de glutamato em camundongos é morfologicamente semelhante àquelas encontradas em estudos *postmortem* de cérebros de pacientes com GA-I, no que diz respeito à perda neuronal com vacuolização pós-sináptica, extensa fibrose gliosa estriatal e degeneração espongiforme da substância branca cerebral (Olney, 1969). Devido a esses achados e também à similaridade estrutural existente entre o glutamato, o GA e o 3-OH-GA (Flott-Rahmel et al., 1997; Lima et al., 1998; Hoffmann e Zschocke, 1999; Wajner et al., 2004; Goodman, 2004; Figura 4), muitos estudos explicam a neurotoxicidade dessa acidemia pela interação desses ácidos orgânicos com receptores e transportadores glutamatérgicos.

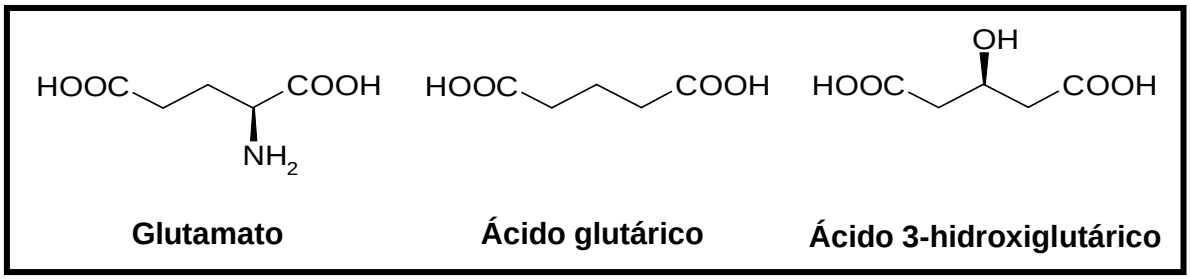

Figura 4. Similaridade estrutural entre glutamato, ácido glutárico e ácido 3-hidroxiglutárico.

Em relação ao 3-OH-GA, Kölker e colaboradores (2000; 2002a) evidenciaram que este ácido orgânico ativa seletivamente receptores do tipo *N*-metil-D-aspartato (NMDA) compostos pelas subunidades NR1/NR2A e NR1/NR2B em culturas neuronais de telencéfalos de embriões de pinto. Colaborando com estes achados, Bjugstad e colaboradores (2001) mostraram que culturas de neurônios de ratos não se mostram sensíveis ao 3-OH-GA antes da expressão de receptores do tipo NMDA. De acordo, Rosa e colaboradores (2004) também demonstraram que o 3-OH-GA interage com receptores glutamatérgicos do tipo NMDA. Nesse contexto, foi

demonstrado que a pré-incubação de culturas de neurônios com antagonistas específicos de receptores NMDA, bem como a pré-administração desses antagonistas *in vivo* reduzem ou mesmo previnem o dano celular provocado pelo 3-OH-GA (Kölker et al., 2000; De Mello et al., 2001). No entanto, Ullrich e colaboradores (1999), utilizando estudos eletrofisiológicos em diferentes sistemas celulares não encontraram evidências de que o 3-OH-GA liga-se diretamente a receptores NMDA, sugerindo que um déficit no metabolismo energético poderia explicar de modo indireto a ativação desses receptores. Desta maneira, considerando que os receptores NMDA NR1/NR2B são predominantemente expressos no cérebro imaturo (McDonald et al., 1988) e que a ocorrência do dano neuronal depende do modelo em estudo, sugere-se a existência de uma dependência da distribuição regional e do período de desenvolvimento na suscetibilidade dos neurônios à toxicidade do 3-OH-GA (Ullrich et al., 1999; Kölker et al., 2004; Goodman, 2004).

Quanto ao GA, foi demonstrado que este metabólito inibe a captação e a ligação do L-[^{3}H]glutamato a seus transportadores em sinaptossomas de cérebro de ratos (Bennett et al., 1973; Porciúncula et al., 2000; Magni et al., 2007; 2009), aumentando assim o risco para superexcitação por uma elevação na concentração de glutamato na fenda sináptica. Também foi demonstrado que o GA reduz a captação de L-[^{3}H]glutamato em vesículas sinápticas, além de interagir com receptores glutamatérgicos metabotrópicos e ionotrópicos do tipo AMPA (não-NMDA) em cérebro de ratos (Porciúncula et al., 2004). De acordo, Dalcin e colaboradores (2007) também demonstraram que o GA interage com receptores ionotrópicos não-NMDA em preparações de membranas de cérebros de ratos. Neste contexto, foi verificado que as convulsões induzidas pela administração intraestriatal de GA foram prevenidas pelo DNQX, um conhecido antagonista de receptores glutamatérgicos não-NMDA (Lima et al., 1998). Entretanto, Kölker e colaboradores (2000) não encontraram evidências de que o GA possa interagir com receptores do tipo não-NMDA e relacionam seus efeitos tóxicos a receptores NMDA. Apesar de diversas evidências da neurotoxicidade destas substâncias relacionadas ao sistema glutamatérgico, recentes trabalhos não confirmam essa hipótese (Lund et al., 2004; Freudenberg et al., 2004), fazendo com que esta questão continue sob intenso debate.

Por outro lado, Olivera e colaboradores (2008) demonstraram o envolvimento dos astrócitos na patogênese da GA-I. Os autores observaram que tanto o GA como o 3-OH-GA induziram a proliferação de astrócitos e causaram despolarização mitocondrial em culturas de astrócitos. Da mesma forma, verificaram que a injeção *in vivo* de GA induziu a proliferação de astrócitos, principalmente na forma imatura. Além disso, esses mesmos autores observaram que a administração de antioxidantes preveniu a disfunção mitocondrial e o aumento da proliferação astrócitária *in vitro* e *in vivo*, sugerindo o envolvimento do estresse oxidativo na indução da astrocitose. Assim, esses resultados sugerem que a disfunção mitocondrial induzida pelos metabólitos da GA-I leva os astrócitos a adotar um fenótipo proliferativo, o que pode ser fundamental para a perda neuronal e as alterações na estrutura cerebral encontradas na GA-I.

Além disso, recentemente Zinnanti e colaboradores (2007) sugeriram o mecanismo envolvido na susceptibilidade depende de idade apresentada pelas crianças glutaricoacidêmicas. Os autores observaram que camundongos nocaute de 4 semanas para a enzima GCDH, suplementados com uma dieta a base de lisina, apresentaram um aumento nos níveis cerebrais de lisina e GA, enquanto os camundongos de 8 semanas não apresentaram esse aumento. Assim, esses autores sugerem que ocorra uma redução na captação de lisina com a idade, compatível com existência de transportadores de aminoácidos básicos de baixa afinidade/alta capacidade no cérebro imaturo e de alta afinidade/baixa capacidade no cérebro maduro (Russell e Taegtmeyer, 1992). De fato, trabalhos têm demonstrado que o transporte e o catabolismo da lisina são reduzidos com a maturidade (Banos et al., 1978; Rao et al., 1992). Portanto, esses dados sugerem que a maior captação de lisina no cérebro imaturo causa um aumento no acúmulo de GA e gera a susceptibilidade aos danos cerebrais observados nas crianças com GA-I.

No entanto, apesar da intensa investigação e da severidade dos sintomas apresentados pelos pacientes afetados por essa doença, os mecanismos que levam à vulnerabilidade estriatal durante os primeiros anos de vida ainda não foram elucidados, constituindo-se no principal desafio da pesquisa da patogênese da GA-I (Goodman, 2004).

3.4. Convulsões na Acidemia Glutárica tipo I

Convulsões são alterações comportamentais e/ou motoras resultantes de descargas episódicas anormais de um grupo de neurônios no cérebro e acarretam enorme prejuízo à qualidade de vida dos pacientes afetados e suas famílias (Engel, 2001).

Na GA-I os pacientes podem apresentar convulsões após crises encepalopáticas, as quais são precipitadas por infecções agudas na infância (Kölker et al., 2004; 2006). Nesse sentido, diversos trabalhos têm relatado a ocorrência de convulsões em crianças glutaricoacidêmicas após encefalopatias agudas (Osaka et al., 1993; Pöge et al., 1997; Hartley et al., 2001; Funk et al., 2005). De acordo, Fujimoto e colaboradores (2000) demonstraram registros eletroencefalográficos (EEG) característicos de episódios convulsivos em uma criança com GA-I. Esses autores encontraram descargas periódicas sincrônicas, caracterizadas por intermitentes ondas delta de 4-6 Hz e 100-200 μv na estrutura cortical do paciente, após um episódio de encefalopatia aguda. Outro relato de caso também demonstrou alterações EEG caracterizadas por uma mistura de descargas de ponta-ondas focais e generalizadas em uma menina com GA-I (McClelland et al., 2009). Este trabalho em particular, é o primeiro que relata o caso de uma criança glutaricoacidêmica que apresenta epilepsia (convulsões recorrentes).

Experimentalmente, tem sido demonstrado que a injeção intraestriatal de GA leva ao aparecimento de episódios convulsivos (Lima et al., 1998; Fighera et al., 2006; Magni et al., 2007). De acordo com os relatos clínicos, Magni e colaboradores (2007) demonstraram que a injeção intraestriatal de GA causou um aumento nas ondas delta estriatais e corticais em registros EEG de ratos que apresentaram convulsões. Corroborando com esses achados, Zinnanti e colaboradores (2006) relataram que os camundongos nocaute deficientes da enzima GCDH submetidos a uma dieta com alta quantidade de lisina, dentre outros aspectos bioquímicos e neurológicos, também apresentam convulsões.

Vários trabalhos têm sugerido que a ocorrência de convulsões se deve a alterações nas neurotransmisssões GABAérgicas (Lima et al., 1998; Fighera et al., 2006), glutamatérgicas (Lima et al., 1998), e também a inibição da atividade da enzima Na^+,K^+-ATPase (Fighera et al., 2006; Magni et al., 2007).

Nesse sentido, Lima e colaboradores (1998) demonstraram que o muscimol, um agonista de receptores $GABA_A$, reduziu as rotações contralaterais, bem como o número e a duração das convulsões induzidas pela injeção intraestriatal de GA em ratos adultos. Também verificaram que o DNQX, um antagonista de receptores glutamatérgicos não-NMDA, diminuiu as rotações contralaterais induzidas pela administração intraestriatal de GA, sugerindo assim a participação de receptores glutamatérgicos e GABAérgicos na neurotoxicidade induzida por este ácido orgânico em ratos. De fato, um desequilíbrio entre os sistemas glutamatérgico/GABAérgico através da estimulação do sistema glutamatérgico e/ou da inibição do sistema GABAérgico são descritas como as principais causas de excitabilidade no SNC, e estão associadas ao aparecimento de convulsões (Fighera et al., 2003; 2006; Royes et al., 2003; 2006).

Neste mesmo contexto, Fighera e colaboradores (2006) verificaram que a administração de muscimol reduziu o número e a duração das convulsões comportamentais e EEG induzidas pela injeção intraestriatal de GA em ratos adultos. Além disso, observaram uma correlação inversa entre a duração dos episódios convulsivos e a atividade da enzima Na^+,K^+-ATPase no estriado injetado, demonstrando assim a participação desta enzima nas convulsões induzidas pelo GA. Magni e colaboradores (2007) também verificaram uma redução na atividade da enzima Na^+,K^+-ATPase após episódios convulsivos, comportamentais e EEG, causados pela injeção intraestriatal de GA em ratos adultos. Além disso, as convulsões e a inibição da atividade da enzima Na^+,K^+-ATPase foram prevenidas pela adminstração oral de creatina, sugerindo que a alteração do potencial de membrana possa ser um fator determinante para o desenvolvimento das convulsões.

A estabilização do potencial de membrana de repouso pela Na^+,K^+-ATPase é essencial para a manutenção do bloqueio de magnésio dependente de voltagem do receptor NMDA, para a função de canais iônicos dependentes de voltagem e também para os transportadores de glutamato dependentes de Na^+. Portanto, a perda da atividade da Na^+,K^+-ATPase pode diminuir o limiar para a superexcitação (Gegelashvili e Schousboe, 1997), e potencializar as ações excitotóxicas do GA.

3.4.1. Convulsões e inflamação

É conhecido que as crises encefalopáticas que levam ao aparecimento dos episódios convulsivos e ao dano estriatal nos pacientes glutaricoacidêmicos são precipitadas por infecções comuns, doenças febris ou imunizações rotineiras (Hoffmann e Zschocke, 1999; Kölker et al., 2006), sugerindo assim um possível envolvimento de citocinas inflamatórias nas convulsões apresentadas pelos pacientes com GA-I.

Nesse sentido, um ponto que merece atenção é a associação entre inflamação no SNC e ocorrência de convulsões. De acordo, estudos epidemiológicos mostraram que até 80% dos pacientes com malária apresentaram convulsões durante a inflamação aguda (Singh e Prabhakar, 2008). Além disso, pacientes com encefalite causadas por diversos agentes apresentam convulsões que podem persistir mesmo após eliminação do agente infeccioso, e que são atenuadas com o tratamento com anti-inflamatórios (Singh e Prabhakar, 2008). Estes dados indicam uma estreita associação entre inflamação no SNC e ocorrência de convulsões, sugerindo que mediadores inflamatórios atuem como prováveis agentes deste mecanismo de excitabilidade aumentada.

Contudo, a precipitação dos episódios convulsivos por processos infecciosos nos pacientes glutaricoacidêmicos ocorre durante um período limitado do desenvolvimento, normalmente entre os 3 meses e 5 anos de idade (Strauss et al., 2003; Kölker et al., 2006). E, crianças com GA-I que não apresentam crises encefalopáticas durante seus primeiros 5 anos de idade normalmente permanecem assintomáticos (sem alterações estruturais e comportamentais) durante toda sua vida, sugerindo que a vulnerabilidade é limitada a um período definido de desenvolvimento do cérebro (Kölker et al., 2006).

De acordo com este ponto de vista, trabalhos relatam que a presença de uma inflamação sistêmica durante períodos críticos do desenvolvimento pode resultar em aumento da vulnerabilidade cerebral e periférica (Hagberg e Mallard, 2005; Godbout e Johnson, 2006). De fato, tem sido demonstrado que a inflamação pós-natal em ratos injetados com lipopolisacarídeo (LPS) no 14° dia de vida os torna mais susceptíveis às convulsões induzidas por lítio-pilocarpina, ácido caínico (KA) e pentilenotetrazol (PTZ) na vida adulta. Indicando que uma injeção de LPS durante um período crítico do desenvolvimento causa um aumento duradouro na

susceptibilidade às convulsões, sendo esse efeito dependente da presença de citocinas (Galic et al., 2008).

Nesse sentido, utiliza-se a administração de LPS como um modelo para produzir neuroinflamação, pois a resposta inflamatória induzida pelo LPS é caracterizada pela geração de mediadores pró-inflamatórios concomitantemente no sistema nervoso periférico e no SNC (Miyake, 2004). O LPS é um componente glicolipídico da membrana externa de bactérias gram-negativas e sua composição varia de acordo com a bactéria de origem. O reconhecimento do LPS pelo sistema imune é complexo e envolve principalmente os receptores *toll-like* do tipo 4 (TLR-4). No entanto, apenas o TLR-4 não é suficiente para uma resposta imune total, e componentes adicionais como as proteínas CD-14 e MD-2 são requeridas para o complexo reconhecimento do LPS. Este apresenta uma das mais potentes atividades imunoestimulantes entre os ligantes dos receptores *toll-like* (Ulevitch e Tobias, 1995), e pequenas quantidades dessa toxina ativam o sistema imune inato produzindo uma série de citocinas como o fator de necrose tumoral α (TNF-α), a interleucina 1 beta (IL-1β) e a interleucina 6 (IL-6), as quais são moduladoras da transmissão neuronal normal e patológica dentro do SNC (Vitkovic et al., 2000). Essas ações do LPS indicam que ele pode desempenhar um papel central na evocação de respostas inflamatórias (Cohen, 2002).

Nesse contexto, evidencias clínicas e experimentais sugerem que processos inflamatórios facilitam à predisposição às convulsões (Vezzani et al., 1999; 2000; Ravizza et al., 2008; Rodgers et al., 2009; Auvin et al., 2010). Tem sido demonstrado que administração de LPS aumenta a susceptibilidade às convulsões em camundongos injetados com PTZ, e que este fenômeno é bloqueado por drogas antiinflamatórias (Sayyah et al., 2003), sugerindo o envolvimento de moléculas pró-inflamatórias na susceptibilidade às convulsões induzidas por PTZ.

Mais evidências do envolvimento de mediadores pró-inflamatórios na susceptibilidade às convulsões vêm de estudos farmacológicos experimentais *in vivo* que suportam o envolvimento da IL-1β na hiperexcitabilidade neuronal (Vezzani et al., 1999; 2000; Vezzani e Granata, 2005). Nesse contexto, foi demonstrado que a administração intrahipocampal de IL-1β aumentou a duração das convulsões induzidas pelo KA, sendo este efeito bloqueado pelo antagonista do receptor da IL-1 (IL-1Ra) e por um antagonista de receptores NMDA (Vezzani et al., 1999). Também foi demonstrado que a aplicação intrahipocampal do IL-1Ra e camundongos

superexpressando o IL-1Ra apresentam uma maior latência e uma menor duração na propagação das convulsões induzidas pela bicuculina (Vezzani et al., 2000). Além disso, tem sido demonstrado que as crises convulsivas também podem ser reduzidas pelo bloqueio da síntese da forma biologicamente ativa da IL-1β, através da inibição seletiva da enzima conversora de interleucina-1 (ICE ou caspase-1) ou pela deleção do gene da enzima (Ravizza et al., 2006). Nessa mesma linha, Auvin e colaboradores (2010) demonstraram que o processo inflamatório induzido pelo LPS aumenta a epileptogênese em ratos imaturos (P14) e que este aumento pode ser parcialmente revertido pelo IL-1Ra.

Em 2008, Ravizza e colaboradores estudaram o envolvimento da via da IL-1β na epileptogênese usando modelos de epilepsia do lobo temporal. Os autores demonstraram uma expressão aumentada da IL-1β em astrócitos e microglia hipocampais após um estado de mal epilético, e verificaram que a ativação da via da IL-1β durante a epileptogênese estava associada à neurodegeneração e à quebra da barreira hematoencefálica. Além disso, os autores observaram que, além da citocina IL-1β, o seu receptor do tipo 1 (IL-1R1) foram amplamente expressos em neurônios, astrócitos e microglia de tecidos cerebrais epiléticos crônicos de ratos e de humanos. De acordo com este estudo, Ravizza e Vezzani (2006) demonstraram que durante o estado de mal epilético, a indução do IL-1R1 ocorre primeiramente em neurônios, e somente várias horas mais tarde em astrócitos, sugerindo que a via da IL-1β medeia a comunicação funcional neuro-glial durante as crises. Dessa maneira, esses dados indicam que vias inflamatórias específicas são ativadas cronicamente durante a epileptogênese, e que elas persistem no tecido epilético, sugerindo a sua participação na patogênese da epilepsia do lobo temporal.

Outro estudo corrobora com as evidências que suportam o envolvimento de processos inflamatórios na patogênese das convulsões, através da descoberta de uma via pró-convulsivante que envolve o grupo caixa-1 de alta mobilidade (HMBG1), liberado de neurônios e glia, e sua interação com o TLR-4, um receptor chave da imunidade inata. Os autores verificaram que antagonistas do HMGB1 e do TLR-4 retardam o aparecimento e diminuem a ocorrência de convulsões agudas e crônicas. Também demonstraram que os efeitos pró-convulsivantes do HMGB1 foram parcialmente mediados pelos receptores NMDA sensíveis ao ifenprodil, e observaram que camundongos deficientes do TLR-4 são resistentes às convulsões induzidas pelo KA. Além disso, observaram um aumento na expressão das proteínas

HMBG1/TLR-4 no hipocampo de camundongos que apresentaram convulsões agudas e crônicas, bem como no hipocampo de pacientes com epilepsia do lobo temporal, sugerindo o envolvimento da sinalização HMBG1/TLR-4 nas epilepsias humanas. Assim, esse estudo indica que a sinalização HMGB1/TLR-4 pode contribuir para geração e para a perpetuação das convulsões em humanos, constituindo um alvo para os efeitos anticonvulsivantes em pacientes que são refratários as drogas disponíveis (Maroso et al., 2010).

Recentemente, um elegante estudo também verificou a participação do processo inflamatório na gênese das convulsões (Rodgers et al., 2009). Foi demonstrado que a resposta imune cortical inata, induzida pela aplicação cortical de LPS, produziu um grande aumento na excitabilidade cerebral resultando em descargas epileptiformes focais (convulsões focais) em ratos. Este achado sugere um importante envolvimento do sistema imune inato na epileptogênese, já que o antagonismo farmacológico dos TLR-4 preveniu a excitabilidade cortical (Rodgers et al., 2009).

Tendo em vista o grande número de trabalhos que relacionam a presença de mediadores inflamatórios e a ocorrência de convulsões em diversos modelos experimentais (Vezzani et al., 1999; 2000; Ravizza et al., 2006) e clínicos (Vezzani e Granata, 2005; Vezzani etal., 2010; Ravizza et al., 2008), é de grande interesse determinar como a ocorrência de um processo inflamatório, durante um determinado período do desenvolvimento, pode contribuir para o aumento da excitabilidade cerebral e o desenvolvimento de convulsões nos pacientes com GA-I.

3.5. Déficit cognitivo na Acidemia Glutárica tipo I

Trabalhos têm demonstrado que as crianças com GA-I apresentam alterações neurológicas relacionadas à aprendizagem (Patil et al., 2004; Boneh et al. 2008; Beauchamp et al., 2009). Neste sentido, Patil e colaboradores (2004) utilizaram um teste psicoeducacional para diagnosticar possíveis distúrbios no aprendizado de uma criança com GA-I. Eles postularam que o acúmulo de GA e dos demais metabólitos seriam os responsáveis pelo déficit de aprendizagem apresentado pela criança glutaricoacidêmica analisada. Outro trabalho, realizado com pacientes que apresentam essa acidemia orgânica revelou deficiências em atividades motoras

finas e diferentes níveis de anormalidades na fala em todos os pacientes (Boneh et al. 2008). Mais evidências do envolvimento da GA-I na aprendizagem vêm de um estudo que demonstrou déficits motores e dificuldades na articulação da fala nas crianças glutaricoacidêmicas. Os autores discutem que tais dificuldades podem ter impacto sobre a capacidade da criança para desenvolver atividades acadêmicas, de lazer, e mesmo as atividades diárias (Beauchamp et al., 2009).

Experimentalmente apenas um trabalho demonstrou que a administração crônica de GA causou um prejuízo na memória espacial de ratos no labirinto aquático de Morris, desde que os ratos não foram capazes de lembrar a localização anterior da plataforma, pois passaram um tempo significativamente menor no quadrante treinado. Ao contrário, a administração crônica de GA não afetou o desempenho dos ratos no campo aberto e no labirinto em cruz elevado, indicando que a atividade motora e ansiedade não foram alteradas pelo GA. Assim, esses resultados fornecem evidências de que a administração crônica de GA induz um déficit duradouro no aprendizado espacial (Da C. Ferreira et al., 2008).

3.5.1. Déficit cognitivo e inflamação

Embora as alterações neurológicas sejam predominantes na GA-I, pouco se sabe sobre o mecanismo pelo qual o GA leva a essas alterações (Lima et al., 1998; Fighera et al., 2006; Rosa et al., 2007; Magni et al., 2007; 2009). Especificamente em relação ao déficit cognitivo, estudos têm relatado que os pacientes com GA-I apresentam prejuízos cognitivos (Patil et al., 2004; Boneh et al., 2008; Beauchamp et al., 2009), que são mais prevalentes após as crises encefalopáticas, as quais são precipitadas por processos infecciosos (Strauss et al., 2003; Kölker et al., 2006). Estes dados indicam uma associação entre inflamação no SNC e a ocorrência de déficit de memória nas crianças glutaricoacidêmicas, sugerindo que mediadores inflamatórios atuem como prováveis agentes deste mecanismo.

A memória é a capacidade que temos de armazenar informações que possam ser recuperadas e utilizadas posteriormente (Lent, 2004). O hipocampo é uma estrutura do lobo temporal mesial sabidamente envolvido na aquisição/consolidação e/ou evocação da memória (Izquierdo e Medina, 1995). O processo de formação da memória envolve eventos moleculares, entre eles, a ativação da proteína ligante do elemento responsivo ao AMPc (CREB), resultando em um aumento da síntese

protéica e na efetividade da transmissão de informações entre neurônios, com os quais o neurônio-alvo se comunica. Tais alterações entre os neurônios têm sido denominadas "plasticidade sináptica" (McGaugh, 2000; 2002; McGaugh e Izquierdo, 2000).

Um dos tipos de memória estritamente relacionada com o hipocampo é a memória espacial, que representa a habilidade para codificar, armazenar e recuperar informações sobre localizações espaciais, configurações ou rotas (Kessels et al., 2001). Ao longo das últimas décadas, através de estudos com pacientes submetidos a remoções das estruturas temporais mesiais, vem sendo demonstrado à relação destas estruturas com a memória espacial através da realização de testes cognitivos com componentes espaciais, como a aprendizagem de labirintos e a evocação de sequências (Milner, 1968). Além disso, estudos com animais demonstraram a grande importância do sistema hipocampal na memória espacial de roedores (Eichenbaum, 2002) e de primatas (Brasted et al., 2003). Neste contexto, Broadbent e colaboradores (2004) mostraram que ratos com lesões no hipocampo, induzidas com ácido ibotênico, apresentam um prejuízo no aprendizado espacial.

Outro processo que causa prejuízo cognitivo é a neuroinflamação (Chen et al., 2008; Min et al., 2009). Interessantemente, um grande número de doenças cognitivas humanas (como a doença de Alzheimer, a demência associada a AIDS e a síndrome de Down) estão associadas a níveis elevados de moléculas pró-inflamatórias como a IL-1β e o TNF-α (Griffin et al., 1989; Perrella et al., 1992; Akiyama et al., 2000; Casadesus et al., 2007; Holmes et al., 2009). Esta observação sugere uma possível relação entre o aumento nos níveis de moléculas pró-inflamatórias no SNC e disfunção de memória em humanos. De acordo, um estudo com 20 homens adultos saudáveis, submetidos a testes neuropsicológicos, demonstrou que administração intravenosa de LPS (0.8 ng/kg) em duas sessões experimentais causou um aumento nos níveis sanguíneos de IL-6, TNF-α, receptores do TNF, IL-1Ra e cortisol, além de uma redução nas funções da memória declarativa, durante todos os períodos testados (1, 3 e 9 horas após a injeção de LPS; Reichenberg et al., 2001). Outro estudo com 12 homens jovens saudáveis, também submetidos a testes neuropsicológicos, verificou que a administração de uma dose muito baixa de LPS (0.2 ng/kg) em duas sessões experimentais aumentou os níveis circulantes de TNF- α, IL-6, receptores do TNF e IL-1Ra, sem nenhuma

alteração no desempenho cognitivo, mas com uma significativa correlação inversa entre a memória declarativa e os níveis de IL-6 (Krabbe et al., 2005).

Além disso, numerosos estudos com modelos animais também têm encontrado uma associação entre níveis elevados de citocinas e déficits de memória após processos neuroinflamatórios (Pugh et al., 1998; 1999; Barrientos et al., 2002; Hein et al., 2007). Tem sido demonstrado que a administração periférica de LPS, um modelo estabelecido de infecção, prejudica o aprendizado e a memória em vários testes experimentais (Pugh et al., 1998; 2000). Foi verificado que a administração periférica de LPS causou um aumento nos níveis de citocinas hipocampais em ratos, prejudicou a memória contextual, mas não ao tom, e que a administração prévia do IL-1Ra aboliu esse efeito sobre a memória (Pugh et al., 1998). Similar a esse achado, foi demonstrado que a injeção intracerebroventricular (i.c.v.) da proteína capsidial gp-120 do vírus HIV em ratos também aumentou os níveis de IL-1β no hipocampo e prejudicou a memória contextual, mas não ao tom, na tarefa do medo condicionado. Além disso, foi verificado que a administração do IL-1Ra imediatamente após a injeção da proteína gp-120 bloqueou o efeito sobre a memória (Pugh et al., 2000).

Corroborando com esses achados tem sido demonstrado que a infecção ou exposição perinatal ao LPS também altera o aprendizado e a memória. De fato, há evidências de que a ocorrência de um processo inflamatório durante o início do desenvolvimento causa déficit cognitivo (Bilbo et al., 2005a,b; 2007; 2008; Fan et al., 2010). Trabalhos têm demonstrado que a infecção periférica induzida pelo LPS em ratos neonatos resultou em déficit de aprendizado e de memória frente a um desafio inflamatório (LPS) na idade adulta (Bilbo et al., 2005a,b; 2007; 2008). Foi verificado ainda que a exposição perinatal ao LPS também prejudicou o aprendizado, a memória e plasticidade neural em ratos adultos (Harré et al., 2008; Kohman et al., 2008; Fan et al., 2010). Também foi demonstrado que o processo inflamatório neonatal estava associado com uma ativação exagerada da microglia e com uma produção hipocampal aumentada de IL-1 na idade adulta (Bilbo et al., 2005a; 2007). Além disso, foi demonstrado que a inibição da caspase-1 preveniu o prejuízo de memória induzido pelo LPS, sugerindo a participação da IL-1 nos efeitos infecciosos e inflamatórios neonatais que resultam nas alterações cognitivas na idade adulta (Bilbo et al., 2005a). Nessa mesma linha, Ikeda e colaboradores (2005) demonstraram que a combinação de LPS e hipóxia-isquemia em ratos neonatos

causou um prejuízo de longa duração no aprendizado e uma diminuição no volume hipocampal, além de que o tratamento com dexametasona preveniu esses efeitos.

É sabido que níveis fisiológicos de citocinas são requeridos para alguns processos de memória e aprendizagem, particularmente para a consolidação de memórias que dependem do hipocampo (Brennan et al., 2004; Goshen et al., 2007; Labrousse et al., 2009), no entanto, níveis elevados de citocinas inflamatórias estão relacionados a déficits cognitivos. Nesse sentido, diversos trabalhos têm demonstrado a influência negativa das citocinas inflamatórias nos processos de aprendizagem e memória (Matsumoto et al., 2002; Song e Horrobin, 2004; Hein et al., 2010).

Em relação a IL-1β, os seus efeitos prejudiciais sobre a memória foram demonstrados pela primeira vez por Oitzl e colaboradores (1993) que verificaram que a administração i.c.v. de IL-1β uma hora antes do labirinto aquático de Morris causou uma redução na memória espacial. Entretanto quando esta foi injetada imediatamente antes do treino nenhum efeito foi encontrado, sugerindo que a IL-1β não afeta a aquisição de memória espacial, mas sim a consolidação dessa aprendizagem, e que os processos desencadeados pela IL-1β exigem algum tempo para exercer sua influência sobre a memória. Estudos subsequentes confirmaram essa conclusão e demonstraram que a administração periférica de IL-1β também prejudica a aprendizagem espacial (Gibertini et al., 1995; Song e Horrobin, 2004). Importantemente, no teste do labirinto aquático a IL-1β prejudica somente a memória espacial, sem alterar a memória não espacial (Gibertini, 1996; Song e Horrobin, 2004). Da mesma forma, foi demonstrado que camundongos injetados com IL-1β foram menos flexíveis para se adaptar a uma mudança na posição da plataforma submersa (Gibertini, 1996). Em conjunto, esses resultados sugerem que a IL-1 interfere especificamente no aprendizado espacial que depende do funcionamento normal do hipocampo.

Para investigar os efeitos da exposição crônica a IL-1, estudos recentes utilizaram camundongos transgênicos que superexpressam a IL-1β no hipocampo. Demonstraram que uma elevação sustentada nos níveis de IL-1β hipocampal resultou em neuroinflamação (verificada pela microgliose e pelo aumento na produção de vários mediadores inflamatórios) e causou prejuízos na memória espacial testada no labirinto aquático, bem como prejudicou a memória de longa duração na tarefa do medo condicionado (Moore et al., 2009; Hein et al., 2010). E é

importante notar que, em ambos os estudos, os efeitos foram restritos a memória dependente do hipocampo.

Quanto ao TNF-α, o seu envolvimento em processos de memória tem sido estudado por vários grupos de pesquisa, utilizando vários modelos. A maioria dos estudos não relata o envolvimento do TNF-α no funcionamento da memória (Albensi e Mattson, 2000; Stellwagen e Malenka, 2006; Kaneko et al., 2008). No entanto, alguns estudos demonstraram um efeito prejudicial dessa citocina sobre a formação da memória. O efeito danoso do TNF-α sobre a memória foi primeiramente demonstrado por um prejuízo no aprendizado no teste da esquiva passiva em camundongos que superexpressavam o TNF-α no SNC (Fiore et al., 1996). De acordo, foi também demonstrado que a administração i.c.v. diária de TNF-α por uma semana antes do treino no labirinto aquático prejudicou a memória e o aprendizado espacial neste teste (Bjugstad et al., 1998). Um efeito prejudicial do TNF-α também foi relatado após a sua administração intra-hipocampal que resultou na redução na memória de trabalho dependente do hipocampo, evidenciada pelo aumento no número de erros e por maiores latências para executar a tarefa do labirinto em Y (Matsumoto et al., 2002).

Os mecanismos básicos dos efeitos das citocinas inflamatórias sobre o aprendizado e a memória dependentes do hipocampo têm-se concentrado na função neuronal através da potenciação de longa duração (LTP; Lynch, 2004), um sistema modelo para o mecanismo neuronal da memória, em várias vias hipocampais (Martin et al., 2000). Sabe-se que níveis fisiopatológicos de IL-1β e TNF-α podem produzir efeitos prejudiciais sobre a memória (Matsumoto et al., 2002; Song e Horrobin, 2004; Hein et al., 2010), e que estes efeitos são específicos para a consolidação das memórias que dependem do hipocampo, enquanto que as memórias independentes do hipocampo não são alteradas (Rachal Pugh et al, 2001; Goshen e Yirmiya, 2007).

Tem sido sugerido que as citocinas pró-inflamatórias produzidas durante processos inflamatórios possam ser as responsáveis pela disfunção sináptica (Chen et al., 2008; Tanaka et al., 2006; Hein e O'Banion, 2009). De fato, existem fortes evidências de que o aumento de IL-1β no hipocampo pode prejudicar a memória devido aos efeitos sobre a transmissão sináptica, através de prejuízos na indução e na manutenção da LTP hipocampal. No primeiro estudo realizado foi demonstrado que a aplicação de IL-1β inibiu a LTP na região CA3 em fatias hipocampais de camundongos (Katsuki et al., 1990). Em seguida, foi demonstrada uma inibição

similar da LTP, induzida pela IL-1β, na região CA1 (Bellinger et al., 1993) e no giro denteado (Cunningham et al., 1996). Também foi relatado que a manutenção da LTP estava negativamente associada com o aumento nos níveis de IL-1β no hipocampo (Murray e Lynch, 1998). De acordo, mais recentemente Ross e colaboradores (2003) encontraram um aumento quantitativo de IL-1β em fatias hipocampais mantidas a 34-36°C quando comparadas as mantidas a 21-24°C, e que esse aumento de citocinas gerou uma inibição na LTP.

Nesse sentido, tem sido sugerido que a sinalização da IL-1 durante a inflamação, provavelmente mediada pela IL-1β, provoca um aumento seletivo e relativamente persistente na inibição GABAérgica, e que essas ações contribuem para diminuir a excitabilidade sináptica (Ikegaya et al., 2003; Hellstrom et al., 2005). Além disso, foi demonstrado o envolvimento das funções colinérgicas e glutamatérgicas no prejuízo da memória de trabalho induzido pela interleucina-1β em ratos (Matsumoto et al., 2001). De acordo outros estudos demonstraram que a exposição aguda a IL-1β em fatias de hipocampo produziu uma redução na transmissão sináptica glutamatérgica basal (Murray et al., 1997) e uma diminuição na transmissão sináptica excitatória (Coogan e O'Connor, 1997; Luk et al., 1999). Além disso, Vereker e colaboradores (2000) demonstraram que a aplicação de LPS induziu um aumento na concentração de IL-1β e inibiu a LTP no giro denteado de ratos através da ativação da caspase-1.

Neste contexto, outros estudos têm demonstrado que a aplicação de TNF-α também inibe a indução da LTP nas áreas CA1 (Tancredi et al., 1992) e no giro denteado do hipocampo (Cunningham et al., 1996). Além disso, Butler e colaboradores (2004) demonstraram que a infusão de TNF-α antes de um estímulo para induzir a LTP inibe a formação desta. Nesse sentido, tem sido proposto que a inibição da LTP pelo TNF-α ocorre via ativação do receptor 1 do TNF (TNFR1) e dos receptores glutamatérgicos metabotrópicos grupo 1 (Cumiskey et al., 2007).

Mais evidências de que as citocinas inflamatórias possam estar envolvidas nas alterações hipocampais vêm de estudos que mostraram que vários processos inflamatórios, que resultam na ativação da microglia e na produção de citocinas pró-inflamatórias, apresentam uma influência prejudicial sobre a neurogênese (Ekdahl et al., 2003; 2009; Kempermann e Neumann, 2003). Pois, embora em condições normais a microglia possa estar envolvida na facilitação da neurogênese (Hanisch e Kettenmann, 2007; Ziv e Schwartz, 2008), a inflamação induzida pela ativação da

microglia tem sido implicada na supressão da neurogênese (Kempermann e Neumann, 2003; Ekdahl et al., 2009). Evidências são fornecidas pela demonstração de que a administração intracortical crônica de LPS dá origem à ativação da microglia na área onde nascem novos neurônios, prejudicando fortemente a neurogênese hipocampal (basal e induzida) em ratos. Além disso, foi demonstrado que administração de um inibidor da microglia bloqueou os efeitos anti-neurogênicos do LPS (Ekdahl et al., 2003). Interessantemente o estímulo inflamatório não precisa estar localizado no cérebro para afetar a neurogênese, pois foi demonstrado que uma única injeção intraperitoneal (i.p.) de LPS aumentou o número de microglia ativada e reduziu a neurogênese hipocampal (Monje et al., 2003). Além disso, a diferenciação neuronal *in vitro* estava diminuída quando os neurônios foram co-cultivados com a microglia ativa, sendo este efeito mediado por fatores solúveis, já que somente o meio condicionado à microglia ativada produziu o mesmo efeito (Cacci et al., 2005). Estes trabalhos sugerem que a ativação da microglia contribui de maneira decisiva para a inibição da neurogênese hipocampal.

Coletivamente, esses estudos, fornecem fortes evidências de que as citocinas pró-inflamatórias em níveis elevados, especialmente a IL-1β e o TNF-α, estão associadas a déficits de memória após processos neuroinflamatórios. Portanto, é de especial interesse determinar como a presença de um processo inflamatório pode contribuir para o desenvolvimento das alterações estruturais e funcionais no hipocampo dos pacientes com GA-I. No entanto, apenas um trabalho na literatura avaliou o desempenho de ratos jovens injetados cronicamente com GA frente a um teste de memória, porém sem a presença de um processo inflamatório. Dessa maneira, não se conhece os efeitos do acúmulo desses ácidos orgânicos no aprendizado de ratos jovens submetidos a um estímulo infeccioso.

Portanto, devido à escassez de medidas terapêuticas efetivas no controle do desenvolvimento das alterações da GA-I, evidencia-se a importância da busca da compreensão da fisiopatogênese da doença para o desenvolvimento de novas estratégicas terapêuticas.

4. RESULTADOS

4. RESULTADOS

Os resultados que fazem parte desta tese estão apresentados neste item, o qual apresenta-se subdividido sob a forma de artigo científico (capítulo I) e manuscrito (capítulo II). Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos mesmos.

4.1. Capítulo I – Lipopolissacarídeo aumenta a susceptibilidade à convulsão induzida pelo ácido glutárico em ratos jovens: uma abordagem comportamental e eletroencefalográfica.

Artigo

LIPOPOLYSACCHARIDE ENHANCES GLUTARIC ACID-INDUCED SEIZURE SUSCEPTIBILITY IN RAT PUPS: BEHAVIORAL AND ELECTROENCEPHALOGRAPHIC APPROACH

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Lipopolysaccharide enhances glutaric acid-induced seizure susceptibility in rat pups: Behavioral and electroencephalographic approach

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Summary Glutaric acidemia type I (GA-I) is an inherited metabolic disease characterized by accumulation of glutaric acid (GA) and seizures. Considering that seizures are precipitated by common infections in children with GA-I, we investigated whether lipopolysaccharide (LPS) modifies GA-induced electrographic and neurochemical alterations in 21 days-old rats. The effect of LPS on convulsive behavior and electroencephalographic (EEG) alterations induced by GA (0.13; 0.4; 1.3 μmol/striatum) was determined in freely moving rats. After EEG recordings, we measured the levels of interleukin 1β (IL-1β) in GA-injected striatum. The injection of LPS (2 mg/kg; i.p.) 6 h before of GA administration, reduced the latency and increased the

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duration of seizures induced by GA (1.3 μmol/site). In addition, LPS administration increased IL-1β striatal levels, which positively correlated with total time in seizures. The intrastriatal injection of an IL-1β antibody (200 ng/2 μl) prevented the facilitation of GA-induced seizures by LPS. These data suggest that inflammatory processes during critical periods of development may decrease GA-induced seizure threshold.

Glutaric acidemia type I (GA-I) is an inherited neurodegenerative disease caused by deficiency of the mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH; EC 1.3.99.7), which is involved in the metabolism of L-lysine, L-hydroxylysine and L-tryptophan. Affected patients accumulate majorly glutaric acid (GA), 3-hydroxyglutaric acid (3-OH-GA) and glutaconic acid in the body fluids (Goodman et al., 1977). Clinical manifestations of GA-I are predominantly neurological, including convulsions, especially after encephalophatic crises, which are accompanied by bilateral and irreversible destruction of susceptible brain regions, i.e. striatum (Hoffmann and Zschocke, 1999). Furthermore, neurological crises are typically precipitated by common infection, febrile illness and after routine immunization, suggesting a role of inflammatory mediators in this organic aciduria (Hoffmann and Zschocke, 1999). A substantial body of evidence indicates that brief systemic inflammation during critical periods of development may result in long-lasting cerebral and peripheral vulnerability (Hagberg and Mallard, 2005). For instance, it has been shown that rats injected at postnatal day 14 (P14) with LPS are more susceptible to seizures induced by lithium–pilocarpine, kainic acid (KA) and pentylenetetrazole (PTZ; Galic et al., 2008).

The inflammatory response brought about by LPS is characterized by innate immune system activation and production of proinflammatory mediators, such as tumor necrosis factor (TNF) and IL-1β (Cohen, 2002). These proinflammatory cytokines are now accepted as bona fide modulators of both normal and abnormal neuronal transmission within the brain (Merrill, 1992).

In this context, previous reports have shown that GA-I metabolites interfere with astrocytic glutamatergic transporters (Porciúncula et al., 2004) and provide evidence that astrocyte proliferation is associated with mitochondrial dysfunction and oxidative stress (Olivera et al., 2008). In line of this view, the appearance of such newborn astrocytes may underlie the subsequent development of gliosis that disrupt brain development, perhaps contributing for the establishment of neurological deficits in GA-I patients (Goodman and Frerman, 2001). In addition, it has been shown that postnatal LPS administration results in a chronic though mild form of astrogliosis, a feature commonly found in a number of seizure models (Somera-Molina et al., 2007; Oberheim et al., 2008) and patients with epilepsy (Eid et al., 2008).

Although clinical and experimental evidence suggest that infection or inflammation facilitates seizure predisposition (Vezzani and Granata, 2005; Auvin et al., 2010; Vezzani and Baram, 2007) the pathogenesis of GA-induced convulsive behavior is still unknown. Therefore, we decided to investigate the involvement of proinflammatory cytokines during critical periods of development (Galic et al., 2008) in electroencephalographic and neurochemical alterations induced by GA. For this purpose we investigated the effect of LPS administration on seizures and alterations of striatal levels of IL-1β induced by GA in rat pups (21 days-old).

Experimental procedures

Animal and reagents

Twenty one days-old male Wistar rats (30–40 g), maintained under controlled light and environment (12:12 h light–dark cycle, 24 ± 1 °C, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) were used. Animal utilization protocols followed the Official Government Ethics guidelines and were approved by the University Ethics Committee. All efforts were made to reduce the number of animals used, as well as to minimize their suffering. All reagents were purchased from Sigma (St. Louis, MO).

Surgical procedure and behavioral evaluation

The animals were anesthetized with ketamine–xylazine (45–9 mg/kg body weight; i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a cannula was inserted unilaterally into the right striatum (coordinates relative to bregma: AP 0 mm, ML 3.0 mm, V 2.5 mm from the dura). Chloramphenicol (200 mg/kg) was administrated intraperitoneally (i.p.) immediately before the surgical procedure. The behavioral evaluation was performed 3 days after surgery when animals did not show any sign of pain, infection or discomfort.

In order to determine the convulsant dose of GA in rat pups, the animals were injected with GA (0.13; 0.4 or 1.3 μmol/2 μl) or saline (1.3 μmol/2 μl) into the right striatum. Glutaric acid solutions were neutralized with NaOH to pH 7.4 and injections were performed over a 1 min interval. Immediately after the intrastriatal injections, the animals were transferred to a round open field (54.7 cm in diameter) with a floor divided into 10 equal areas. During 20 min, the animals were videomonitored for the appearance of seizures. The latency to clonic and generalized convulsions and time in seizures were recorded. Clonic convulsions were characterized by typical partial clonic activity affecting the face, head, vibrissae and forelimbs. Generalized convulsive episodes were characterized by generalized whole-body clonus involving all four limbs and tail, rearing, wild running and jumping, sudden loss of upright posture and autonomic signs, such as hypersalivation and defecation, respectively.

To investigate whether inflammation alters seizure activity induced by GA, LPS (2 mg/kg; E. coli 055 B5; Eriksson et al., 2000; Maeda et al., 2008) or vehicle was infused i.p. (single administration) 3 or 6 h before the administration of GA (0.13; 0.4 or 1.3 μmol/site) or saline (1.3 μmol/site). The animals were then transferred to a round open field and convulsive behavior was evaluated as described above. Immediately after the behavioral evaluation, the animals were sacrificed by decapitation to determine IL-1β levels in the injected striatum.

Since LPS administration 6 h before GA intrastriatal injection facilitated convulsions and increased IL-1β levels, we investigated whether this cytokine is involved LPS-induced facilitation of GA-induced seizures. The animals were subjected to intrastriatal injection of IL-1β antibody (200 ng/2 μl) or IL-1β denatured antibody 45 min before injection of LPS (2 mg/kg; i.p.) or vehicle

(2 mg/kg, i.p.). This dose of IL-1β antibody was chosen because it neither caused seizures nor altered motor activity in a pilot experiment. Six hours after LPS or vehicle injection, GA (0.13, 0.4 or 1.3 μmol/site) or saline (1.3 μmol/site) was administrated and the appearance of seizures monitored for 20 min by behavioral and electroencephalographic recordings (EEG).

Placement of cannula and electrodes for EEG recordings

A subset of animals (n=5–6) was anesthetized with ketamine–xylazine and surgically implanted with a cannula and electrodes under stereotaxic guidance. The guide cannula (27 gauge) was glued to a multipin socket and inserted into the right striatum through a previously opened skull orifice. One screw electrode was placed over the right parietal cortex (coordinates in mm: AP −4.5; ML 2.5; and V 2) along with a ground lead positioned over the nasal sinus. Bipolar nichrome wire Teflon-insulated depth electrodes (100 μm) were implanted into the right striatum (coordinates in mm: AP 0; ML 3; V 2.5). The electrodes were connected to the multipin socket and, together with the injection cannula, were fixed to the skull with dental acrylic cement. Electroencephalographic recordings were performed 3 days after the surgery.

Intrastriatal injection of drugs and EEG recording

The procedures for EEG recordings were carried out as previously described by Magni et al. (2007). Routinely, the animals were allowed to settle for habituation in a plexiglas cage (25 cm × 25 cm × 60 cm) for at least 20 min. Baseline EEG recordings were obtained 10 min prior to drugs administration in order to establish an adequate control brain electrical activity. The drug injection protocol used in this set of experiments was the same used in those experiments that evaluated the effect of LPS on GA-induced behavioral seizures, except that EEG was concomitantly recorded using digital encephalographic equipment (Neuromap EQSA260, Neuromap LTDA, Brazil). During 20 min the animals were videomonitored for the appearance of clonic or tonic seizures. The latency to seizures and time in seizures were recorded.

EEG signals were amplified, filtered (0.1–70.0 Hz, bandpass), digitalized (sampling rate 250 Hz) and stored in a PC for off-line analysis, as described below.

EEG analyses

Seizures were defined by the occurrence of episodes consisting of a pattern of continuous high-amplitude sharp spikes in low-frequency rhythmic spiking activity in ipsilateral striatum and cortex (≥2× baseline amplitude, ≥5 s). Handling for drug injection caused artifacts in the EEG (Figs. 1, 3 and 7). However, rhythmic scratching of the electrode headset by the animal rarely caused artifacts. These recordings were easily identified and discarded.

Rectal temperature measurement

In order to investigate the effect of infection state on the basal rectal temperature (T_r) of 21-days old rats, vehicle or LPS was infused intraperitoneally and T_r changes were recorded every hour for 3 or 6 h, and expressed as absolute value.

Rectal temperature was measured with a lubricated thermocouple, which was inserted into the rectum of the animal, for 1 min. The probe was linked to a digital device, which displayed the temperature at the tip of the probe with a 0.1 °C precision. The values displayed were manually recorded. In order to minimize the effects of the stress associated with handling and injecting on rectal temperature, all rats were habituated to the measuring procedure for two consecutive days. In these sessions, the animals were subjected to the same temperature measuring procedure described above.

IL-1β immunoassay

The content of IL-1β was determined in injected striatum (right). After the behavioral evaluation of seizures, the injected striatum was dissected rapidly out at 4 °C and frozen at −70 °C. The striatum was weighed and homogenized in a solution containing bovine serum albumin (BSA 10 mg/ml), EGTA (2 mM), EDTA (2 mM) and PMSF (0.2 mM) in phosphate-buffered saline (PBS, pH 7.4) using a Potter homogenizer. The striatum homogenate was centrifuged (3000 × g for 10 min) and cytokines were determined in supernatant. Cytokine levels were measured using a commercially available ELISA Kit from R&D Systems (Minneapolis, MN) using a selective antibody against rat IL-1β, according to the manufacturer's protocol, and are expressed as pg/mg of protein (detection limit: 4 ng/ml).

Protein determination

Protein content was measured colorimetrically by the method of Bradford (1976) using bovine serum albumin (1 mg/ml) as a standard.

Statistics

The behavioral data were analyzed by non-parametric test (Kruskal–Wallis analysis of variance). Data from IL-1β levels and rectal temperature measurements were analyzed by a two-way ANOVA. Post hoc analysis was carried out by the Student–Newman–Keuls test. P<0.05 was considered significant. The Pearson's correlation coefficient was used for correlation analyses.

Results

GA-induced seizures

In the present study, the intrastiatal injection saline (1.3 μmol/site) and GA (0.13 μmol/site) did not induces convulsive behavior. On the other hand, statistical analysis revealed that intrastriatal injection of GA (1.3 μmol/site) decreased the latency to the first convulsive episode [164.88 ± 18.18 s; $H(3)$ = 24.08, P < 0.01; Fig. 1F] and increased time in seizures 40.88 ± 4.5 s; [$H(3)$ = 25.58; P < 0.01; Fig. 1G] as compared to GA (0.4 μmol/site) group. The EEG recordings confirmed these behavioral alterations elicited by the intrastriatal injection of GA (Figs. 1A–D). The electroencephalographically recorded GA-induced seizures were behaviorally accompanied by clonus of the left forelimb and/or hind limb and head, rotational behavior and full lateralization toward the left side of the body. In addition, EEG recordings of the ispilateral striatum revealed that the GA administration caused the appearance of an epileptogenic focus in the injected striatum that spread to the ipsilateral cortex. The EEG seizure activities were characterized by the occurrence of multispikes and major seizure activity. Multispikes correlated with myoclonic jerks, which are characteristic of clonic convulsions. Generalized convulsions appeared in the electroencephalographic recordings as the major seizure activity, and were characterized by 2–3 Hz high-amplitude activity (Figs. 1D–E). The animals that were

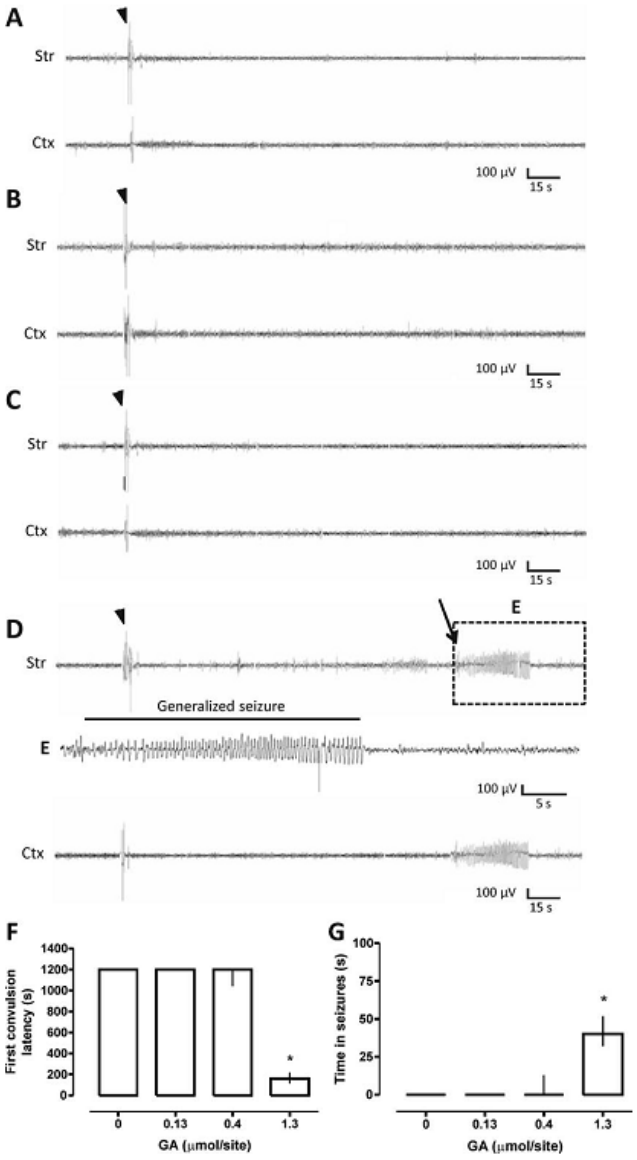

Figure 1 Representative electroencephalographic recordings: intrastriatal administration of saline (control; A), intrastriatal administration of GA (0.13 µmol/site; B), (0.4 µmol/site; C), and (1.3 µmol/site; D) which were accompanied by pattern of continuous high-amplitude sharp spikes in low-frequency rhythmic spiking activity in ipsilateral striatum and cortex. (E) The expanded waveforms from box of the EEG recording in D. In all traces the arrowhead indicates the saline or GA intrastriatal administration; the arrow indicates the EEG pattern associated with generalized seizures. Str, ipsilateral striatum; Ctx, ipsilateral cortex. Calibration bars, 100 µV and 15 s. Effect of intrastriatal injection of GA (0.13; 0.4; 1.3 µmol/site) on the first convulsion latency (F) and time in seizures (G) in rat pups. Data are median and interquartile range for $n = 6–8$ in each group.*$P < 0.01$ as compared to GA (0.4 µmol/site) group; Kruskal–Wallis analysis of variance.

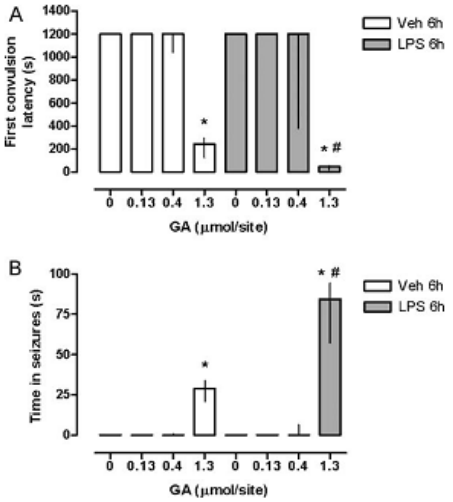

Figure 2 Effect of injection LPS (i.p.) 6 h before of the intrastriatal administration of GA (0.13; 0.4; 1.3 μmol/site) on the first convulsion latency (A) and time in seizures (B) in rat pups. Data are median and interquartile range for $n = 6-8$ in each group. $^{*}P < 0.05$ when compared to vehicle 6 h or LPS 6 h/GA (0.4 μmol/site) groups (A, B); $^{\#}P < 0.05$ when compared to vehicle 6 h/GA (1.3 μmol/site) group (A, B); Kruskal–Wallis analysis of variance.

injected with GA (1.3 μmol/site) showed 3.0 ± 0.55 seizures during the 20 min period of observation.

LPS facilitates GA-induced seizures

Statistical analysis revealed that administration of LPS 3 h before the intrastriatal injection of GA (all doses) had no effect on the latency to convulsions [119.62 ± 53.61 s; $H(7) = 5.46$; $P > 0.05$] and time in seizures [28.75 ± 8.04 s; $H(7) = 7.66$; $P > 0.05$] as compared to vehicle 3 h/GA (1.3 μmol/site) group (data not shown). On the other hand, statistical analysis revealed that the administration of LPS 6 h before intrastriatal injection of GA (1.3 μmol/site), decreased the latency to convulsions [39.7 ± 6.69 s; $H(7) = 66.54$; $P < 0.05$; Fig. 2A], and increased the time in seizures [80.7 ± 7.17 s; $H(7) = 66.05$; $P < 0.05$; Fig. 2B] as compared to vehicle 6 h/GA (1.3 μmol/site) group. Prior administration of LPS (2 mg/kg) did not cause behavioral changes.

LPS (2 mg/kg) injection did not affect baseline (248.21 ± 5.40 μV) or interictal mean amplitude, when compared with control group (234.88 ± 1.16 μV, $n = 6-8$ in each group; data not shown). EEG recordings showed that animals injected with LPS 3 h before GA injection (Fig. 3C) did not present significant alterations in EEG recordings when compared with the group that received LPS 6 h before GA injection (Fig. 3E). However, the injection of LPS 6 h before GA decreased the latency to seizures [38.55 ± 7.37 s; $H(2) = 7.05$; $P < 0.05$; Fig. 3G] and increased the time in seizures [67.33 ± 10.82 s; $H(2) = 10.97$; $P < 0.05$; Fig. 3H] as compared to vehicle/GA (1.3 μmol/site; Fig. 3A) and LPS 3 h/GA (1.3 μmol/site; Fig. 3C) groups. Furthermore, LPS (2 mg/kg) injection did not alter the number of convulsive episodes induced by GA (1.3 μmol/site, data not shown). No animals died due to GA-induced convulsions.

Effect of LPS on basal rectal temperature

LPS causes an initial drop in basal temperature (1–2 h) followed by a temperature increase in the coming hours (3–6 h). Statistical analysis (two-way ANOVA with repeated measures) showed that LPS administration increased rectal temperature after a period of ~3 h [$F(5,100) = 7.64$; $P < 0.01$; Fig. 4] when compared with vehicle/saline and vehicle/GA (1.3 μmol/site) groups. Post hoc analysis (F test for simple effect) revealed that the LPS-induced increase of rectal temperature in 3 h persisted up to 6 h [$F(3,20) = 64.4$; $P < 0.05$] when compared with vehicle/saline and vehicle/GA (1.3 μmol/site) groups.

IL-1β levels in the striatum

Since LPS injection increased the GA-induced convulsive behavior, we evaluated the effect of administrating this toxin on IL-1β levels after GA (1.3 μmol/site) injection in the striatum of rat pups. Statistical analysis showed that LPS administration increased IL-1β levels after 3[$F(3,11) = 11.49$; $P < 0.01$; Fig. 5A] and 6 h [$F(3,10) = 8.26$; $P < 0.01$; Fig. 5B] in the injected striatum. Post hoc analysis (Student–Newman–Keuls test) revealed that this increase was higher in 6[$F(3,13) = 4.58$; $P < 0.03$] than in 3 h.

Correlation analyses (Pearson's correlation coefficient) revealed a strong positive correlation between time in seizures induced by GA administration (1.3 μmol/site) and striatal IL-1β levels ($r = 0.922$; $P < 0.03$; Fig. 6) in animals injected with LPS (2 mg/kg; i.p.).

IL-1β antibody treatment

Fig. 7G–H shows that injection of the IL-1β antibody (200 ng/2 μl), 45 min before LPS prevented the cytokine-mediated reduction in the latency [187.44 ± 27.3 s; $H(3) = 17.26$; $P < 0.05$] and the total time increase of seizures [26.88 ± 1.9 s; $H(3) = 20.43$; $P < 0.05$] as compared to IL-1β denatured antibody/LPS 6 h/GA (1.3 μmol/site) group. Thus, the administration of IL-1β antibody inhibited the LPS-mediated increase in the duration of GA-induced seizures. These results are in agreement with the view that inflammatory process plays a role in GA-induced seizures (Fig. 7A–H).

Discussion

The dose–effect curve for GA revealed that the dose of 1.3 μmol/site caused seizures in rat pups, measured as decreased seizure latency and increased time in seizures. In this context, there is a significant body of evidence suggesting that GA accumulation may play a role in the convulsions and neurological impairment seen in patients with glutaric acidemia (Lima et al., 1998; Kolker et al., 2002; Fighera

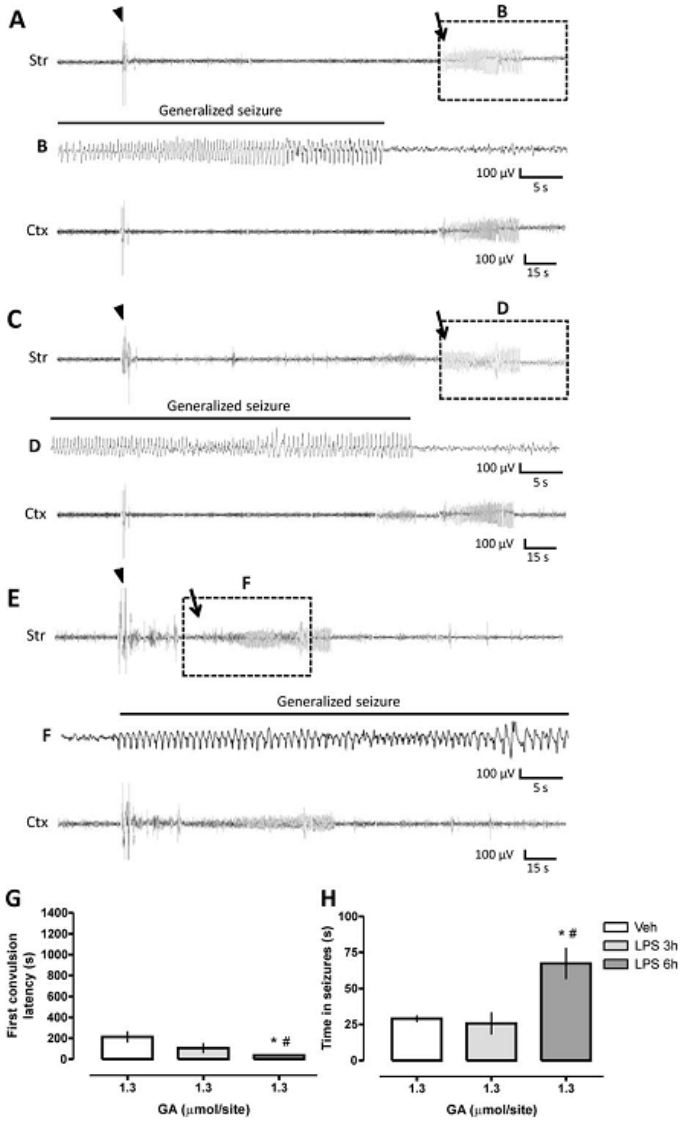

Figure 3 Representative electroencephalographic recordings: (A) intrastriatal administration of GA (1.3 µmol/site); (B) expanded waveforms from box of the EEG recording in A; (C) injection LPS (i.p.) 3 h before intrastriatal administration of GA (1.3 µmol/site); (D) expanded waveforms from box of the EEG recording in C; (E) injection LPS (i.p.) 6 h before intrastriatal administration of GA (1.3 µmol/site); (F) expanded waveforms from box of the EEG recording in E. All seizures were accompanied by pattern of continuous high-amplitude sharp spikes in low-frequency rhythmic spiking activity in ipsilateral striatum and cortex. In all traces the arrowhead indicates the GA intrastriatal administration; the arrow indicates the EEG pattern associated with generalized seizures. Str, ipsilateral striatum; Ctx, ipsilateral cortex. Calibration bars, 100 µV and 15 s. Effect of injection LPS (3 or 6 h; i.p.) before of the intrastriatal injection of GA (1.3 µmol/site) on the first convulsion latency (G) and time in seizures (H) according EEG recordings. Data are median and interquartile range for $n = 6–8$ in each group. $^{*}P < 0.05$ when compared to vehicle/GA (1.3 µmol/site) group; $^{\#}P < 0.05$ when compared to LPS 3 h/GA (1.3 µmol/site) group; Kruskal–Wallis analysis of variance.

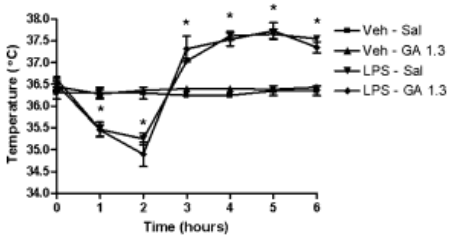

Figure 4 Effect of injection LPS (i.p.) following the intrastriatal administration of saline or GA (1.3 μmol/site) on rectal temperature from rat pups. Data are mean ± S.E.M. from absolute rectal temperatures for $n = 6$ in each group. $^{*}P < 0.01$ as compared to control group and vehicle/GA (1.3 μmol/site) group. Two-way ANOVA (Student–Newman–Keuls test).

et al., 2006). Moreover, experimental findings in vivo and in vitro suggest that energy metabolism impairment and decrease in the L-[^{3}H] glutamate uptake play an important role in the convulsive behavior elicited by GA (Porciúncula et al., 2004; Magni et al., 2009). In fact, an impairment of glutamate metabolism and homeostasis has been proposed to underlie several brain diseases, including epilepsy (Kelly and Stanley, 2001).

Since patients with GA-I are more prone to seize when presenting an infectious/inflammatory disease, we decided to investigate whether LPS administration (3 h and 6 h before GA administration) facilitated the seizures induced by this

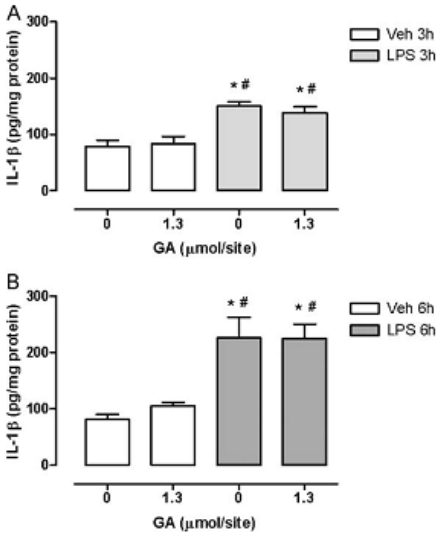

Figure 5 Effect on the IL-1β levels after administration of LPS (3 h; A and 6 h; (B) in the GA-injected striatum (1.3 μmol/site) from rat pups. Data are mean ± S.E.M from IL-1β levels for $n = 4$–5 in each group. $^{*}P < 0.01$ as compared to control group and $^{\#}P < 0.01$ as compared to vehicle/GA (1.3 μmol/site) group (A, B); Two-way ANOVA (Student–Newman–Keuls test).

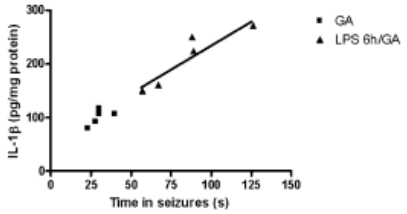

Figure 6 Increase of time in seizures induced by LPS (i.p.) 6 h before GA (1.3 μmol/site) correlates with increase IL-1β levels in the striatum (Pearson's correlation coefficient). Data are individual values for $n = 6$ in each group.

organic acid. LPS injection 6 h before intrastriatal GA injection reduced seizure latency and increased time in seizures when compared with GA alone. In this context, it has been shown that LPS administration enhances seizure susceptibility in mice injected with PTZ. Interestingly, this phenomenon is blocked by antiinflammatory drugs (Sayyah et al., 2003), suggesting the involvement of proinflammatory molecules in LPS-induced seizure susceptibility. It is worth noting that the facilitatory effect of LPS on GA-induced seizures was seen only when LPS was administered 6 h before GA, but did not at 3 h.

In addition, the LPS-induced increase of rectal temperature at 3 h was of similar magnitude of that observed at 6 h, indicating that changes in GA-induced convulsive parameters observed after LPS injection (at 6 h) is not related to an increase in body temperature.

Since LPS is a general stimulator of innate immune system to produce proinflammatory and/or cytotoxic factors as IL-1β, IL-6, TNF-α (Cohen, 2002), it has been used to induce neuroinflammation in animals (Eriksson et al., 2000; Iliev et al., 2001; Turrin et al., 2001; Turrin and Rivest, 2004; Maeda et al., 2008). Therefore, we determined the IL-1β levels in the GA-injected striatum at 3 and 6 h after LPS administration, and it observed that LPS treatment increased IL-1β levels at 3 and 6 h in a time-dependent manner.

This study does not provide a mechanism by which increased IL-1β levels facilitates GA-induced seizures in LPS-injected animals. However, our results are in agreement with previous studies that have shown that cerebral IL-1β levels are significantly higher in animals treated with LPS and kainic acid (KA) which developed seizures than in those that did not seize (Heida and Pittman, 2005). Thus, it is plausible to propose that increased neuronal excitability elicited by higher striatal IL-1β level leads to appearance of electroencephalographic and behavioral seizures induced by administration of GA and LPS. In addition, we observed a strong correlation between time in GA-induced seizures and striatal IL-1β levels in LPS-injected animals (LPS injected 6 h before GA administration). Taken together these observations, we may suggest that there should be a higher striatal IL-1β level to generate neuronal excitability and to increase the time in seizures as observed here.

In this context, it has been demonstrated that administration of IL-1β prolongs the duration of electroencephalographic and behavioral seizures induced by

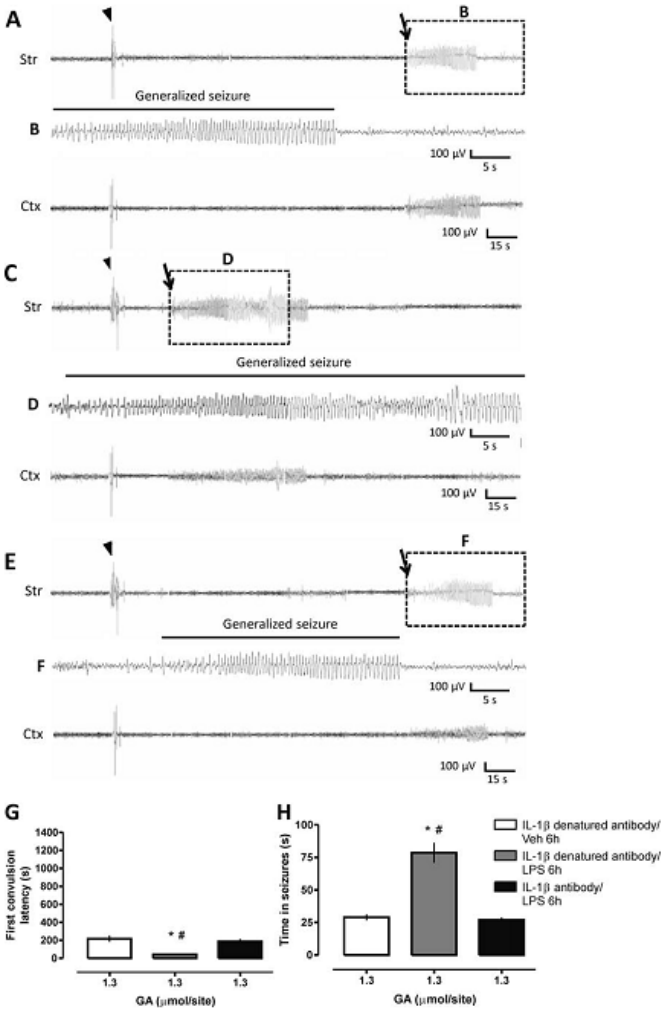

Figure 7 Representative electroencephalographic recordings: (A) intrastriatal injection IL-1β denatured antibody 45 min before vehicle (i.p.) and 6 h before intrastriatal administration of GA (1.3 μmol/site); (B) expanded waveforms from box of the EEG recording in A; (C) intrastriatal injection IL-1β denatured antibody 45 min before injection LPS (i.p.) and 6 h before intrastriatal administration of GA (1.3 μmol/site); (D) expanded waveforms from box of the EEG recording in C; (E) intrastriatal injection IL-1β antibody 45 min before LPS (i.p.) and 6 h before intrastriatal administration of GA (1.3 μmol/site), which delayed the appearance of pattern of continuous high-amplitude sharp spikes in low-frequency rhythmic spiking activity in ipsilateral striatum and cortex; (F) expanded waveforms from box of the EEG recording in E. In all traces the arrowhead indicates the GA intrastriatal administration; the arrow indicates the EEG pattern associated with generalized seizures. Str, ipsilateral striatum; Ctx, ipsilateral cortex. Calibration bars, 100 μV and 15 s. Effect of IL-1β antibody on the first convulsion latency (G) and time in seizures (H) in rat pups that received LPS 6 h before GA (1.3 μmol/ striatum). Data are median and interquartile range for $n = 6–8$ in each group. $^{*}P < 0.05$ as compared to IL-1β denatured antibody/vehicle 6 h/GA (1.3 μmol/site) group (D, E) and $^{\#}P < 0.05$ as compared to IL-1β antibody/LPS 6 h/GA (1.3 μmol/site) group (D, E); Kruskal–Wallis analysis of variance.

intracerebral application of KA in rodents (Vezzani et al., 1999). On the other hand, the intracerebral injection of IL-1-receptor antagonist (IL-1Ra) has powerful anticonvulsant effects (Vezzani et al., 2002), and transgenic mice overexpressing IL-1Ra have a reduced susceptibility to seizures (Vezzani et al., 2000), suggesting that cerebral IL-1β increase contributes to the maintenance of seizures in these models.

Current evidence indicates that cytokines, particularly IL-1β, increase neuronal excitability by activating IL-1 receptors (Vezzani et al., 1999; Bernardino et al., 2005). In this context, it has been suggested that IL-1R1-mediated modulation of glutamatergic transmission may contribute to excitotoxicity and spontaneous seizures since IL-1R1 and N-methyl-D-aspartate (NMDA) receptors colocalize in the striatum (Lawrence et al., 1998; Kwon et al., 2008) and in hippocampal pyramidal neurons (Viviani et al., 2003). In addition, the neuronal IL-1R1 stimulation induces Src kinase-mediated tyrosine phosphorylation of the NR2B subunit of the NMDA receptor. As a consequence, IL-1β facilitates NMDA receptor-mediated Ca^{2+} influx into neurons, promoting excitotoxicity (Viviani et al., 2003) and, possibly, seizure generation (Vezzani and Baram, 2007). IL-1β can also inhibit glutamate uptake in astrocytes (Hu et al., 2000) and increase its glial release possibly via TNF-α production (Bezzi et al., 2001), thus resulting in elevated extracellular glutamate levels and hyperexcitability. It is also worth pointing out that IL-1β has been shown to reduce γ-aminobutyric acid $(GABA)_A$ receptor-mediated chloride currents in hippocampal cell cultures (Wang et al., 2000), which may further add to seizure generation. Thus, since increased glutamatergic transmission and/or inhibited GABAergic function may induce seizures (Fighera et al., 2003, 2006), it is plausible that the dual actions of IL-1β on excitatory and inhibitory neurotransmission could facilitate GA-induced seizures. However, this explanation remains speculative in nature, and further studies are necessary to clarify this point.

Although the tissue concentrations of GA after intrastriatal administration are unknown, we may speculate that reduction of glutamate uptake by GA may facilitate the activation of excitatory amino acids receptors and, consequently, to generate seizures. Of note, it has been shown that glutamate transporters inhibitors do not produce seizures by themselves, but they are capable of facilitating seizures in the presence of inflammation (Liu et al., 2009). Since GA enhances synaptic turnover of glutamate and neuronal hyperexcitability (Nanitsos et al., 2004), it is plausible to propose that LPS-induced increase in IL-1β levels facilitates seizures by increasing neuronal glutamate availability. Taken together these observations, and previous reports demonstrating that GA inhibits glutamate uptake (Porciúncula et al., 2004) and that IL-1β may induce excitotoxicity by glutamatergic mechanisms (Viviani et al., 2003; Vezzani and Baram, 2007), it is conceivable that our results may be related to these findings.

We also found that anti-IL-1β antibody prevented the IL-1β-induced reduction of seizure latency and increase of time in seizures, suggesting the participation of this proinflammatory cytokine in the increased seizures susceptibility. These data suggest that IL-1β signaling present in inflammatory process contributes critically to neuronal hyperexcitability and consequently in GA-induced reduction of seizure latency and increase of time in seizures. This finding is reinforced by the evidence that impairment of the endogenous production of IL-1β by using selective blockade, or gene knockout of caspase-1, the enzyme producing the biologically active form of IL-1β, significantly reduces seizures (Ravizza et al., 2006).

In conclusion, since IL-1β can exacerbate seizures, inhibit neurogenesis and increase blood–brain barrier permeability (Quagliarello et al., 1991; Ekdahl et al., 2003), pharmacologic approaches specifically targeted to block the overproduction of IL-1β or its function(s) in glutaric acidemia may represent new nonconventional strategies for the treatment of seizure in this disorder. However, clinical studies shall be conducted in order to evaluate its clinical efficacy in glutaric acidemic patients.

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We confirm that we have read the journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. In addition, we would like to state that all authors have seen and approved the study and that no part of the work has been published or is under consideration for publication elsewhere. Moreover, the present study was supported by government funding and has no financial or other relationship that might lead to a conflict of interest. We also would like to declare that all experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and that the University Ethics Committee approved the respective protocols.

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4.2. Capítulo II – Lipopolissacarídeo aumenta o prejuízo na memória espacial induzido pelo tratamento crônico pós-natal com ácido glutárico em ratos.

Manuscrito

LIPOPOLYSACCHARIDE ENHANCES THE SPATIAL MEMORY IMPAIRMENT INDUCED BY CHRONIC EARLY POSTNATAL GLUTARIC ACID IN RATS

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Lipopolysaccharide enhances the spatial memory impairment induced by chronic early postnatal glutaric acid in rats

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ABSTRACT

Glutaric aciduria type I is an inherited deficiency of glutaryl-coenzyme A dehydrogenase characterized by accumulation of glutaric acid and neurological symptoms. It has been reported that patients with GA-I have cognitive impairment after encephalopathic crises, which are precipitated by infectious processes during an age-dependent susceptibility period. In the present work, we investigated whether chronic exposure of GA during early development (5 µmol g of body weight $^{-1}$, twice per day, from the 5th to the 28th day of life), in the absence and presence of LPS (2 mg/Kg, once per day, from the 25th to the 28th day of life), could alter the cognitive performance of rats in the Barnes maze and evaluate if the alterations can be related to structural or functional changes in the hippocampus. We showed that GA-chronic treatment caused a deficit of spatial learning in pups rats, and that the presence of an inflammatory process induced by LPS increased the spatial memory impairment induced by GA. Since none of the treatments affected the weight or locomotor activity of the animals, the effect of treatments on impairment of spatial learning is not due the these alterations, but is possibly related to the increase in proinflammatory cytokines, the reduction of hippocampal volume and the decreased in α1 subunit activity of Na^+,K^+-ATPase enzyme as observed. In addition, we found that the worsening in spatial memory observed in GA-LPS group, as compared with other groups, was α2/3 isoform specific of enzyme, since only this group showed no compensatory response in this subunits, and consequently presented a decreased in total activity of Na^+,K^+-ATPase enzyme. The results provide evidence that early chronic GA treatment induces long-lasting spatial behavioral deficit, and that the presence of an inflammatory process potentiates the deficit in spatial learning.

Keywords: glutaric acid; LPS; IL-1β; TNF-α; spatial memory; hippocampus**.**

1. Introduction

Glutaric acidemia type I (GA-I) is an inherited neurodegenerative disease caused by deficiency of mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH; EC 1.3.99.7) involved in the metabolism of L-lysine, L-hydroxylysine and L-tryptophan. Affected patients usually present a major accumulation of glutaric acid (GA), and a lesser amount of 3-hydroxyglutaric acid (3-OH-GA) and glutaconic acid in the body fluids (Goodman et al., 1977; Goodman and Freman, 2001; Strauss and Morton, 2003; Strauss et al., 2003). Following a period of normal development, affected children may experience irreversible striatal injury during encephalopathic crisis commonly precipitated by a non-specific illness, usually before the age of 36 months. After encephalopathic crises appear the clinical manifestations of GA-I which are predominantly neurological and include seizures, dystonia, dyskinesia and memory deficit (Morton et al. 1991; Hoffmann and Zschocke, 1999; Patil et al., 2004; Beauchamp et al., 2009).

However, children with GA-I who escape encephalopathy in their first 5 years typically remain asymptomatic, suggesting that the cerebral vulnerability is restricted to a defined period of brain development (Kölker et al., 2006). Since neurological crises are typically precipitated by common infection and febrile illness, this indicates a potentiating role of inflammatory cytokines in the neurological disorders this organic aciduria (Hoffmann et al., 1996; Hoffmann and Zschocke, 1999). In agreement with this view, a substantial body of evidence indicates that brief systemic inflammation during critical periods of development may result in long-lasting cerebral and peripheral vulnerability (Eklind et al., 2005; Hagberg and Mallard, 2005; Godbout and Johnson, 2006).

Although the neurological disorders are prevalent in GA-I, little is known about the mechanism by which the accumulated organic acids lead to these alterations (Lima et al., 1998; Fighera et al., 2006; Rosa et al., 2007; Magni et al., 2007; 2009). Specifically in relation to cognitive deficits, studies have reported that patients with GA-I show cognitive impairment (Patil et al., 2004; Boneh et al., 2008, Beauchamp et al., 2009) especially after encephalopathic crises (Kölker et al., 2006), suggesting a close association between inflammation in the central nervous system and the occurrence of memory deficits in GA-I children.

In this context, studies have reported a correlation between elevated levels of proinflammatory cytokines and memory deficits after neuroinflammatory processes

(Barrientos et al., 2002, Hein et al., 2007). In agreement a large number of cognitive disorders in humans are associated with elevated levels of proinflammatory molecules such as interleukin 1 beta (IL-1β) and tumor necrosis factor α (TNF-α; Griffin et al., 1989; Perrella et al., 1992; Casadesus et al., 2007; Olcese et al., 2009). In addition, numerous studies in animal models have also found an association between elevated levels of IL-1β and TNF-α with memory deficits after neuroinflammatory processes (Pugh et al., 1998; 1999; Barrientos et al., 2002; Sparkman et al., 2005; Hein et al. 2007). Thus, the central or peripheral administration of lipopolisacarideo (LPS) has been used as a model to produce neuroinflammation in animals (Hauss-Wegrzyniak et al., 2000; Liu et al., 2000a,b; Turrin et al., 2001). LPS is a glycolipid component of the outer membrane of Gram-negative bacteria able to activate the innate immune system, particularly of microglia, leading to an array of proinflammatory mediators being produced, such as IL-1β and TNF-α (Cohen, 2002). These proinflammatory cytokines are now accepted as bona fide modulators of both normal and abnormal neuronal transmission within the brain (Merrill, 1992; Vitkovic et al., 2000).

Since it has been reported that patients with GA-I have cognitive impairment (Patil et al., 2004; Boneh et al., 2008, Beauchamp et al., 2009), especially after encephalopathic crises which are precipitated by infectious processes during an age-dependent susceptibility period (Kölker et al., 2006), the objective of this work was to evaluate the performance of pups rats injected chronically with GA, in the absence and presence of LPS, in a test of spatial memory and evaluate if the alterations may be related to structural or functional changes in the hippocampus.

2. Experimental Procedures

2.1. Reagents

Unless otherwise stated, reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Animals

Pregnant rats were housed in individual cages and left undisturbed during gestation. Forty-eight hours after delivery, litters were culled to eight male pups; rats were weaned at 21 days of life. The animals were divided so that in each cage there

was the same number of rats for each treatment. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (24 ± 1◦C, 55% relative humidity) colony room. The “Principles of Laboratory Animal Care” (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics and Animal WelFare Committee of the Federal University of Santa Maria, Santa Maria, Brazil. All efforts were made to minimize the number of animals used and their suffering.

2.3. In vivo treatment

Buffered GA, pH 7.4 (5 µmol.g of body weight^{-1}) was administered subcutaneously, twice per day, from the 5th to the 28th day of life (Da C. Ferreira et al., 2008) to produce brain concentrations of GA of around 0.6 µmol g^{-1}, ~0.72 mM (Ferreira et al., 2005) similar to concentrations those found in glutaric acidemic patients. Control animals received saline subcutaneously in the same volumes and frequency. All solutions were prepared so that each animal received 10 µL solution. g of body weight $^{-1}$.

2.4. LPS-injection

The pups rats were injected intraperitoneally (i.p.) with lipopolysaccharide (LPS 2 mg/kg; E.coli 055 B5; Eriksson et al., 2000; Maeda et al., 2008) or vehicle (saline 0.9%), once per day, from 25th to 28th day of life to mimic an infections state.

2.5. Cognitive tasks

Behavioral experiments were carried between 9.00 am and 2.00 pm. Animals were assessed on the Barnes maze from the 29th to the 32th postnatal day, and after Barnes maze test, they were then tested in the open field task on the 32th postnatal day.

2.6. Barnes Maze

One day after treatment finished, animals were trained to solve the Barnes maze. The Barnes maze is a validated test often used for the assessment of spatial

learning and memory in rodents (Barnes 1979). The Barnes maze paradigm exploits the natural inclination of small rodents to seek escape to a darkly lit, sheltered environment when placed in an open arena under bright, aversive illumination. Our maze consists of a 120 cm diameter circular wooden table, 3.5 cm-thick and elevated 90 cm above the floor.

Twenty holes, 6 cm diameter, were equidistantly located around the perimeter and centered 5 cm from it. The apparatus was located in a 4m×4m test room where four visuospatial cues made of rigid black paper (rectangle, circle, cross, triangle) were affixed to the walls but not directly over any one maze hole; this increases the spatial component of the Barnes maze during training (Bach et al. 1995). A black wooden escape tunnel (15 cm×10 cm×30 cm) was placed beneath one hole, selected randomly for each rat but remained constant throughout the training sessions for a given rat. The remaining 19 holes led only to a false escape box (15 cm×10 cm×10 cm) which, from the platform, appeared indistinguishable from an escape box but was too small to be entered; false boxes removed visual cues that might be observed through an open hole. Above the platform (height 45 cm) there is an incandescent lamp (200 W), which gave bright illumination of the maze as the aversive stimuli.

On the first day of the experiment, the rats were moved to testing room and left undisturbed for 60 min. Following this habituation period, the rats were trained to find the escape hole; they were placed in the escape box for 1 min, then into a cylindrical opaque chamber (start box) in the center of the maze. With light on, the start box was removed and the rat allowed exploring freely and finding the escape box. A maximum latency of 180 s to find it was allowed. Each rat was given three trials per day, over four consecutive days. In each trial, we scored the time to reach the escape tunnel and the number of wrong holes visited. The arena as well boxes were wiped clean using distilled water both between each training session for a given rat and between each rat.

2.7. Physical development

All rats used in the experiments had assessed their physical development. For this, the weight of animals was daily determined during all treatment.

2.8. Open field task

After the second day of the Barnes maze test, the locomotor activity was measured for 5 minutes in the open field. The apparatus consisted of a wooden box measuring 60 cm×40 cm×50 cm with a glass front wall, whose floor was divided by black lines into 12 equal squares. The animals were gently placed facing the rear left corner of the arena and the number of squares crossed with the four paws recorded for 5 minutes to evaluate motor activity (Walsh, 1976). The testing room was dimly illuminated with indirect white lighting.

2.9. IL-1β and TNF-α immunoassay

After the second day of the Barnes maze test, the content of IL-1β and TNF-α were determined in the hippocampus. After behavioral assay, the hippocampus was dissected out rapidly at 4°C and frozen at -70°C. The hippocampus was weighed and homogenized in a solution containing bovine serum albumin (BSA 10 mg/ml), EGTA (2 mM), EDTA (2 mM) and PMSF (0.2 mM) in phosphate-buffered saline (PBS, pH 7.4) using a Potter homogenizer. The hippocampus homogenate was centrifuged (3000 g for 10 min) and cytokines were determined in the supernatant. Cytokine levels were measured using a commercially available ELISA Kit from RandD Systems (Minneapolis, MN) using an antibody selective against rat IL-1β or TNF-α, according to the manufacturer's protocol. The results were expressed as pg/mg of protein. Absorbance was read at 405 nm. The detection limit was 4 ng/ml.

2.10. Hippocampal volume

After the second day of the Barnes maze test, the animals were deeply anesthetized with thiopental (40 mg/kg, i.p.) and transcardially perfused with saline solution followed by a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed from the skulls, post-fixed in the same solution at room temperature for 24 h and cryoprotected by immersion in 30% sucrose solution in phosphate buffer at 4 °C until they sank. The brains were then quickly frozen in isopentane that was previously cooled in liquid nitrogen (-70 °C). Thirty-µm serial coronal sections obtained using a cryostat (Leica, Germany) were mounted on poly-L-lysine coated slides, stained with a solution of 0.01% cresyl violet and acetic acid 0.01%, washed in distilled water, cleared in a graded series of ethanol and xylene, and coverslipped for observation by light microscopy [objects (20X), Olympus AX 70

photomicroscope coupled to a digital Leica DC 300F camera and Image-Pro Plus Software 6.1 (Media Cybernetics, San Diego, CA, USA)]. Coronal sections from the hippocampus (stratum pyramidale of the CA1 and CA3 subfields) and hilus of the dentate gyrus were identified according to Paxinos and Watson.

The Cavalieri method (Gundersen et al., 1987; Rodrigues et al., 2004) was used to estimate the volume of the hippocampus. Cross sectional areas of the hippocampus were captured (with 150 mm/intervals) using the image analysis system previously described. The hippocampal formation was outlined and measured. The boundaries of these regions were defined in accordance with criteria of Amaral and Witter (1995) whereby the hippocampal formation ‘‘hippocampus’’ was composed of three layered structures, including the dentate gyrus, the hippocampus proper (CA1, CA2 and CA3 subfields) and the subiculum. The hippocampal volume was calculated by summation of the areas analyzed and multiplication of the distance between the sections (150 mm). At least 10 areas were measured in each analyzed brain.

2.11. Na^+, K^+-ATPase activity measurements

After the second day of the Barnes maze test, the Na^+,K^+-ATPase activity was measured according to Wyse et al. (2000) in the hippocampus. Briefly, the assay medium consisted of 30 mM Tris-HCl buffer, pH 7.4; 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$ and 50 µg of protein in the presence or absence of ouabain (1 mM), in a final volume of 350 µL. The reaction was started by the addition of adenosine triphosphate to a final concentration of 5 mM. After 30 min at 37 °C, the reaction was stopped by the addition of 70 µL of 50% (w/v) trichloroacetic acid. Saturating substrate concentrations were used, and reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified by the colorimetric method described by Fiske and Subbarow (1925), using KH_2PO_4 as reference standard. Specific Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and expressed in nmol Pi/mg protein/min.

In a separate set of experiments we investigated whether some Na^+,K^+-ATPase isoform is selectively inhibited. For this purpose, we used a classical pharmacological approach, based on the isoform-specific sensitivity to ouabain (Nishi

et al., 1999). We determined whether some treatments inhibited ouabain-sensitive ATPase activity using 3 µM (that inhibits Na^+,K^+-ATPase isoforms containing α2 and α3 subunits) or 4 mM ouabain (that inhibits all isoforms).

2.12. Protein determination

Protein content was measured colorimetrically by the method of Bradford (1976) using bovine serum albumin (1 mg/ml) as a standard.

2.13. Statistics

Statistical analysis was carried out by two-way analysis of variance (ANOVA) and only F-values of $P<0.05$ are presented. Post hoc analysis was carried out by the Student-Newman-Keuls test, when appropriate. All data were expressed as mean ± S.E.M. Statistical analyses were performed utilizing the SPSS software in a PC-compatible computer. $P<0.05$ was considered significant.

3. Results

Barnes Maze test

The effect of GA-chronic treatment, in the presence and absence of LPS, on cognitive performance is shown in Fig. 1A. At the second day of test, statistical analysis demonstrated a learning deficit in GA-Veh, Sal-LPS and GA-LPS [$F(3,43)=8.82$; $P<0.01$; Fig. 1B] treated groups when compared to Sal-Veh-treated group. This lack of learning was showed by an absence in the reduction of the latency for escape in these groups when compared to Sal-Veh-treated group. Furthermore, the latency for escape of GA-LPS-treated group was statistically higher as compared to GA-Veh and Sal-LPS-treated groups. At the third day of test, statistical analysis also showed an absence in the reduction of the latency for escape of GA-LPS [$F(3,43)=4.56$; $P<0.01$; Fig. 1C] treated group as compared with other groups.

Physical development of animals

Figure 2 indicates the physical development of animals determined by their weight during the treatment. Statistical analysis showed no difference in the animal weight between all tested groups during the treatments.

Open field task

Exploratory and locomotor activity of animals is shown in Fig. 3. Statistical analyses showed no difference between the number of crossings (Fig. 3A) and rearings (Fig. 3B) of the animals.

IL-1β and TNF-α levels in the hippocampus

Since numerous studies have found an association between elevated levels of proinflammatory cytokines and memory deficits after neuroinflammatory processes (Barrientos et al., 2002; Hein et al., 2007) we decide to determine the levels of IL-1β and TNF-α in hippocampus at the second day of Barnes maze test (day where there was the spatial learning deficit) in pups rats-treated.

IL-1β levels in the hippocampus are shown in Fig. 4A. In this context, statistical analyses showed a significant increase on IL-1β levels in GA-Veh, Sal-LPS and GA-LPS [$F(1,35)=9.70$; $P<0.01$] treated groups as compared to Sal-Veh-treated group.

In addition, we evaluated TNF-α levels in the hippocampus (Fig. 4B). Satistical analyses showed a significant increase on TNF-α levels in GA-Veh, Sal-LPS and GA-LPS treated groups [$F(1,37)=8.44$; $P<0.01$] as compared to Sal-Veh group. Thus, these results suggest the participation of IL-1β and TNF-α cytokines in the impairment of spatial performance.

Hippocampal volume

Figure 5 shows the total hippocampal volume of pups rats treated with Sal-Veh, GA-Veh, Sal-LPS and GA-LPS at the second day of Barnes maze test. Statistical analysis showed a significant reduction in hippocampal volume in GA-Veh, Sal-LPS and GA-LPS-treated groups [$F(1,12)=28.13$; $P<0.001$] when compared to Sal-Veh group, suggesting a negative regulation of hippocampal cellular plasticity in those groups.

Na^+, K^+-ATPase activity measurements

Figure 6A shows the effect of GA-chronic treatment, in the presence and absence of LPS, on Na^+,K^+-ATPase total activity in rat hippocampus at the second day of Barnes maze test (day where there was the spatial learning deficit). Statistical

analysis revealed that treatment with GA-LPS decreased Na^+,K^+-ATPase total activity in rat hippocampus [$F(1,20)=16.00$; $P<0.01$].

We also investigated whether some α isoform is selectively inhibited by treatments. For this purpose, we used a classical pharmacological approach, based on the isoform-specific sensitivity to ouabain (Nishi et al. 1999).

Figure 6B shows the effect of different treatments on α1 subunit activity of Na^+,K^+-ATPase enzyme in rat hippocampus. In this experimental condition, statistical analysis demonstrated that treatments with GA-Veh, Sal-LPS and GA-LPS [$F(1,20)=13.18$; $P<0.01$] decreased α1 subunit activity of Na^+,K^+-ATPase enzyme, suggesting the participation of this subunit in the spatial memory deficit observed in these groups at the second day of Barnes Maze test.

Besides, we evalueted the effect of different treatments on α2/3 subunits activity of Na^+,K^+-ATPase enzyme in rat hippocampus (Fig. 6C). We found that treatments with GA-Veh and Sal-LPS [$F(1,20)=22.46$; $P<0.001$] increased α2/3 subunits activity of Na^+,K^+-ATPase enzyme, suggesting a compensatory response. However, GA-LPS-treated group showed no this compensatory response, and we belive that the absence of this response may be the cause for the largest spatial memory impairment observed in this group.

4. Discussion

In this work we used a model that does not exactly mimic human GA-I, in which, besides GA, other metabolites accumulate in lesser amounts. However, it reproduces the main biochemical feature of this disorder, which is high sustained tissue levels of GA (~0.72mM) in the brain of pups rats similar to those found in human GA-I (Ferreira et al., 2005). Glutaric acid was administered from the 5th to the 28th day of life of pups rats, that corresponds to the age-dependent susceptibility period of glutaric acidemic children (Kölker et al., 2006). In addition, GA chronic treatment period represents a phase of great cellular proliferation and synaptogenesis in various cerebral structures involved in learning/memory in rats, as hippocampus (Winick and Noble, 1965; Dutra et al., 1993; Morgane et al., 2002).

In the present study we showed that GA-chronic treatment caused a deficit of spatial learning in pups rats, and that the presence of an inflammatory process induced by LPS potentiated the impairment in spatial memory induced by GA alone. Since none of the treatments affected the weight or locomotor activity of the animals,

the effect of treatments on spatial memory is not due to the these alterations. Then, we decided to investigate the involvement of proinflammatory cytokines, hippocampal volume and Na^+,K^+-ATPase enzyme activity on the deficit of spatial learning induced by GA and LPS in rat pups.

We demonstrated that pups rats chronically treated with GA presented spatial learning deficit at the second day of Barnes maze test. In agreement with results, studies have reported that GA-I patients showed memory impairment (Patil et al., 2004; Boneh et al., 2008, Beauchamp et al., 2009). Our finding is consistent with a previous study where GA chronic administration provoked an impairment of spatial performance of rats in the water maze (Da C. Ferreira et al., 2008). Moreover, we showed that Sal-LPS-treated group also presented a memory impairment at the second day of learning test. According to our results, it has been suggested that neonatal inflammation causes cognitive impairment (Ikeda et al., 2005; Bilbo et al., 2005; Fan et al., 2008). For instance, we demonstrated that in the same way that GA-I children have functional brain damage after a infectious process (Kölker et al., 2006), pups rats GA-LPS-treated showed a greater impairment of cognitive performance than caused by GA-Veh or Sal-LPS alone, at the second and third days of test.

In addition to the spatial learning deficit, GA chronic treatment produced a hippocampal increase in IL-1β and TNF- α cytokines. Our findings suggest that GA chronic treatment generates an inflammatory process within SNC, since this treatment produced an increase in hippocampal cytokines. This observation suggests a possible link between increased levels of proinflammatory molecules in the CNS and the dysfunction of memory in pups rats, because animals treated with Sal-LPS and GA-LPS showed increased levels of cytokines along with the impairment of spatial memory.

In line with this view, a large number of cognitive disorders in humans are associated with elevated levels of proinflammatory molecules such as IL-1β and TNF-α (Griffin et al., 1989, 1994; Perrella et al. 1992; Casadesus et al. 2007). The mechanisms underlying the effects of inflammatory cytokines on hippocampal-dependent learning and memory have focused on neuronal function (long-term potentiation or LTP; Lynch, 2004). It is known that pathophysiological levels of IL-1 can produce detrimental effects on memory. These effects are specific for the consolidation of memories that depend on the hippocampus, whereas hippocampal-

independent memories are not altered (Rachal Pugh et al., 2001; Goshen and Yirmiya, 2007).

It has been suggested that proinflammatory cytokines produced during inflammation may be responsible for synaptic dysfunction (Chen et al., 2008, Tanaka et al., 2006, Hein and O'Banion, 2009). In fact, there is strong evidence that increased levels of IL-1β in the hippocampus can impair memory by its effects on synaptic transmission. It has been suggested that the IL-1 signaling during inflammation, probably mediated by IL-1β causes a selective and relatively persistent increase in GABAergic inhibition, and these actions contribute to decrease the synaptic excitability (Ikegaya et al, 2003; Hellstrom et al. , 2005). In addition, it has been shown the involvement of cholinergic and glutamatergic functions in working memory impairment induced by IL-1β in the hippocampus of rats (Matsumoto et al., 2001). Accordingly, other studies have demonstrated that acute exposure to IL-1β in hippocampal slices produces a significant reduction of basal synaptic glutamatergic transmission (Murray et al., 1997) and a reduction in excitatory synaptic transmission (Coogan and O'Connor, 1997 ; Luk et al, 1999). In addition, Vereker and co-works (2000) showed that the LPS induced an increase in IL-1β concentration and inhibited LTP in the rat dentate gyrus by activating caspase-1. Likewise, it also has been shown that exogenous application of IL-1β inhibits LTP (Tancredi et al. 2000, Ross et al, 2003). In this context, studies have shown that TNF-α also inhibits the induction of LTP in areas CA1 (Tancredi et al., 1992) and in the dentate gyrus of the hippocampus (Cunningham et al., 1996). Moreover, Butler and colleagues (2004) showed that the infusion of TNF-α before a stimulus to induce LTP inhibits the formation of LTP.

It is well known that pathological levels of inflammatory cytokines alter hippocampal function, but it is also possible that excessive production of inflammatory cytokines leads to a change in neurogenesis. In this context, has been shown that LPS-induced inflammation, which gives rise to microglia activation in the area where the new neurons are born, strongly impaired basal hippocampal neurogenesis in rats (Ekdahl et al., 2003). In addition, it is important to note that pups rats were injected during a phase of great cellular proliferation and synaptogenesis in various cerebral structures involved in learning/memory in rats, as hippocampus (Winick and Noble, 1965; Dutra et al., 1993; Morgane et al., 2002). In fact, there are evidence that inflammation in early development causes cognitive deficit. It has been

shown that a combination of LPS and hypoxia-ischemia in neonatal rats caused a long-lasting learning impairment plus a decreased in hippocampal volume, and that the previous treatment with dexamethasone prevented these effects (Ikeda et al., 2005). In addition, studies have demonstrated that neonatal infection induced by LPS caused memory impairment in adulthood (Bilbo et al., 2005a,b; Fan et al., 2008).

It is known that environmental enrichment improves learning and memory in part by promoting neurogenesis (van Praag et al., 1999) and increasing the structural complexity of existing networks (Greenough et al., 1973; Green et al., 1983; Wallace et al., 1992; Moser et al., 1994; Silva-Gomez et al., 2003). Likewise, conditions that inhibit neurogenesis and reduce dendrite complexity (e.g., inflammation, stress) also inhibit learning and memory (McEwen, 2007).

To examine this possibility we determined the total hippocampal volume in pups rats GA-Veh, Sal-LPS and GA-LPS-treated, and observed a reduction in hippocampal volume in these groups as compared to Sal-Veh-treated group. Thus, the present findings indicate that a peripheral infection during early development caused an increased inflammatory cytokine response in the hippocampus and an atrophy of hippocampal neurons, here observed by a reduction in the total volume of hippocampus.

In addition, it has been shown that proinflammatory cytokines are involved in inhibition of Na^+,K^+-ATPase enzyme activity (Kreydiyyeh et al., 2004; Kreydiyyeh and Al-Sadi, 2004). Na^+,K^+-ATPase is a crucial enzyme responsible for the active transport of sodium and potassium ions in the nervous system necessary to maintain the ionic gradient for neuronal excitability (Skou and Esmann, 1992). In fact, pups GA-Veh, Sal-LPS and GA-LPS-treated rats showed an inhibition in α1 subunit activity of Na^+,K^+-ATPase enzyme along with the impairment of cognitive performance, suggesting the participation this subunit in the spatial memory deficit observed in these groups at the second day Barnes Maze test.

All GA-Veh, Sal-LPS and GA-LPS-treated pups rats showed a deficit of spatial learning, an increase in inflammatory cytokine levels, a reduction in hippocampal volume and the inhibition in α1 subunit activity of Na^+,K^+-ATPase enzyme at the second day of Barnes maze test (day where there was the spatial learning deficit). Why only the GA-LPS-treated pups rats showed a worsening in spatial memory when compared to rats treated with GA or LPS alone?

Although all groups showed an inhibition in α1 subunit activity of Na^+,K^+-ATPase enzyme, GA-Veh and Sal-LPS-treated groups presented an increase in α2/3 subunits activity of Na^+,K^+-ATPase enzyme in rat hippocampus, suggesting a compensatory response, and showed no changes in the total activity of the enzyme. Interestingly, GA-LPS-treated group demonstrated no change in α2/3 subunits activity of Na^+,K^+-ATPase enzyme and consequently a decrease in total activity of Na^+,K^+-ATPase.

Therefore, our results suggest that worsening in spatial memory deficit observed in GA-LPS-treated group when compared to GA-Veh and Sal-LPS-treated groups, was due to the inhibitory effect on Na^+,K^+-ATPase total activity, that is α2/3 isoform specific. This was verified since all treated groups cause spatial memory impairment at the second day of testing (and reduced the α1 subunit activity), but only GA-LPS-treated group presented an inhibition on Na^+,K^+-ATPase total activity and a worsening in spatial memory impairment as compared to other groups, probably because this group showed no compensatory response in α2/3 subunits activity. In this context, some works have shown that the inhibition of Na^+,K^+-ATPase enzyme activity induces spatial learning deficits (Zhan et al., 2004; Lima et al., 2008), and impairs retention of an inhibitory avoidance task in rats (Dos Reis et al., 2002). Moreover, Moseley and co-works (2007) showed that deficiency in Na^+,K^+-ATPase α isoform genes impairs spatial learning and increases anxiety-related behavior.

In summary, in this study we showed that GA-chronic treatment caused a deficit of spatial learning in pups rats, and that the presence of an inflammatory process potentiated the impairment in spatial memory induced by GA alone. Accordingly, we also observed an increase in IL-1β and TNF-α cytokines, a reduction in hippocampal volume and an inhibition in α1 subunit activity of Na^+,K^+-ATPase enzyme, in all groups that presented spatial learning impairment. In addition, we found that GA-LPS-treated group presented a worsening in spatial memory, and this effect was α2/3 isoform specific, since only this group showed no change in α2/3 subunits activity and consequently presented a decreased in total activity of Na^+,K^+-ATPase enzyme.

Thus, due to the limitations that GA-I brings to children with this condition, the understanding of the mechanisms involved in the neurological changes induced by the accumulation of GA is important for the development of new therapies to treat this condition.

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Figure Legends

Figure 1. Effect of GA-chronic treatment (5 µmol/g), in the presence and absence of LPS (2 mg/Kg), immediately after the end of first session on escape latency (A) of pups rats and in the Barnes maze. Figures 1B and 1C shows an amplification of the second and third days of test respectively. Data are mean ± S.E.M. for *n*=12 in each group. **P*<0.01 as compared to Sal-Veh group. $^{\#}$*P*<0.01 as compared to GA-Veh or Sal-LPS groups. Two-way ANOVA (Student-Newman-Keuls test).

Figure 2. Effect of GA-chronic treatment (5 µmol/g), the in presence and absence of LPS (2 mg/Kg), on weight of animals. Data are mean ± S.E.M. for *n*=12 in each group. Two-way ANOVA.

Figure 3. Effect of GA-chronic treatment (5 µmol/g), in the presence and absence of LPS (2 mg/Kg), on the number of crossings (A) and rearings (B) of the animals. Data are mean ± S.E.M. for *n*=12 in each group. Two-way ANOVA.

Figure 4. Effect of GA-chronic treatment (5 µmol/g), in the presence and absence of LPS (2 mg/Kg), on hippocampal levels of IL-1β (A) and TNF-α (B) of pups rats at the second day of Barnes maze test. Data are mean ± S.E.M. for *n*=8 in each group. **P*<0.01 as compared to Sal-Veh group. Two-way ANOVA (Student-Newman-Keuls test).

Figure 5. Effect of GA-chronic treatment (5 µmol/g), in the presence and absence of LPS (2 mg/Kg), on the total hippocampal volume of pups rats at the second day of Barnes maze test. Data are mean ± S.E.M. for *n*=8 in each group. **P*<0.01 as compared to Sal-Veh group. Two-way ANOVA (Student-Newman-Keuls test).

Figure 6. Effect of GA-chronic treatment (5 µmol/g), in the presence and absence of LPS (2 mg/Kg), on Na^+,K^+-ATPase total activity (A); on α1 subunit activity of Na^+,K^+-ATPase enzyme (B); and on α2/3 subunits activity of Na^+,K^+-ATPase enzyme (C), in rat hippocampus at the second day of Barnes maze. Data are mean ± S.E.M. for *n*=8 in each group. **P*<0.01 as compared to Sal-Veh, GA-Veh and Sal-LPS groups. $^{\#}P$<0.01 as compared to Sal-Veh group. ◆*P*<0.001 as compared to Sal-Veh and GA-LPS groups. Two-way ANOVA (Student-Newman-Keuls test).

Figure 1

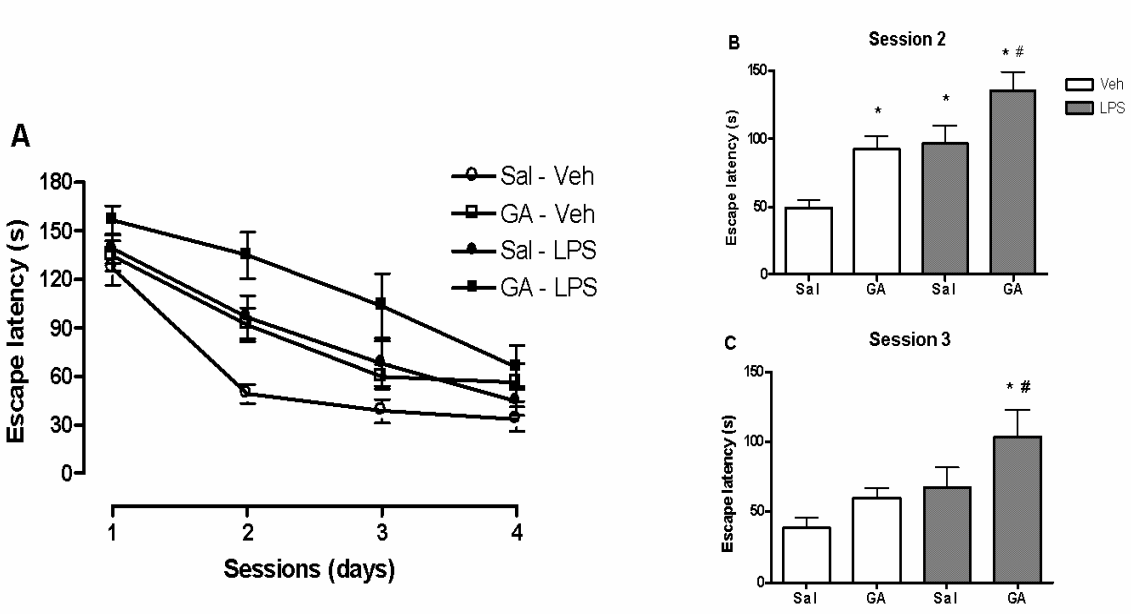

A
Escape latency (s)
180
150
120
90
60
30
0
1
2
3
4
Sessions (days)
Sal - Veh
GA - Veh
Sal - LPS
GA - LPS
B
Session 2
Escape latency (s)
150
100
50
0
Sal
GA
Sal
GA
Veh
LPS
C
Session 3
Escape latency (s)
150
100
50
0
Sal
GA
Sal
GA

Figure 2

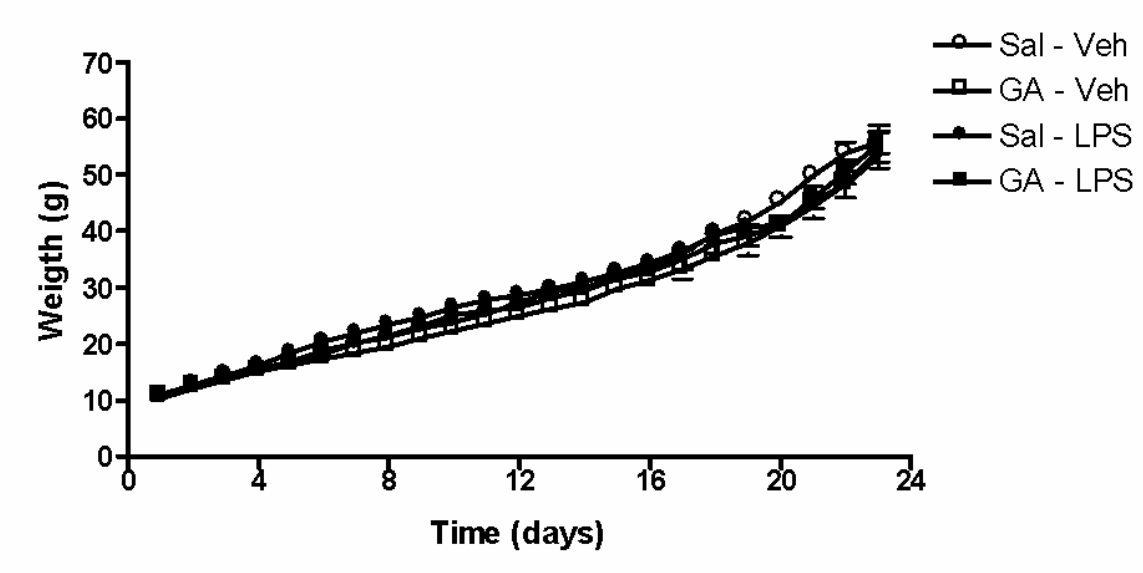

Sal - Veh
GA - Veh
Sal - LPS
GA - LPS
Weigth (g)
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
Time (days)

Figure 3

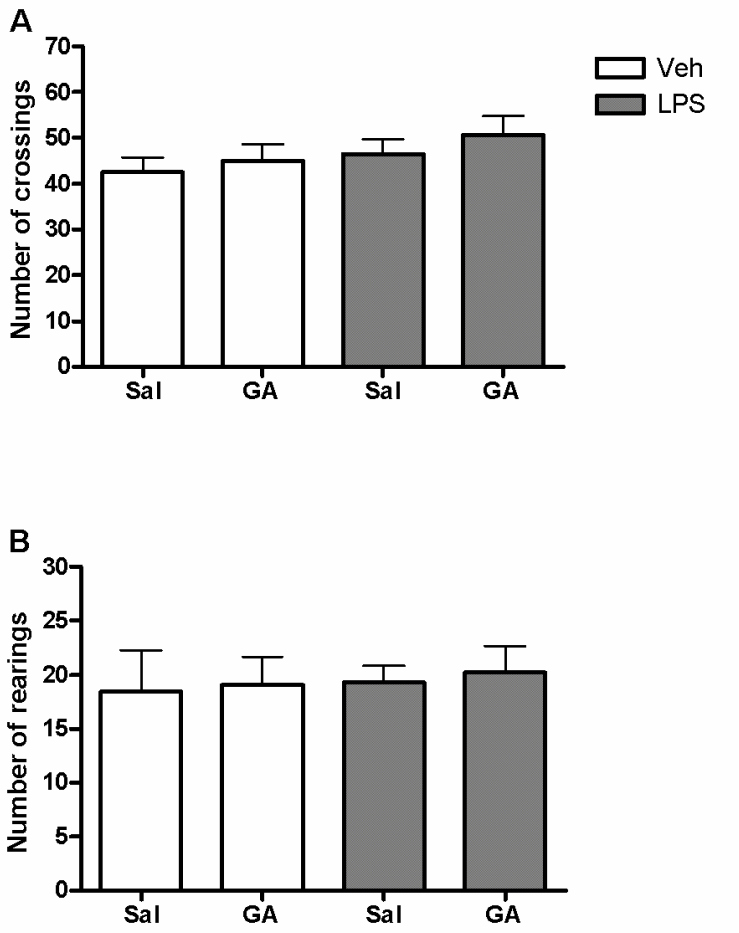

A
Number of crossings
70
60
50
40
30
20
10
0
Sal
GA
Sal
GA
Veh
LPS
B
Number of rearings
30
25
20
15
10
5
0
Sal
GA
Sal
GA

Figure 4

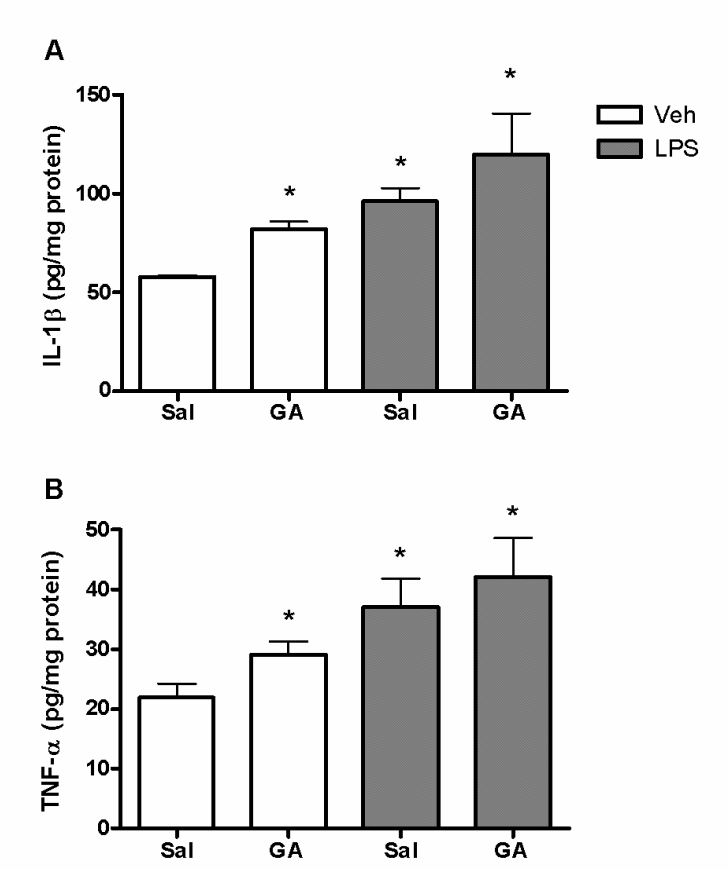

A
IL-1β (pg/mg protein)
150
100
50
0
Sal
GA
Sal
GA
Veh
LPS
B
TNF-α (pg/mg protein)
50
40
30
20
10
0
Sal
GA
Sal
GA

Figure 5

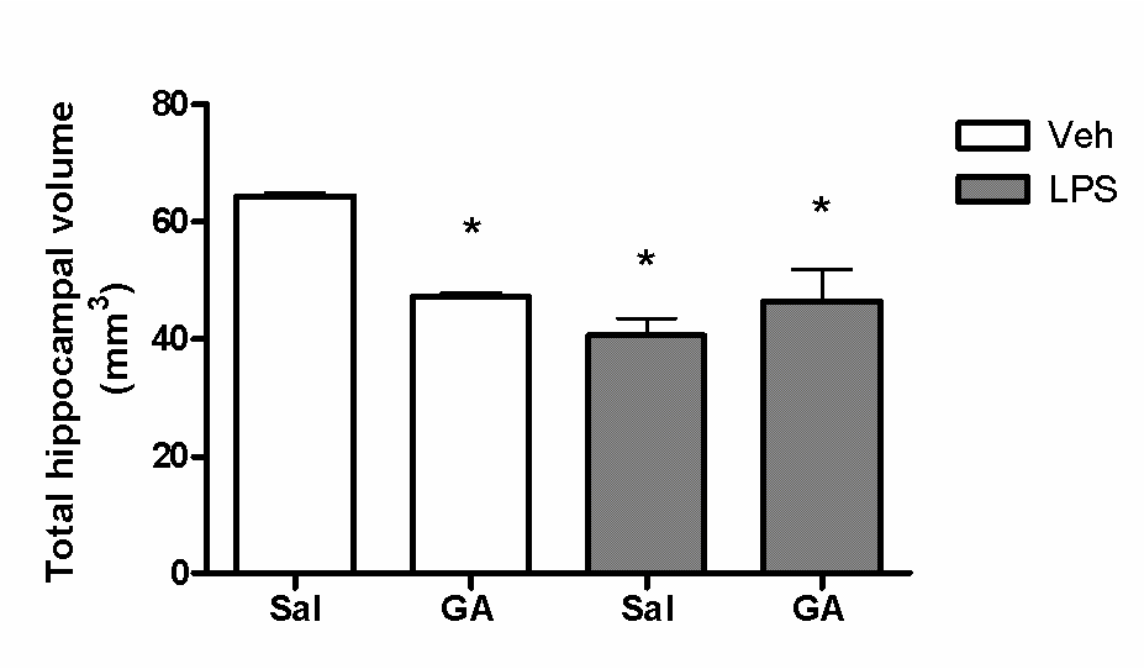
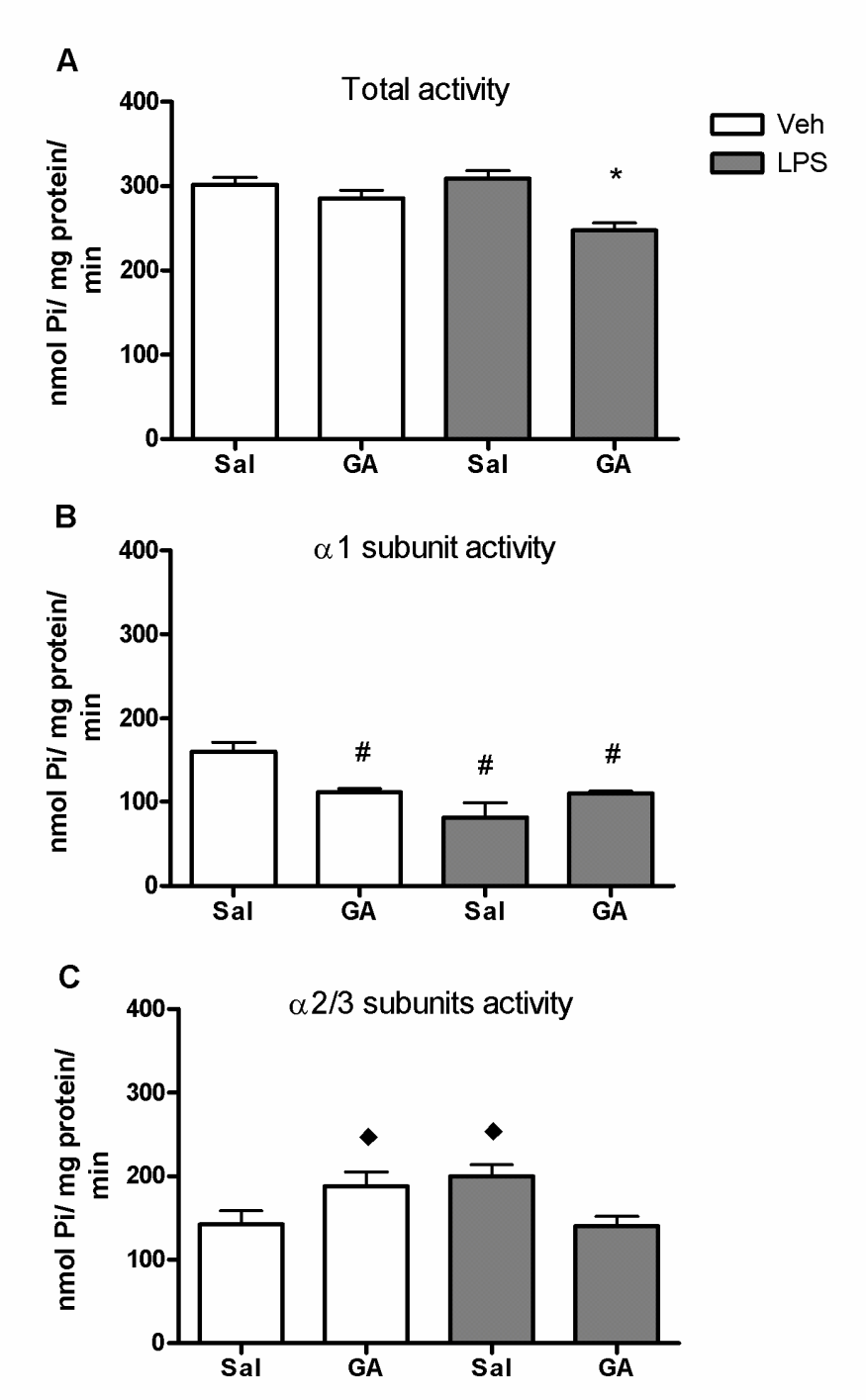

Total hippocampal volume (mm3)
80
60
40
20
0
Sal
GA
Sal
GA
*
*
*
Veh
LPS

Figure 6


A
Total activity
Veh
LPS
nmol Pi/ mg protein/ min
400
300
200
100
0
*
Sal
GA
Sal
GA
B
α1 subunit activity
nmol Pi/ mg protein/ min
400
300
200
100
0
#
#
#
Sal
GA
Sal
GA
C
α2/3 subunits activity
nmol Pi/ mg protein/ min
400
300
200
100
0
◆
◆
Sal
GA
Sal
GA

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5. DISCUSSÃO

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A GA-I é uma doença metabólica hereditária caracterizada bioquimicamente por um acúmulo principal de GA (Goodman et al., 1977; Goodman e Freman, 2001; Strauss e Morton, 2003; Strauss et al., 2003), e clinicamente por alterações neurológicas como as convulsões (Morton et al., 1991; Hoffmann e Zschocke, 1999). Aproximadamente 90% das crianças afetadas desenvolvem graves sintomas clínicos antes dos 36 meses de idade, e a ocorrência de crises encefalopáticas, as quais levam ao início das convulsões, não tem sido reportadas após os 5 anos de vida, sugerindo que a vulnerabilidade é restrita a um período limitado de desenvolvimento do cérebro (Hoffmann et al., 1996; Bjugstad et al., 2000; Kölker et al., 2006).

Nesse contexto, diversos trabalhos têm reportado a ocorrência de convulsões em crianças glutaricoacidêmicas após episódios de encefalopatias agudas (Osaka et al., 1993; Pöge et al., 1997; Hartley et al., 2001). De acordo, estudos experimentais têm demonstrado que a injeção intraestriatal de GA causa o aparecimento de episódios convulsivos (Lima et al., 1998; Fighera et al., 2006; Magni et al., 2007). No entanto, esses trabalhos utilizaram ratos adultos e sabe-se que as convulsões ocorrem principalmente em crianças, por isso se utilizou ratos jovens para mimetizar as alterações neurológicas observadas em crianças com GA-I. Inicialmente, realizou-se uma curva de dose de GA baseados em um estudo prévio de Lima e colaboradores (1998), e foi encontrado que o GA na dose de 1.3 µmol/estriado causou o aparecimento de episódios convulsivos (comportamentais e EEG) em ratos jovens.

Desde que as crianças glutaricoacidêmicas podem apresentar convulsões quando têm uma doença infecciosa/inflamatória, utilizou-se o LPS (3 e 6 horas antes da administração de GA) para avaliar se a presença de um estado infeccioso alteraria os parâmetros convulsivos induzidos por este ácido orgânico. Foi observado que a administração i.p. de LPS (2 mg/kg; Maeda et al., 2008) 6 horas antes da injeção intraestriatal de GA (1.3 µmol/estriado) reduziu a latência para a primeira convulsão e aumentou a duração dos episódios convulsivos, quando comparado ao GA sozinho. Nesse sentido, tem sido demonstrado que a administração prévia de LPS em ratos aumenta a susceptibilidade as convulsões por PTZ, sendo esse efeito bloqueado por drogas antiinflamatórias (Sayyah et al., 2003),

sugerindo o envolvimento de moléculas pró-inflamatórias na susceptibilidade às convulsões induzidas pelo LPS. No entanto, o efeito do LPS sobre as alterações nos parâmetros convulsivos induzidos pelo GA foi verificado apenas quando o LPS foi administrado 6 horas antes do GA, e não em 3 horas. Interessantemente, o aumento da temperatura retal induzido pelo LPS em 3 horas foi o mesmo que em 6 horas, sugerindo que as mudanças induzidas pelo LPS nos parâmetros convulsivos não são devidas ao aumento na temperatura corporal. Por isso, determinou-se os níveis da IL-1β no estriado injetado 3 e 6 horas após a administração de LPS, e foi verificado que o LPS aumentou os níveis de IL-1β em 3 e 6 horas, mas que esse aumento foi estatisticamente maior em 6 horas do que em 3 horas.

Não pode ser determinado por este estudo exatamente como os níveis de IL-1β facilitam as convulsões induzidas pelo GA em 6 horas, mas não em 3 horas, após a injeção de LPS. No entanto, os resultados estão de acordo com achados anteriores que demonstraram que os animais injetados com LPS e KA que desenvolvem convulsões, apresentam níveis cerebrais de IL-1β significativamente maiores do que os animais injetados de forma idêntica que não tiveram convulsões (Heida e Pittman, 2005). Isto é possível porque os níveis estriatais de IL-1β tem uma maior produção com o passar do tempo. De fato, observou-se uma correlação positiva entre a duração das convulsões e o nível estriatal de IL-1β nos animais que receberam LPS 6 horas antes do GA. Dessa maneira, sugere-se que deve haver uma maior concentração de IL-1β no estriado para gerar a excitabilidade neuronal e a maior duração dos episódios convulsivos.

De fato, estudos farmacológicos suportam o envolvimento da sinalização da IL-1β-IL-1R1 na hiperexcitabilidade da rede neuronal e na geração de convulsões. Neste contexto, achados experimentais demonstraram que a injeção intrahipocampal de IL-1β antes do KA em ratos prolongou a duração das convulsões (Vezzani et al., 1999). Além disso, foi demonstrado que a injeção intracerebral do IL-1Ra apresentou fortes efeitos anticonvulsivantes (De Simoni et al., 2000; Vezzani et al., 2002), e que camundongos transgênicos superexpressando o IL-1Ra em astrócitos apresentavam uma sensibilidade reduzida às convulsões (Vezzani et al., 2000). Estes achados são reforçados pela evidência que um prejuízo na produção endógena de IL-1β através do uso de um bloqueador seletivo ou de um animal nocaute para a caspase-1 (a enzima responsável por produzir a forma biologicamente ativa da IL-1β) reduziu significativamente as convulsões (Ravizza et

al., 2006). Estes dados indicam que os níveis elevados de IL-1β no cérebro devido a um estado inflamatório resultam em efeitos pró-convulsivos. De acordo, foi observado que o aumento nos níveis de IL-1β, 6 horas após a injeção de LPS, levou a uma precipitação e a um aumento na duração das crises convulsivas.

Os mecanismos pelos quais as citocinas levam à excitabilidade neuronal têm sido explorados em diversos estudos, focados sobre as propriedades ictogênicas e neurotóxicas da IL-1β, as quais são mediadas pelo IL-1R1 (Vezzani et al., 1999; Bernardino et al., 2005). Evidências demonstraram que o IL-1R1 se colocaliza no estriado (Lawrence et al., 1998; Kwon et al., 2008) e em neurônios piramidais hipocampais (Viviani et al., 2003) com o receptor NMDA, um subtipo de receptores de glutamato crucialmente envolvido no aparecimento e na disseminação das convulsões. A IL-1β através da ativação do IL-1R1 neuronal induz fosfoforilação, mediada pela quinase Src, do resíduo de tirosina 1472 da subunidade NR2B do receptor NMDA (Balosso et al., 2008). Como consequência desta ação, o influxo de cálcio que ocorre no receptor NMDA em neurônios é aumentado pela IL-1β e este efeito desempenha um papel na promoção da excitotoxicidade (Viviani et al., 2003) e, possivelmente, na geração das convulsões (Vezzani e Baram, 2007). Além disso, a IL-1β pode também inibir a recaptação astrocitária de glutamato (Hu et al., 2000) e induzir a liberação de glutamato (Mascarucci et al., 1998), resultando em níveis elevados de glutamato extracelular e hiperexcitabilidade. Além disso, foi demonstrado que a IL-1β reduz as correntes de cloreto mediadas pelo receptor GABA em culturas de células hipocampais (Wang et al., 2000), a qual pode contribuir para a geração das convulsões. Assim, é plausível que uma ação dupla da IL-1β na neurotransmissão excitatória e inibitória poderia levar à geração das convulsões induzidas pelo GA.

De fato, existem evidências de que substâncias que aumentam a transmissão glutamatérgica e/ou inibem a transmissão GABAérgica possam induzir a geração de convulsões (Karpiak et al., 1981; Amato et al., 1999; Fighera et al., 2003; 2006; Royes et al., 2003; 2006). Neste contexto, muitos estudos têm investigado o envolvimento da neurotransmissão glutamatérgica na neurotoxicidade induzida pelo GA (Wajner et al., 2004). Tem sido sugerido que a redução da captação de glutamato induzida pelo GA poderia facilitar a ativação de receptores de aminoácidos excitatórios, e consequentemente causar as convulsões (Porciúncula et al., 2004; Magni et al., 2007; 2009). Além disso, foi demonstrado que um antagonista

de receptores glutamatérgicos não-NMDA, diminuiu as rotações contralaterais induzidas pela administração intraestriatal de GA, sugerindo assim a participação de receptores glutamatérgicos na neurotoxicidade induzida por este ácido orgânico (Lima et al., 1998). Em relação a IL-1β, tem sido demonstrado que esta citocina induz um aumento na duração das convulsões frente a agentes convulsivantes, sendo que este aumento foi bloqueado pelo uso de antagonistas de receptores glutamatérgicos (Vezzani et al., 1999; 2008), indicando a participação desses receptores na manutenção das convulsões. Também, tem sido demonstrado que inibidores dos transportadores de glutamato não produzem convulsões, mas que são capazes de facilitar as convulsões na presença de um processo inflamatório (Liu et al., 2009). Desde que o GA aumenta a concentração de glutamato nas sinapses e a excitabilidade neuronal (Porciúncula et al., 2004; Magni et al., 2007), é plausível propor que o aumento nos níveis de IL-1β induzidos pelo LPS pode facilitar as convulsões por aumentar a disponibilidade do glutamato neuronal. Essas observações em conjunto com trabalhos prévios que demonstram que o GA inibe a captação de glutamato (Porciúncula et al., 2004; Magni et al., 2007; 2009), e que a IL-1β pode causar excitotoxicidade por mecanismos glutamatérgicos (Viviani et al., 2003; Vezzani e Baram, 2007), sugerem que os resultados desse trabalho se relacionem a esses achados.

Também verificou-se que a aplicação do anticorpo da IL-1β preveniu a redução da latência para a primeira convulsão e o aumento da duração dos episódios convulsivos, observados pela administração de LPS 6 horas antes do GA, demonstrando assim a participação desta citocina pró-inflamatória no aumento da susceptibilidade às convulsões induzidas pelo GA. Estes dados sugerem que a sinalização da IL-1β presente no processo inflamatório contribui decisivamente para a hiperexcitabilidade neuronal e, consequentemente, para as alterações nos parâmetros convulsivos induzidos pelo GA.

Neste contexto, Ravizza e colaboradores (2008) tem proposto que a inflamação persistente pode ser um mecanismo fundamental da epilepsia. Os achados desse trabalho indicam que a inflamação precoce associada ao acúmulo intrastriatal de GA levam a excitabilidade neuronal. A resposta inflamatória à infecção, gerada neste estudo pela ativação da imunidade inata pelo LPS, pode resultar na liberação estriatal de citocinas pró-inflamatórias (IL-1β). A rápida modulação da excitabilidade cerebral induzida pela inflamação resultou em redução

do limiar convulsivo e também no aumento da duração das convulsões, confirmadas comportamentalmente e EEG, da mesma maneira como observada por outros em uma variedade de modelos experimentais de convulsão (Vezzani et al., 2008). No entanto, esta rápida modulação pode também servir como uma fonte aguda de neuro-excitabilidade com ativação suficiente da resposta imune inata. Dessa maneira, a resposta imune inata pode ser vista, não apenas como uma consequência das convulsões, mas sim como uma potencial precursora para ocorrência dos episódios convulsivos. Por fim, os resultados aqui demonstrados sugerem um importante papel da imunidade inata através da liberação de mediadores pró-inflamatórios, neste caso da citocina IL-1β, no aumento da suscetibilidade às convulsões induzida pelo GA, assim como o observado nos pacientes com GA-I após uma doença infecciosa.

Assim, nessa primeira parte desse trabalho foi verificado que o aumento de IL-1β, induzido pelo LPS, potencializou as convulsões causadas pelo GA, e que o uso do anticorpo contra esta citocina preveniu essa potencialização. Dessa maneira, pode-se sugerir que o uso de tratamentos farmacológicos específicos com o objetivo de bloquear a superprodução ou as funções da IL-1β na GA-I, pode representar uma estratégia não convencional para o tratamento dessa patologia. Entretanto, estudos clínicos devem ser realizados a fim de avaliar a eficácia desse tratamento nos pacientes glutaricoacidêmicos que apresentam convulsões.

Embora as convulsões se constituam de um grave problema para as crianças com GA-I, elas também apresentam outras alterações neurológicas importantes como déficits de memória e aprendizagem. De acordo, trabalhos na literatura relatam prejuízos cognitivos nas crianças glutaricoacidêmicas (Patil et al., 2004; Boneh et al., 2008; Beauchamp et al., 2009), especialmente após processos infecciosos (Kölker et al., 2006). Portanto, investigou-se se o tratamento crônico com GA causaria déficit na memória espacial de ratos jovens, bem como se a presença de um processo inflamatório produzido pelo LPS potencializaria o déficit cognitivo induzido pelo GA. Além disso, também foi avaliado se esses tratamentos poderiam contribuir para o desenvolvimento de alterações funcionais e estruturais no hipocampo desses animais.

Para essa finalidade, utilizou-se um modelo que não mimetiza exatamente a GA-I em humanos, na qual, além do GA outros metabólitos são acumulados em menores quantidades. No entanto, ele reproduz a principal característica bioquímica

dessa doença, que são altos níveis teciduais de GA (~0.72 mM) no cérebro de ratos jovens, em concentração semelhante à encontrada em pacientes com GA-I (Ferreira et al., 2005). O GA foi administrado do 5° a 28° dia de vida dos ratos jovens, que corresponde ao período de susceptibilidade dependente da idade nas crianças glutaricoacidêmicas (Kölker et al., 2006). Além disso, o período de tratamento crônico com o GA representa uma fase de grande proliferação celular e sinaptogênese em várias estruturas cerebrais envolvidas na aprendizagem e na memória de ratos, como o hipocampo (Winick e Noble, 1965, Dutra et al., 1993).

No presente estudo foi demonstrado que o tratamento crônico com GA causou um déficit no aprendizado espacial em ratos jovens, e que a presença de um processo inflamatório induzido pelo LPS potencializou o prejuízo na memória induzida pelo GA sozinho. Como nenhum dos tratamentos afetou a atividade locomotora ou o peso dos animais, o efeito dos tratamentos sobre a memória espacial não se deve a estas alterações. Dessa forma, decidiu-se investigar se o déficit cognitivo induzido pelo GA e/ou LPS estaria relacionado ao aumento de citocinas pró-inflamatórias, a redução do volume hipocampal e a diminuição na atividade da enzima Na^+,K^+-ATPase.

De acordo com os trabalhos que têm relatado que os pacientes com GA-I apresentaram prejuízos de memória (Patil et al, 2004; Boneh et al., 2008; Beauchamp et al., 2009), foi observado que os ratos jovens injetados cronicamente com GA apresentaram déficit no aprendizado espacial no segundo dia de teste do labirinto de Barnes. Esse resultado está de acordo com um estudo experimental prévio que demonstrou que a administração crônica GA provocou um prejuízo no aprendizado espacial de ratos no labirinto aquático (da Costa Ferreira et al., 2008). Além disso, verificou-se que o grupo tratado com LPS também apresentou um déficit de memória no segundo dia do teste de aprendizado. De acordo com esse resultado, estudos têm demonstrado que a inflamação neonatal provoca prejuízos nas funções cognitivas (Ikeda et al., 2005; Bilbo et al., 2005a,b; Fan et al., 2008). Também foi verificado que, da mesma forma que as crianças com GA-I apresentam alterações neurológicas após um processo infeccioso (Kölker et al., 2006), os ratos jovens tratados com GA-LPS apresentaram um maior prejuízo no desempenho cognitivo do que o causado pelo GA ou LPS sozinho, no segundo e no terceiro dias de teste.

Além do tratamento crônico com GA em ratos jovens causar um déficit no aprendizado espacial, ele também produziu um aumento nas citocinas (IL-1β e TNF-α) no hipocampo dos ratos. Este achado sugere uma possível relação entre níveis aumentados de moléculas pró-inflamatórias no SNC e disfunção de memória em ratos jovens, já que os animais tratados com LPS e GA-LPS também apresentaram um aumento nos níveis de citocinas e uma redução na memória espacial.

De fato, um grande número de doenças cognitivas em humanos está associado a níveis elevados de moléculas pro-inflamatórias, como a IL-1β e o TNF-α (Griffin et al., 1989; Perrella et al., 1992; Akiyama et al., 2000; Casadesus et al., 2007; Holmes et al., 2009). Os mecanismos básicos dos efeitos das citocinas inflamatórias sobre a aprendizagem e a memória dependentes do hipocampo têm-se centrado na função neuronal através da LTP (Lynch, 2004). Sabe-se que os níveis fisiopatológicos da IL-1 podem produzir efeitos prejudiciais sobre a memória (Matsumoto et al., 2002; Song e Horrobin, 2004; Hein et al., 2010), e que estes efeitos são específicos para a consolidação das memórias que dependem do hipocampo, enquanto memórias independentes do hipocampo não são alteradas (Rachal Pugh et al., 2001; Goshen e Yirmiya, 2007).

Tem sido sugerido que as citocinas pró-inflamatórias produzidas durante processos inflamatórios possam ser as responsáveis pela disfunção sináptica o SNC (Chen et al., 2008; Tanaka et al., 2006; Hein e O'Banion, 2009). De fato, existem fortes evidências de que o aumento de IL-1β no hipocampo pode prejudicar a memória devido aos efeitos sobre a transmissão sináptica, através de prejuízos na indução e na manutenção da LTP hipocampal (Cunningham et al., 1996; Murray e Lynch, 1998; Vereker et al., 2000; Ross et al., 2003). Nesse sentido, tem sido sugerido que a sinalização da IL-1 durante a inflamação, provavelmente mediada pela IL-1β, provoca um aumento seletivo e relativamente persistente na inibição GABAérgica, e que essas ações contribuem para diminuir a excitabilidade sináptica (Ikegaya et al., 2003; Hellstrom et al., 2005). Além disso, foi demonstrado o envolvimento das funções colinérgicas e glutamatérgicas no prejuízo da memória de trabalho induzido pela interleucina-1β em ratos (Matsumoto et al., 2001). De acordo outros estudos demonstraram que a exposição aguda a IL-1β em fatias de hipocampo produziu uma redução na transmissão sináptica glutamatérgica basal (Murray et al., 1997) e uma diminuição na transmissão sináptica excitatória (Coogan e O'Connor, 1997; Luk et al., 1999).

Neste contexto, outros estudos têm demonstrado que a aplicação de TNF-α também inibe a indução da LTP nas áreas CA1 (Tancredi et al., 1992) e no giro denteado do hipocampo (Cunningham et al., 1996). Além disso, Butler e colaboradores (2004) demonstraram que a infusão de TNF-α antes de um estímulo para induzir a LTP inibe a formação desta. Nesse sentido, tem sido proposto que a inibição da LTP pelo TNF-α ocorre via ativação do receptor 1 do TNF (TNFR1) e dos receptores glutamatérgicos metabotrópicos do grupo 1 (Cumiskey et al., 2007).

É bem conhecido que níveis patológicos de citocinas inflamatórias alteram a função hipocampal, mas também é possível que a produção excessiva de citocinas inflamatórias leve a uma mudança na neurogênese. Neste contexto, tem sido demonstrado que a inflamação induzida pelo LPS dá origem a ativação da microglia na área onde nascem os novos neurônios, prejudicando fortemente a neurogênese basal em ratos (Ekdahl et al., 2003). Além disso, é importante observar que os ratos jovens foram injetados durante uma fase de grande proliferação celular e sinaptogênese em várias estruturas cerebrais envolvidas na aprendizagem/memória em ratos, como o hipocampo (Winick e Noble, 1965, Dutra et al., 1993). De fato, há evidências de que a inflamação no início do desenvolvimento causa déficit cognitivo (Bilbo et al., 2005a,b; 2007; 2008; Fan et al., 2010). Tem sido demonstrado que a combinação de LPS e hipóxia-isquemia neonatal em ratos causou um prejuízo de longa duração na aprendizagem e uma redução no volume do hipocampo, e que o tratamento com dexametasona preveniu esses efeitos (Ikeda et al., 2005). Além disso, trabalhos têm demonstrado que a infecção neonatal induzida pelo LPS causou prejuízo de memória na idade adulta (Bilbo et al., 2005a,b; Fan et al., 2008).

Neste contexto, é sabido que o enriquecimento ambiental aumenta a aprendizagem e memória, em parte, através da promoção da neurogênese (van Praag et al., 1999) e do aumento da complexidade estrutural das redes neuronais existentes (Greenough et al., 1973; Green et al., 1983; Wallace et al., 1992; Moser et al., 1994; Silva-Gomez et al., 2003). Da mesma forma, condições que inibem a aprendizagem e a memória (inflamação, stress) também inibem a neurogênese e reduzem a complexidade dendrítica (McEwen, 2007).

Para examinar esta possibilidade determinou-se o volume total do hipocampo em ratos jovens tratados com GA, LPS e GA-LPS, e observou-se uma redução no volume hipocampal nestes grupos em relação ao grupo controle. Assim, esses resultados sugerem que um processo inflamatório durante o desenvolvimento inicial

pode causar um aumento de citocinas no hipocampo e uma atrofia dos neurônios hipocampais, aqui observado por uma redução no volume total do hipocampo.

Além disso, existem trabalhos demonstrando que as citocinas pró-inflamatórias estão envolvidas na inibição da atividade da enzima Na^+,K^+-ATPase (Kreydiyyeh et al., 2004; Kreydiyyeh e Al-Sadi, 2004), uma proteína de membrana que apresenta um envolvimento chave na manutenção da homeostase elétrica das células e, portanto, regula a excitabilidade neuronal (Skou e Esmann, 1992). De fato, foi observado que os ratos jovens tratados com GA, LPS e GA-LPS demonstraram uma inibição na atividade da subunidade α1 da enzima Na^+,K^+-ATPase quando apresentaram prejuízo no desempenho cognitivo (no segundo dia de teste do labirinto de Barnes), sugerindo a participação desta subunidade no déficit de memória espacial observada nesses grupos.

Todos os ratos jovens tratados com GA, LPS e GA-LPS que apresentaram déficit no aprendizado espacial tiveram um aumento nos níveis de citocinas inflamatórias, uma redução no volume hipocampal e uma inibição na atividade da subunidade α1 da enzima Na^+,K^+-ATPase no segundo dia de teste do labirinto de Barnes (dia em que houve o prejuízo no aprendizado espacial). Embora todos os grupos tenham demonstrado uma inibição na atividade da subunidade α1 da enzima Na^+,K^+-ATPase, os grupos tratados somente com GA ou LPS apresentaram um aumento na atividade das subunidades α2/3 da enzima no hipocampo dos ratos, sugerindo uma resposta compensatória, e portanto não alterando a atividade total da enzima. Interessantemente, o grupo tratado com GA-LPS não apresentou nenhuma mudança na atividade das subunidades α2/3 da enzima e, consequentemente, demonstrou uma redução na atividade total da enzima Na^+,K^+-ATPase.

Portanto, os resultados sugerem que a piora no déficit de memória espacial observada no grupo tratado com GA-LPS quando comparado aos grupos tratados somente com GA ou LPS, foi devido ao efeito inibitório sobre a atividade total da enzima Na^+,K^+-ATPase, provavelmente por não apresentar uma resposta compensatória na atividade das subunidades α2/3 da enzima. Neste contexto, alguns trabalhos relataram que a inibição da atividade enzimática total da Na^+,K^+-ATPase induz déficit no aprendizado espacial (Zhan et al., 2004; Lima et al., 2008), e prejudica a retenção de uma tarefa na esquiva inibitória em ratos (Dos Reis et al., 2002). Além disso, Moseley e colaboradores (2007) demonstraram que a deficiência

de isoformas α da enzima Na^+,K^+-ATPase prejudicou o aprendizado espacial, reduziu a atividade motora e aumentou a ansiedade em camundongos.

Em resumo, esta segunda parte do estudo demonstrou que o tratamento crônico com GA causou um déficit no aprendizado espacial de ratos jovens, e que a presença de um processo inflamatório potencializou o prejuízo na memória espacial induzido pelo GA sozinho. Também foi observado um aumento nas citocinas IL-1β e TNF-α, uma redução no volume hipocampal e uma inibição na atividade da subunidade α1 da enzima Na^+,K^+-ATPase, em todos os grupos que apresentaram déficit no aprendizado espacial. Além disso, verificou-se que o grupo tratado com GA-LPS apresentou uma piora na memória espacial, e que este efeito foi específico das isoformas α2/3, uma vez que apenas esse grupo não apresentou nenhuma mudança na atividade destas subunidades e, consequentemente, apresentou uma redução na atividade total da enzima Na^+,K^+-ATPase.

Assim, dado o elevado grau de limitação que a GA-I traz para a criança portadora desta patologia, o entendimento dos mecanismos envolvidos nas alterações neurológicas induzidas pelo acúmulo deste acido orgânico é importante para o desenvolvimento de novas estratégias terapêuticas para o tratamento dessa patologia.

6. CONCLUSÕES

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De acordo com os resultados obtidos no presente estudo, pode-se concluir que:

Capítulo I

1. O GA na dose de 1.3 µmol/estriado causou convulsões comportamentais e EEG em ratos jovens.

2. A administração de LPS 6 horas antes, mas não 3, da injeção de GA potencializou as convulsões (comportamentais e EEG) induzidas pelo GA em ratos jovens.

3. A injeção de LPS aumentou os níveis de IL-1β no estriado em 3 e 6 horas de uma maneira tempo dependente.

4. Existe uma correlação positiva entre o tempo total de convulsões e os níveis estriatais de IL-1β.

5. A facilitação das convulsões induzidas pelo LPS 6 horas antes do GA não se deve ao aumento da temperatura, já que esse aumento foi similar em 3 e 6 horas.

6. Observou-se a participação da via da IL-1β na potencialização das convulsões causadas pela administração de LPS 6 horas antes do GA nos ratos jovens.

Capítulo II

7. O tratamento com GA-LPS potencializou o prejuízo na memória espacial causado tanto pelo GA quanto pelo LPS em ratos jovens.

8. O déficit na memória espacial não se deve a desnutrição, já que nenhum dos tratamentos alterou o peso dos animais.

9. O prejuízo na memória não ocorreu devido a uma alteração locomotora, pois os tratamentos não alteraram a atividade locomotora nem exploratória dos animais.

10. Os tratamentos com GA, LPS e GA-LPS aumentaram os níveis de IL-1β e TNF-α no hipocampo dos ratos jovens.

11. Os tratamentos com GA, LPS e GA-LPS reduziram o volume hipocampal total dos ratos jovens.

12. Os tratamentos com GA, LPS e GA-LPS causaram uma redução na atividade da subunidade α1 da enzima Na^+,K^+-ATPase. Por outro lado, os tratamentos somente com GA ou LPS apresentaram um aumento na atividade das subunidades α2/3 da enzima. Assim, apenas o tratamento com GA-LPS causou uma redução na atividade total da enzima Na^+,K^+-ATPase no hipocampo dos ratos jovens.

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8. APÊNDICE

8. APÊNDICE – Artigos do mestrado

8.1. Creatine decreases convulsions and neurochemical alterations induced by glutaric acid in rats

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Research Report

Creatine decreases convulsions and neurochemical alterations induced by glutaric acid in rats

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ABSTRACT

Glutaric acidemia type I (GA-I) is an inherited metabolic disease characterized by striatal degeneration, seizures, and accumulation of glutaric acid (GA). Considering that GA impairs energy metabolism and induces reactive species generation, we investigated whether the acute administration of creatine, an amino acid with antioxidant and ergogenic properties, protects against the seizures and neurochemical alterations (inhibition of Na^+,K^+-ATPase and increased protein carbonylation) induced by the intrastriatal injection of GA (4 μmol/striatum). We also investigated whether creatine protected against the GA-induced inhibition of glutamate uptake *in vitro*. Creatine administration (300 mg/kg, p.o.) decreased seizures (evidenced by electrographic changes), protein carbonylation and Na^+,K^+-ATPase inhibition induced by GA. However, creatine, at a dose capable of fully preventing GA-induced protein carbonylation (50 and 150 mg/kg, p.o.), did not prevent convulsions and Na^+,K^+-ATPase inhibition, suggesting that the anticonvulsant activity of creatine in this experimental model is not related to its antioxidant action. Creatine also protected against the GA-induced inhibition of L-[^{3}H]glutamate uptake in synaptosomes, suggesting that creatine may reduce the deleterious effects of GA by maintaining glutamate uptake in the synaptic cleft. Therefore, considering that creatine significantly attenuates the deleterious effects of GA assessed by behavioral and neurochemical measures, it is plausible to propose the use of this amino acid as an adjuvant therapy in the management of glutaric acidemia.

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1. Introduction

Glutaric acidemia type I (GA-I) is an inherited organic acid cerebral disorder caused by the impairment of the mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH; EC 1.3.99.7), which catalyzes the dehydrogenation and decarboxylation of glutaryl-CoA to crotonyl-CoA in the catabolic pathway of L-lysine, L-hydroxylysine and L-tryptophan (Goodman et al., 1977; Liesert et al., 1999). Deficient GCDH activity gives rise to an accumulation of organic acids and their derivatives in body fluids and brain tissue of affected patients, i.e. glutaric acid (GA), 3-hydroxyglutaric (3-OH-GA) and glutaconic acids (Baric et al., 1998; Goodman et al., 1977). Clinical manifestations of GA I are predominantly neurological, including generalized convulsions, which are accompanied by bilateral destruction of caudate and putamen (Hoffmann and Zschocke, 1999; Morton et al., 1991) and acute loss of motor skills and dystonia (Freudenberg et al., 2004). Furthermore, postmortem examination of the basal ganglia and cerebral cortex of patients with GA I show postsynaptic vacuolization characteristic of glutamate-mediated brain injury (Goodman et al., 1977). Recently, it has been proposed that GA and 3-OH-GA induce striatal degeneration by disrupting mitochondrial energy metabolism (Ullrich et al., 1999; Das et al., 2003; Ferreira et al., 2005), promoting oxidative stress (de Oliveira Marques et al., 2003; Latini et al., 2002, 2005), and increasing glutamatergic neurotransmission (Wajner et al., 2004), which ultimately causes secondary excitotoxicity (Kolker et al., 1999; 2000a,b, 2002a,b; Porciuncula et al., 2004; Rosa et al., 2004). In this context, it has been shown that the intrastriatal injection of GA causes convulsions, increases oxidative damage markers (total protein carbonyl and thiobarbituric acid-reactive substances) and decreases striatal Na^+,K^+-ATPase activity in rats, mimicking, in various aspects, the neurological alterations of the disease in humans (Lima et al., 1998; Fighera et al., 2006).

Creatine (*N*-[aminoiminomethyl]-*N*-methyl glycine) is an endogenous amino acid produced from glycine, methionine and arginine in the liver, kidney and pancreas (Wyss and Kaddurah-Daouk, 2000). Recent experimental findings have demonstrated that creatine affords significant neuroprotection against hypoxia, amyotrophic lateral sclerosis, ischemia, oxidative insults and excitotoxicity (Holtzman et al., 1998; Klivenyi et al., 1999; Wick et al., 1999; Michaels and Rothman, 1990; Malcon et al., 2000). It is interesting that creatine supplementation also restores creatine phosphate levels and decreases mitochondrial generation of reactive oxygen species (ROS) induced by 3-OH-GA (Kolker et al., 2001; Das et al., 2003), one of the metabolites that accumulates in glutaric acidemia.

Although creatine reduces 3-OH-GA-induced production of reactive species, little is known about the protective effects of this amino acid against the convulsions and the deleterious neurochemical alterations induced by GA. In this context, considering that ROS are involved in the convulsive behavior induced by GA, it is feasible to propose that drugs, such as creatine, which prevents phosphocreatine (PCr) depletion (Wyss and Kaddurah-Daouk, 2000) and acts as scavenger of ROS (Lawler et al., 2002), may protect against GA-induced behavioral, electrographic and neurochemical deleterious effects. Therefore, in the present study, we decided to investigate whether the administration of creatine protects against GA-induced seizures and some known deleterious neurochemical effects of GA, such as protein oxidative damage, Na^+,K^+-ATPase activity inhibition (Fighera et al., 2006) and synaptosomal L-[^{3}H] glutamate uptake inhibition (Porciuncula et al., 2000).

2. Results

In the present investigation, we showed that administration of creatine (300 mg/kg, p.o.) increased the latency for the first convulsive episode [$F(3,54)=16.89$; $P<0.05$; Fig. 1A] and decreased the duration of convulsive episodes [$F(3,54)=13.60$; $P<0.05$; Fig. 1B] induced by the unilateral (right) intrastriatal injection of GA (4 μmol/striatum). This result was confirmed by electrographic recordings (Fig. 2). GA-induced seizures were characterized by clonus of the left forelimb and/or hindlimb and head (myoclonic jerks), rotational behavior and full lateralization toward the left side of the body, which were accompanied by the occurrence of multispikes plus slow waves and major seizure activity in the EEG. Generalized convulsions appeared in the electroencephalographic recordings as the major seizure activity (2–3 Hz high-amplitude activity). After the ictal discharge, postictal EEG suppression and slow waves were observed, correlating with behavioral catalepsy. The intrastriatal injection of GA induced the appearance of high-voltage synchronic clusters in the EEG followed by increase of striatal discharges (basal: 161±26.9 μV; after GA injection: 537±41.2 μV). GA injection increased cortical discharges basal: 124±24.9 μV; after GA injection: 461±45.9 μV; Fig. 2B). In addition, electrographic recordings of the ipsilateral striatum and cerebral cortex revealed that the intrastriatal injection of GA induced the appearance of an epileptogenic focus in the right striatum, which spread to the ipsilateral cortex.

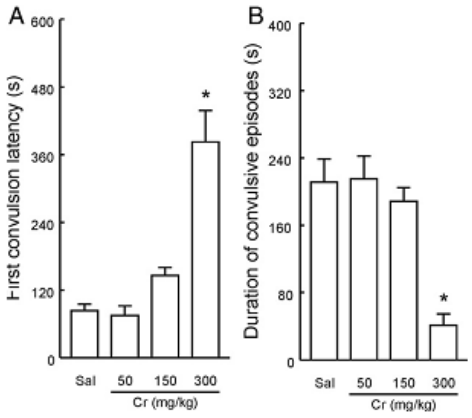

Fig. 1 – Creatine pretreatment (300 mg/kg, p.o.) increases the latency for the first convulsive episode (A) and decreases the duration (B) of convulsive episodes induced by GA (4 μmol/striatum). Data mean±S.E.M. for n=8–10 in each group. *P<0.05 compared with Vehicle-GA group (F test for simple effect).

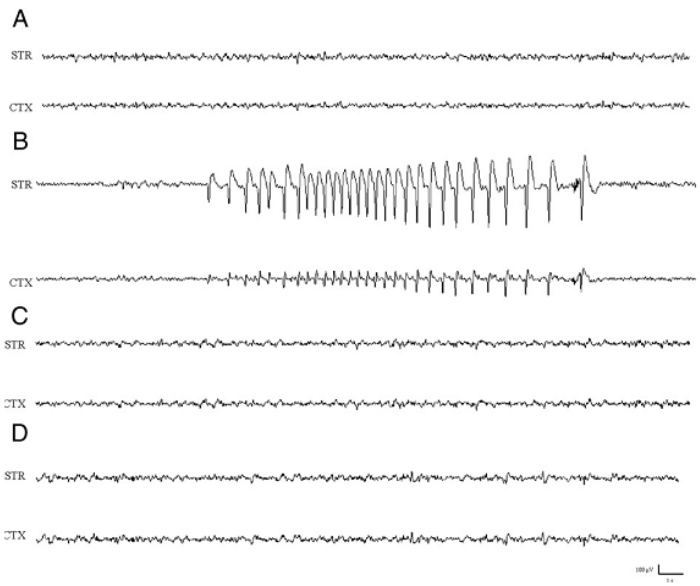

Fig. 2 – Representative electroencephalographic recordings before (A) and after intrastriatal GA administration (4 μmol/striatum; B) which were accompanied by pattern of continuous high-amplitude sharp spikes in low-frequency rhythmic spiking activity in ipsilateral cortex. Typical electroencephalographic recordings after injection of creatine (300 mg/kg, p.o.) (C) and after intrastriatal GA administration (D); STR, ipsilateral striatum; CTX, ipsilateral cortex. Scale bars, 100 μV and 1 s.

To quantify the epileptic effect of intrastriatal GA administration, we calculated the power densities of the different EEG frequencies. As shown in Figs. 3A and B, the field activity in control conditions or after creatine was similar and characterized by intermittent delta–beta oscillations. The injection of GA into striatum increased striatal (71%) and ipsilateral cortex (81%) delta frequency, and decreased beta oscillation in the striatum (37%) and ipsilateral cortex (50%), when compared with control conditions.

Electroencephalographic recordings also showed that creatine (300 mg/kg, p.o.) prevented ictal activity induced by intrastriatal injection of GA during all observation period, followed by a decrease of GA-induced spike amplitude between 65 and 70% with a mean of 151 ± 25 μV (Fig. 2D). Quantitative analysis of EEG revealed that creatine pretreatment decreases striatal [$F(1,10) = 9.81$; $P < 0.03$; Fig. 3A] and ipsilateral cortex delta power density [$F(1,10) = 6.42$; $P < 0.05$; Fig. 3B]. In addition, creatine administration was effective against GA-induced striatal [$F(1,10) = 20.90$; $P < 0.05$; Fig. 3A] and ipsilateral cortex [$F(1,10) = 24.88$; $P < 0.05$; Fig. 3B] beta oscillation decrease.

The effects of GA and creatine injection on the total protein carbonylation in the injected and in the contralateral striatum were also determined. Statistical analysis revealed that intrastriatal injection of GA increased protein carbonyl content in the injected striatum [$F(1,59) = 57.48$; $P < 0.05$] and that creatine (50 to 300 mg/kg, p.o.) prevented the protein carbonylation increase induced by GA [$F(3,59) = 18.69$; $P < 0.05$; Fig. 4].

Fig. 5 shows the effect of creatine (50–300 mg/kg, p.o.) on GA-induced Na^+,K^+-ATPase activity decrease. Statistical analyses showed that the decrease in Na^+,K^+-ATPase activity [$F(1,59) = 95.58$; $P < 0.05$] induced by GA (4 μmol/striatum) was prevented by creatine at the dose of 300 mg/kg (p.o.). Creatine doses lower than 300 mg/kg did not alter GA-induced decrease in Na^+,K^+-ATPase activity.

Considering that acute creatine administration protected against GA-induced behavioral, electrographic and neurochemical deleterious effects, we decided to investigate whether creatine protects against GA-induced decrease of glutamate uptake in synaptosomes (Porciuncula et al., 2000). Statistical analysis revealed that creatine (10 μM; Fig. 6A) prevented GA-induced decrease of glutamate uptake [$F(3,16) = 8.49$; $P < 0.05$; Fig. 6B].

3. Discussion

In the present study, we confirm and extend our previous findings that GA elicits behavioral and electrographic seizures and increases reactive species generation *in vivo* (Fighera et al., 2006). In addition, we show, for the first time, that a single administration of creatine affords significant protection against the acute deleterious effects of GA: increase in protein carbonylation, decrease of Na^+,K^+-ATPase activity and electrographic convulsions *in vivo*. Creatine also protected against GA-induced decrease of L-[^{3}H] glutamate uptake in synaptosomes.

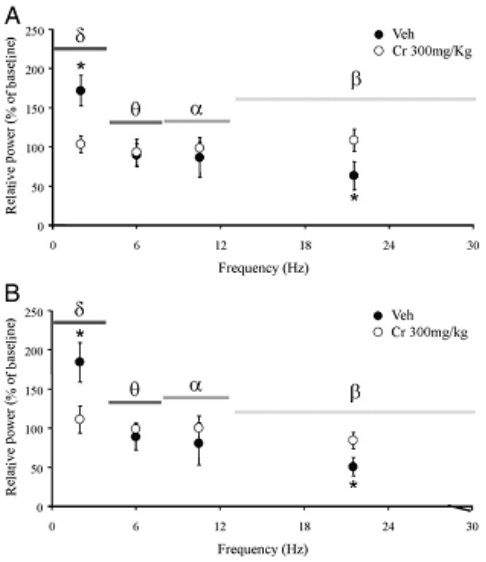

Fig. 3 – Quantitative analysis of EEG recording demonstrated that creatine pretreatment (300 mg/kg, p.o.) attenuated GA-induced delta increase and beta depression power density (4 μmol/2 μl) in the striatum (A) and ipsilateral cortex (B). Data mean ± S.E.M. for n = 5–6 in each group. *P < 0.05 when compared with basal conditions.

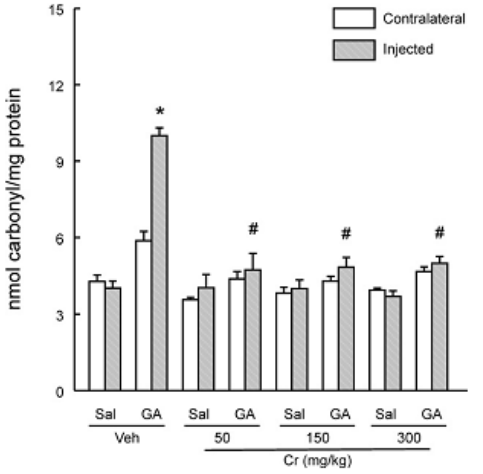

Fig. 4 – Creatine pretreatment (50 to 300 mg/kg, p.o.) prevents the striatal GA-induced protein carbonylation increase. Data are mean + S.E.M. for n = 8–10 in each group. *P < 0.05 compared with Vehicle-Sal group. #P < 0.05 compared with Vehicle-GA group (Student–Newman–Keuls test).

There is a significant body of evidence suggesting that GA accumulation may play a role in the convulsions and neurological impairment seen in patients with glutaric acidemia (Lima et al., 1998; Kolker et al., 1999, 2001, 2002b). Moreover, experimental findings *in vivo* and *in vitro* suggest that energy metabolism impairment and oxidative stress play an important role in the convulsive behavior elicited by GA (Flott-Rahmel et al., 1997; Lima et al., 1998; Frizzo et al., 2004; Porciuncula et al., 2000, 2004; Rosa et al., 2004, Ferreira et al., 2005). It is also worth remarking that a number of GA I patients excrete increased amounts of lactate, 3-hydroxybutyrate, acetoacetate and dicarboxylic acids, further indicating a mitochondrial dysfunction in this organic acidemia (Gregersen and Brandt, 1979; Floret et al., 1979).

In this context, the currently reported increase of striatal and cortical delta frequency induced by GA, to a certain degree, agrees with the study of Fujimoto et al. (2000), who have found periodic synchronic discharges, characterized by intermittent 4–6 Hz positive and 100–200 μV delta waves in cortical structure in a patient with glutaric aciduria type 1 during an episode of acute encephalopathy.

An impairment of glutamate metabolism and homeostasis has been suspected to underlie several brain diseases, including epilepsy (Kelly and Stanley, 2001). In fact, recent studies have demonstrated that inhibition of glutamate transporters leads to recurrent neuronal activity, characterized by periodic cell depolarization and bursts of action potentials (Demarque et al., 2004). This oscillatory effect alters the background pattern of bilateral recurrent paroxysmal bursts followed by cortical beta-gamma oscillations (Milh et al., 2007). In this context, the results presented in this report suggest that cellular glutamate uptake impairment elicited by GA (Porciuncula

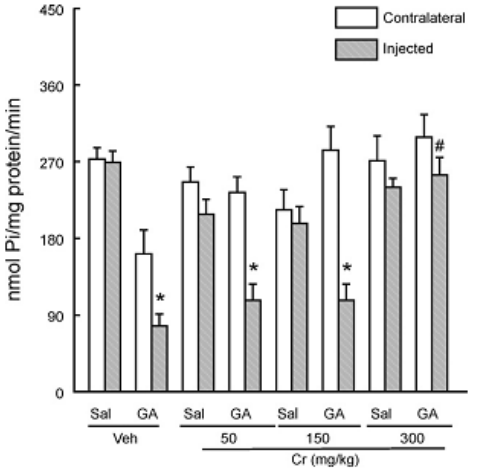

Fig. 5 – Effect of creatine pretreatment (50 to 300 mg/kg, p.o.) on the GA-induced Na^+,K^+-ATPase activity inhibition. Data are mean ± S.E.M. for n = 8–10 in each group. *P < 0.05 compared to contralateral striatum. #P < 0.05 compared with Vehicle-GA group.

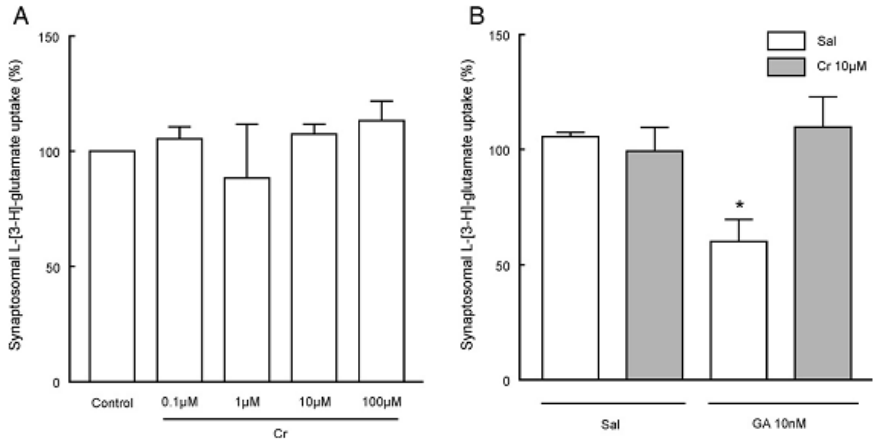

Fig. 6 – Effect of creatine on synaptosomal [^{3}H] glutamate uptake. Creatine (0.1 to 100 μM) addition to membrane preparation had not effect on L-[^{3}H] glutamate uptake (A). The incubation with creatine (10 μM) into synaptosomal membrane preparation reversed GA-induced L-[^{3}H] glutamate uptake inhibition (B). *P < 0.05 compared with control. #P < 0.05 compared with GA group. Data are the means ± SEM of n = 6–8 animals in each group.

et al., 2000, 2004) may lead increased excitatory activity, which ultimately results in electrographic and behavioral seizures (Fighera et al., 2006). Furthermore, since substantial evidence supports that mitochondrial dysfunction and consequent ATP depletion are a major cause of seizures and oxidative stress (Cassarino et al., 1999), it is plausible to propose that a disturbed energy metabolism elicited by this organic acid (Funk et al., 2005; Ferreira et al., 2005) leads to neuronal hyperexcitability, seizures and oxidative stress. In line with this view, and based in the previously reported positive correlation between duration of GA-induced seizures and inhibition of Na^+,K^+-ATPase activity (Fighera et al., 2006), we also suggest that an inhibition of selected targets to free radicals, such as Na^+,K^+-ATPase, may play an important role in the hyperexcitability and concomitant oxidative damage elicited by GA. On the other hand, it is critical to emphasize that, in this model of organic acidemia, a primary metabolic inhibition induced by intrastriatal injection of GA may alter ATP availability for several regulatory processes, disrupting normal Na^+,K^+-ATPase regulation. In this context, previous studies have proposed that nitric oxide, carbon monoxide and NMDA glutamate receptors modulate Na^+,K^+-ATPase activity through activation of regulatory proteins kinase G and C, in which free radicals play modulatory role (Nathanson et al., 1995; Petrushanko et al., 2006). However, further studies are necessary to clarify this point.

In the present study, we demonstrated that creatine pretreatment (300 mg/kg, p.o.) attenuated-GA induced convulsive behavior and protected against protein carbonylation and Na^+, K^+-ATPase activity inhibition. On the other hand, this report also revealed that creatine, at doses capable of preventing GA-induced protein carbonylation, does not prevent GA-induced seizures and Na^+,K^+-ATPase activity decrease, suggesting that protein carbonylation may occur separately from convulsive episodes (Fighera et al., 2006) and that the ability of creatine to reduce protein carbonylation is not related to its anticonvulsant action. However, one must also consider that selected targets, such Na^+,K^+-ATPase, which could not contribute significantly to the total protein carbonyl content, could be responsible for the GA-induced convulsions. Thus, if these targets were more sensitive to oxidative damage, they would require additional antioxidant protection, which could be afforded by increasing creatine doses. Therefore, it is not possible to rule out antioxidant mechanisms in the anticonvulsant action of creatine.

In agreement with this view, it has been claimed that the neuroprotective effect exerted by creatine in several neurodegenerative processes involves buffering of intracellular energy, preventing the increase of Ca^{2+} and ROS intramitochondrial levels, which lead to excitotoxic and cell death (O'Gorman et al., 1997; Leist and Nicotera, 1998; Dolder et al., 2003; Klivenyi et al., 2003; Andres et al., 2005). In addition, recent findings have indicated that creatine exerts cytoprotection via direct antioxidant activity (Sestili et al., 2006). Therefore, it is also possible that stabilization of buffering of intracellular energy and antioxidant properties may underlie its recurrent protection evidenced in this model of organic acidemia.

There is evidence that substances that increase GABAergic function and/or inhibit glutamatergic transmission may decrease convulsive episodes and oxidative stress (Karpiak et al., 1981; Amato et al., 1999; Fighera et al., 2003; Royes et al., 2003, 2006). In line of this view, many studies have investigated the role of glutamatergic and GABAergic neurotransmission on 3-OH-GA and glutaric acid-induced neurotoxicity (for a review see: Wajner et al., 2004). In the present study, we showed that GA inhibits glutamate uptake in striatal synaptosomes. Our results are in agreement with previous data from the literature, demonstrating that GA reduces glutamate uptake in forebrain synaptosomes or cultured astrocytes (Porciuncula et al., 2000, 2004).

Although tissue concentration of GA after intrastriatal administration is unknown, we may speculate that reduction of glutamate uptake by GA may facilitate the activation of excitatory amino acids receptors and, consequently, seizures. Of note, it has been demonstrated that glutamate transporters inhibitors do not produce seizures by themselves, but they are capable of facilitating seizures during energetic failure (Sepkuty et al., 2002; Demarque et al., 2004). Since GA induces cellular energy impairment and oxidative stress (Ferreira et al., 2005), creatine may have acted as an antioxidant by maintaining mitochondrial bioenergetics and protect neurons from excitotoxic damage caused by GA by increasing glutamate uptake and therefore, reducing the concentration of this amino acid in the synaptic cleft.

The exact mechanism by which GA reduces glutamate uptake is still unknown. It has been suggested that the inhibition of glutamate uptake could be due to a direct interaction of GA with glutamate transporters (Porciuncula et al., 2004). However, the activity of glutamate transporters can also be reduced by several indirect mechanisms, including reactive species formation and reduction in Na^+,K^+-ATPase activity (Volterra et al., 1994; Nanitsos et al., 2004). Thus, the reduction of glutamate uptake by GA could be related with its ability to induce oxidative stress and reduce Na^+,K^+-ATPase activity. Our results are in agreement with this view, since creatine reverted the oxidative stress and Na^+,K^+-ATPase activity reduction induced by GA.

In summary, the current study reports that striatal GA administration induces convulsive behavior, protein carbonylation and decrease of Na^+,K^+-ATPase activity, which are prevented by creatine. In addition, creatine protected against GA-induced synaptosomal glutamate uptake inhibition. Although the precise mechanism underlying the striatal degeneration and convulsive behavior of GAI patients is not known, it is plausible to propose that compounds, such as creatine, that decrease GA-induced toxicity assessed by behavioral and neurochemical parameters may be useful as adjuvant therapeutic measures against GA accumulation. However, clinical studies shall be conducted in order to evaluate its clinical efficacy in glutaric acidemic patients.

4. Experimental procedures

4.1. *Animal and reagents*

Adult male Wistar rats (270–300 g) maintained under controlled light and environment (12:12 h light–dark cycle, 24±1 °C, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water were used. Animal utilization protocols followed the Official Government Ethics guidelines and were approved by the University Ethics Committee. All reagents were purchased from Sigma (St. Louis, MO).

4.2. *Behavioral evaluation and surgical procedure*

Animals were anesthetized with Equitesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3 ml/kg, i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a cannula was inserted unilaterally into the striatum (coordinates relative to bregma: AP, 0 mm; ML, 3.0 mm; V, 3.0 mm from the dura). Chloramphenicol (200 mg/kg, i.p.) was administrated immediately before the surgical procedure. The experiments were performed 7–9 days after surgery when animals did not show any sign of pain, infection or discomfort.

Creatine (50, 150 or 300 mg/kg) or vehicle (0.1% carboxymethylcellulose) was infused by intragastric gavage (single administration) 45 min before the intrastriatal administration of GA (4 μmol/2 μl) or saline (5.5 μmol/2 μl). The intrastriatal administration (2 μl) was performed over a 2 min period using a 10 μl Hamilton syringe attached to a 30 gauge needle, whose tip protruded 2 mm from the cannula, allowing injection the dorsal striatum. All intrastriatal injections were made in unanesthetized rats and an additional minute was allowed to elapse before removal of needle to avoid backflow of drug through the cannula. Immediately after the intrastriatal injections, the animals were transferred to a round open field (54.7 cm in diameter) with a floor divided into 10 equal areas. During 20 min, the animals were video-monitored for the appearance convulsive episodes, according to (de Mello et al., 1996). Accordingly, clonic convulsions are episodes characterized by typical partial clonic activity affecting the face, head, vibrissae and forelimbs. Such clonic events are short, typically lasting 1–2 s and can occur either individually or in multiple discrete episodes before generalization and over time. Generalized convulsive episodes are characterized by generalized whole-body clonus involving all four limbs and tail, rearing, wild running and jumping, sudden loss of upright posture and autonomic signs, such as hypersalivation and defecation, respectively. Immediately after the behavioral evaluation, the animals were sacrificed for cannula placement or biochemical analysis.

4.3. *Placement of cannula and electrodes for EEG recordings*

A subset of animals (n=5–6) were anesthetized with Equitesin and surgically implanted with a cannula and electrodes under stereotaxic guidance. The guide cannula (27 gauge) was glued to a multipin socket and inserted into the right striatum through a previously opened skull orifice. One screw electrode was placed over the right parietal cortex (coordinates in mm: AP, −4.5; L, 25; V, 2) along with a ground lead positioned over the nasal sinus. Bipolar nichrome wire Teflon-insulated depth electrodes (100 μm) were implanted into the right striatum (coordinates in mm: AP, 0; L, 3; V, 4.2). The electrodes were connected to the multipin socket and, together with the injection cannula, were fixed to the skull with dental acrylic cement. Electroencephalographic recordings were performed for at least 7 days after the surgery.

4.4. *Intrastriatal injection of drugs and EEG recording*

Routinely, the animals were allowed to settle for habituation in a Plexiglas cage (25×25×60 cm) for at least 20 min. Baseline EEG recordings were obtained 10 min prior to drugs administration in order to establish an adequate control brain electrical activity. The drug injection protocol used in this set of experiments was the same used in those experiments that evaluated the effect of creatine on GA-induced behavioral seizures, except

that EEG was concomitantly recorded using digital encephalographic equipment (Neuromap EQSA260, Neuromap LTDA, Brazil). EEG signals were amplified, filtered (0.1 to 70.0 Hz, bandpass), digitalized (sampling rate 250 Hz) and stored in a PC for off-line analysis, as described below.

4.5. *EEG analyses*

In order to quantify the GA-induced seizure activity, as well as the possible anticonvulsant effect of creatine, we calculated the power densities of the different EEG frequencies obtained from freely moving rats. Digitalized data from basal, preinfusion and seizure periods were divided in 30 s segments and a 4 s sample from each segment was converted into frequency domain by fast Fourier transformation (FFT) method (Dringenberg et al., 2003). The resultant power values displayed for each frequency were grouped into 4 bands represented by delta (0–4 Hz), theta (>4–8 Hz), alpha (>8–13 Hz) and beta (>13–30 Hz). EEG recordings were visually analyzed for the appearance of seizure activity. Seizures were defined by the occurrence of episodes consisting of the following alterations in the recording leads: isolated sharp waves (≥1.5× baseline); multiple sharp waves (≥2× baseline) in brief spindle episodes (≥1 s≥5 s); multiple sharp waves (≥2× baseline) in long spindle episodes (≥5 s); spikes (≥2× baseline) plus slow waves; multispikes (≥2× baseline, ≥3 spikes/complex) plus slow waves; major seizure (repetitive spikes plus slow waves obliterating background rhythm, ≥5 s). Data from seizure periods were expressed as percent of baseline values. Rhythmic scratching of the electrode headset by the animal rarely caused artifacts. These recordings were easily identified and discarded.

4.6. *Protein carbonyl assay*

Immediately after the behavioral evaluation, the animals were killed by decapitation and had their brain exposed by the removal of the parietal bone. A punch of the injected and noninjected striata was rapidly obtained. Striatal tissue was homogenized in 10 volumes (w/v) of 10 mM Tris–HCl buffer pH 7.4 using a glass homogenizer and its carbonyl protein content was determined by the method described by Levine et al. (1990) adapted for brain tissue (Schneider Oliveira et al., 2004).

4.7. *Na^+,K^+-ATPase activity measurement*

The measurement of Na^+,K^+-ATPase activity was performed in the same fresh, diluted, noncentrifuged homogenates used for determination of the striatal protein carbonyl content. Assay of enzyme activity was performed according by Wyse et al. (2000). Briefly, the incubation medium consisted of 30 mM Tris–HCl buffer, pH 7.4; 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$ and 50 μg of protein in the presence or absence of ouabain (2 mM), in a final volume of 350 μl. The reaction was started by the addition of adenosine triphosphate to a final concentration of 5 mM. After 30 min at 37 °C, the reaction was stopped by the addition of 70 μl of trichloroacetic acid (TCA, 50%). Saturating substrate concentrations were used, and reaction was linear with protein and time. Appropriate controls were included in the assays for nonenzymatic hydrolysis of ATP. The amount of inorganic phosphate released was quantified by the colorimetric method described by Fiske and Subbarow (1925), and Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-sensitive activity from the overall activity (in the absence of ouabain).

4.8. *Synaptosomal preparation*

The animals were sacrificed by decapitation and had their brain exposed by the removal of the parietal bone. A punch of the striatum was rapidly removed and synaptosomal preparation was obtained by isotonic Percoll/sucrose discontinuous gradients at 4 °C, as previously described (Dunkley et al., 1986). Briefly, homogenates (10%, w/v) from striatum were made in 5 mM HEPES and 320 mM sucrose (pH 7.4), and centrifuged twice at 1000×*g* for 5 min to produce a pellet (P1) and a supernatant (S1). The S1 were centrifuged twice at 10,000×*g* for 20 min to produce P2 and S2 that were discarded. P2 was resuspended in buffer HEPES/sucrose and was subjected to 16, 10 and 7.5% Percoll solution density gradient centrifugation at 15,000×*g* for 20 min. The synaptosomal fractions were isolated, suspended and homogenized in Krebs' buffer (pH 7.4), containing in mM 145 NaCl, 5 KCl, 1.2 $KH_2PO_4.2H_2O$, 1.3 $MgSO_4.6H_2O$, 20 HEPES, 10 glucose and 1.2 $CaCl_2$, and centrifuged twice at 15,000×*g* for 20 min. The supernatant was removed and the pellet resuspended in Krebs' buffer. The synaptosomal fraction used contained approximately 1 mg of protein/ml. This fraction also contained approximately 5% contamination with fragments of the inner and outer mitochondrial membranes, microsomes, myelin as well as neural and glial plasma membranes (Dunkley et al., 1986; Nagy et al., 1986; Migues et al., 1999). In order to evaluate the integrity of the synaptosomes, lactate dehydrogenase (LDH; EC 1.11.27) release was monitored by incubating the preparation with GA for 15 min. The LDH activity in the incubation medium was assayed spectrophotometrically at a wavelength of 340 nm (Labtest reagents, Brazil). Under the experimental conditions used, no changes in LDH were observed.

4.9. *Synaptosomal [^{3}H] glutamate uptake*

Experiments were performed in a final volume of 500 μl in a standard incubation medium composed of Krebs' buffer, and 5 μM (0.1 μCi) of L-[H^3]-glutamate (49 Ci/mmol, Amersham International, UK). Synaptosomal [^{3}H] glutamate uptake was measured in the presence of physiological concentrations of creatine (0.1 to 100 μM) (Ipsiroglu et al., 2001).

The effect of creatine (10 μM) (a concentration that had no effect *per se*) on synaptosomal [^{3}H]glutamate uptake was evaluated in the presence of GA (10 nM). This concentration of GA was previously reported to reduce [^{3}H] glutamate uptake in forebrain synaptosomes (Porciuncula et al., 2000). Controls did not contain glutaric acid or creatine. The uptake was carried out for 5 min at 37 °C after the addition of synaptosomes (100 μg of protein/tube) and stopped by centrifugation (16,000×*g* for 1 min at 4 °C). Radioactivity in the synaptosomal pellet was measure after the addition of scintillation liquid in a Packerd Model 1409 scintillation counter. The specific glutamate uptake was calculated as the difference between total uptake at 37 °C and the uptake at 4 °C (nonspecific uptake). Under our experimental conditions, the specific uptake was 95±13 pmol/min/mg protein.

4.10. *Protein determination*

Protein content was measured colorimetrically by the method of Bradford (1976) using bovine serum albumin (1 mg/ml) as a standard.

4.11. *Statistics*

The latency for convulsion, total time spent convulsioning and electroencephalographic recordings were analyzed by one- or two-way ANOVA, depending on the experimental design, followed by a Student–Newman–Keuls test. Data from *in vivo* total carbonyl and Na^+,K^+-ATPase activity determinations were analyzed by a 2 (carboxymethylcellulose or creatine)×2 (saline or GA)×2 (injected or contralateral hemisphere) factorial ANOVA (analysis of variance), with the hemisphere factor treated as a within-subject factor. *Post hoc* analyses were carried out by the F test for simple effect or the Student–Newman–Keuls test, when appropriate. Data from *in vitro* Synaptosomal [^{3}H] glutamate uptake were analyzed by one-way ANOVA. All data are expressed as mean±S.E.M.. $P<0.05$ was considered significant.

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8.2. Kinetic characterization of L-[^{3}H]glutamate uptake inhibition and increase oxidative damage induced by glutaric acid in striatal synaptosomes of rats

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Kinetic characterization of L-[^{3}H]glutamate uptake inhibition and increase oxidative damage induced by glutaric acid in striatal synaptosomes of rats

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ABSTRACT

Glutaric acidemia type I (GA-I) is an inherited metabolic disease characterized by accumulation of glutaric acid (GA) and striatal degeneration. Although growing evidence suggests that excitotoxicity and oxidative stress play central role in the neuropathogenesis of this disease, mechanism underlying striatal damage in this disorder is not well established. Thus, we decided to investigate the *in vitro* effects of GA 10 nM (a low concentration that can be present initial development this disorder) on L-[^{3}H]glutamate uptake and reactive oxygen species (ROS) generation in synaptosomes from striatum of rats. GA reduced L-[^{3}H]glutamate uptake in synaptosomes from 1 up to 30 min after its addition. Furthermore, we also provided some evidence that GA competes with the glutamate transporter inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC), suggesting a possible interaction of GA with glutamate transporters on synaptosomes. Moreover, GA produced a significant decrease in the V_{MAX} of L-[^{3}H]glutamate uptake, but did not affect the K_D value. Although the GA did not show oxidant activity *per se*, it increased the ROS generation in striatal synaptosomes. To evaluate the involvement of reactive species generation in the GA-induced L-[^{3}H]glutamate uptake inhibition, trolox (0.3, 0.6 and 6 μM) was added on the incubation medium. Statistical analysis showed that trolox did not decrease inhibition of GA-induced L-[^{3}H]glutamate uptake, but decreased GA-induced reactive species formation in striatal synaptosomes (1, 3, 5, 10, 15 and 30 min), suggesting that ROS generation appears to occur secondarily to glutamatergic overstimulation in this model of organic acidemia. Since GA induced DCFH oxidation increase, we evaluate the involvement of glutamate receptor antagonists in oxidative stress, showing that CNQX, but not MK-801, decreased the DCFH oxidation increase in striatal synaptosomes. Furthermore, the results presented in this report suggest that excitotoxicity elicited by low concentration of GA, could be in part by maintaining this excitatory neurotransmitter in the synaptic cleft by non-competitive inhibition of glutamate uptake. Thus the present data may explain, at least partly, initial striatal damage at birth, as evidenced by acute bilateral destruction of caudate and putamen observed in children with GA-I.

1. Introduction

Glutaric acidemia type I (GA-I) is an autosomal recessive inherited neurometabolic disease caused by deficiency of the activity of the mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH; EC 1.3.99.7), characterized biochemically by an accumulation of glutaric acid (GA), and 3-hydroxyglutaric acid (3-OH-GA) in

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the body fluids and brain tissue (GA, 500–5000 μM; 3-OH-GA, 40–200 μM) of affected patients (Goodman et al., 1977; Liesert et al., 1999; Goodman and Frerman, 2001; Strauss and Morton, 2003; Strauss et al., 2003). Clinical manifestations of GA-I are predominantly neurological, including generalized convulsions, progressive dystonia and dyskinesia, especially after encephalophatic crises, which are accompanied by bilateral and irreversible destruction of vulnerable brain regions, *i.e.* striatum and cortex (Morton et al., 1991; Hoffmann and Zschocke, 1999).

In this scenario, a considerable body of evidence have indicated that GA and 3-GA can induce brain damage by energy depletion (Ullrich et al., 1999; Silva et al., 2000; Das et al., 2003; Ferreira et al., 2005; Latini et al., 2005a), oxidative stress (Latini et al., 2002, 2005b; de Oliveira Marques et al., 2003; Fighera et al., 2006) and primary or secondary excitotoxicity (Kölker et al., 1999, 2000a, 2002a,b; de Mello et al., 2001; Porciúncula et al., 2004; Rosa et al., 2004). Furthermore, it has been suggested that these mechanisms might cooperate in a synergistic way to cause the neuropathological alterations found in GA-I patients (Kölker et al., 2004a; Wajner et al., 2004).

With regard to excitotoxicity, postmortem examination of the basal ganglia and cerebral cortex of patients with GA-I revealed post-synaptic vacuolization characteristic of glutamate-mediated brain damage indicating that this process may represent an important mechanism underlying the pathophysiology of this disorder (Goodman et al., 1977; Forstner et al., 1999; Hoffmann and Zschocke, 1999). Nevertheless, although there is convincing evidence of the participation of glutamate in the toxicity of GA, the primary cause of striatum degeneration in GA-I is still not well defined (Kölker et al., 2001, 2002a,b; Koeller et al., 2002; Latini et al., 2005a; de Oliveira Marques et al., 2003; Ferreira et al., 2005). While some experimental findings demonstrate that GA and 3-OHGA are excitotoxic to cultured neurons and may interact with glutamate receptors or transporters (Flott-Rahmel et al., 1997; Kölker et al., 1999, 2002a,b, 2004b; Rosa et al., 2004; Wajner et al., 2004), recent works did not show excitotoxic actions by 3-OHGA (Lund et al., 2004; Freudenberg et al., 2004). Thus, the role of excitotoxicity in GA-I neuropathophysiology is still under intense debate.

Glutamate is the main excitatory neurotransmitter in the brain, and its interaction with specific membrane receptors is responsible for many functions such as cognition, memory and movement (Ozawa et al., 1998). The role of glutamate in mammalian brain is mediated by activation of ionotropic receptors and metabotropic receptors (Nakanishi, 1992; Hollmann and Heinemann, 1994; Ozawa et al., 1998). Ionotropic receptors can be divided into *N*-methyl-D-aspartate (NMDA: NR1 and NR2A-D) and non-NMDA, the latter including the a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA: GluR1-4) and kainate (GluR5-9 and KA1-2) receptors. Metabotropic glutamate receptors (mGluRs) have been divided into groups I, II and III (Conn and Pin, 1997; Ozawa et al., 1998). Glutamate receptors are involved in a variety of physiological processes during brain development, including synaptogenesis and synaptic plasticity, and present a unique profile of susceptibility to toxicity mediated by differential activation of the receptor subtypes (McDonald and Johnston, 1990). Ionotropic receptor ontogeny is characterized by rapid maturational changes in various forebrain structures in the rat. NMDA receptor expression reaches the highest level in hippocampus and neocortex in the first postnatal week, whereas AMPA receptors density peaks occur in the second postnatal week (Insel et al., 1990; Petralia et al., 1999). This variable receptor expression profile generates a regional- and age-specific window of susceptibility to many neurotoxins and diseases (Kölker et al., 2000a; Haberny et al., 2002; Jensen, 2002).

The synaptic actions of glutamate are terminated by its removal from the synaptic cleft by a high-affinity sodium-dependent excitatory amino acid transporter (EAAT) system, mainly located in the astrocytic membranes (Danbolt, 2001; Amara and Fontana, 2002). The astroglial glutamate transporters GLAST (EAAT1) and GLT1 (EAAT2) are mainly responsible for the clearance of extracellular glutamate (Rothstein et al., 1996; Danbolt, 2001).

Besides its physiological effects, glutamate is also a potent neurotoxin and the presence of high amounts of this neurotransmitter in the synaptic cleft may lead to excitotoxicity by overstimulation of glutamate receptors, a process related to neuropathology of acute (brain hypoxia, ischemia and trauma) and chronic (Parkinson's disease, Alzheimer's disease and organic acidemias) brain disorders (Lipton and Rosenberg, 1994; Maragakis and Rothstein, 2001; Wajner et al., 2004; Kölker et al., 2008).

In models of glutaric acidemia, a considerable body of evidence suggest that neurotoxic actions elicited by GA and 3-OHGA are due the similar chemical structure of this organic acids with glutamate (Flott-Rahmel et al., 1997; Lima et al., 1998; Kölker et al., 1999, 2000a,b, 2002a,b; Ullrich et al., 1999; Porciúncula et al., 2000, 2004; de Mello et al., 2001; Rosa et al., 2004). In this context, it has been demonstrated that 3-OH-GA decreases cell viability by *N*-methyl-D-aspartate (NMDA) receptors stimulation (Kölker et al., 2000a,b) and ROS generation (Kölker et al., 2001). Furthermore, recent studies have suggested the GA-induced neurotoxicity can be due inhibition of glutamate uptake related with an interaction of GA with glutamate transporters, leading to an increase of the glutamate levels in synaptic cleft and overstimulation glutamate receptors (Porciúncula et al., 2000, 2004; Magni et al., 2007). On the other hand, the activity of glutamate transporters can also be reduced by indirect mechanisms, including reactive species formation (Volterra et al., 1994; Nanitsos et al., 2004).

Thus, considering that mechanism involved the primary striatal damage in the GA-I is not well established, we decided investigate whether low concentration of GA (that can be present in the initial development of this disorder −10 nM) alter L-[^{3}H]glutamate uptake. In this context, we evaluated the kinetic parameters dissociation constant (K_D) and maximum velocity (V_{MAX}) of glutamate transport to evaluate whether GA-induced reduction on L-[^{3}H]glutamate uptake could be due to competitive or non-competitive mechanism. Furthermore, we investigated the involvement of striatal ROS generation induced by GA on the L-[^{3}H]glutamate uptake. In addition, we also evaluated the effects of the glutamate receptor antagonists and transporters inhibitor on the oxidative stress and L-[^{3}H]glutamate uptake, respectively.

2. Experimental procedures

2.1. Animal and reagents

Adult male Wistar rats (270–300 g) maintained under controlled light and environment (12:12 h light-dark cycle, 22 ± 1 °C, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water were used. Animal utilization protocols followed the Official Government Ethics guidelines and were approved by the University Ethics Committee. L-[^{3}H]glutamate (49 Ci/mmol) was purchased from Amersham International, UK. All other reagents, including glutaric acid free acid (GA, 99% pure), were purchased from Sigma (St. Louis, MO, USA).

2.2. Synaptosomal preparation

The animals were sacrificed by decapitation and had their brain exposed by the removal of the parietal bone. A punch of the striatum was rapidly removed and synaptosomal preparation was obtained by isotonic Percoll/sucrose discontinuous gradients at 4 °C as previously described (Dunkley et al., 1988). Briefly, homogenates (10%, w/v) from striatum were made in 5 mM HEPES and 320 mM sucrose (pH 7.4), and centrifuged twice at 800 × *g* for 10 min to produce a pellet (P1) and a supernatant (S1). P1 was discarded and S1 was subjected to 16, 10, 7.5% Percoll solution density gradient centrifugation at 24,000 × *g* for 10 min. The

synaptosomal fractions were isolated, suspended and homogenized in Krebs' buffer (pH 7.4), containing in mM: 145 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 20 HEPES, 10 glucose and 1.2 $CaCl_2$ and centrifuged at 15,000 × g for 10 min. The supernatant was removed and the pellet resuspended in Krebs' buffer. The synaptosomal fraction used contained approximately 1 mg of protein/ml. This fraction also contained approximately 5% contamination with fragments of the inner and outer mitochondrial membranes, microsomes, myelin, as well as neural and glial plasma membranes (Dunkley et al., 1988; Nagy et al., 1986; Migues et al., 1999).

2.3. Synaptosomal L-[^{3}H]glutamate uptake

Assays were performed in a final volume of 500 μl in a standard incubation medium composed of Krebs' buffer and 5 μM (3700 Bq) of L-[^{3}H]glutamate (1.81 GBq/mol, Amersham International, UK).

The synaptosomal L-[^{3}H]glutamate uptake was performed in the presence of GA (10 nM). This concentration of GA was previously reported to reduce L-[^{3}H]glutamate uptake in forebrain synaptosomes (Porciúncula et al., 2000). Controls did not contain glutaric acid. The uptake was carried out for 1, 3, 5, 10, 15 and 30 min at 37 °C after the addition of synaptosomes (100 μg of protein/tube) and stopped by centrifugation (16,000 × g for 1 min at 4 °C). Radioactivity in the synaptosomal pellet was measured in a Wallac 1409 liquid scintillation counter. The specific L-[^{3}H]glutamate uptake was calculated as the difference between total uptake at 37 °C and the uptake at 4 °C (non-specific uptake).

Kinetic analysis assay of L-[^{3}H]glutamate uptake was performed in an incubation medium composed of Krebs' buffer in a final volume of 500 μl containing labeled and unlabeled glutamate at final concentrations ranging from 0.625 to 320 μM. The synaptosomal L-[^{3}H]glutamate uptake was performed in the presence of GA (10 nM). Controls did not contain GA. The uptake was carried out for 10 min at 37 °C after the addition of synaptosomes from striatum (100 μg of protein/tube) and followed how above described. Kinetic parameters K_D and V_{MAX} for synaptosomal L-[^{3}H]glutamate uptake were determined by nonlinear regression analysis (GraphPad Software, San Diego, CA).

In order, to evaluate the involvement of glutamate transporters on the GA-induced L-[^{3}H]glutamate uptake reduction, the experiments were done in the presence of 50 μM L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC), which is a substrate inhibitor of glutamate transporters.

To evaluate whether oxidative stress could alter GA-induced L-[^{3}H]glutamate uptake reduction, other experiment was done in the presence of the synthetic antioxidant Trolox (0.3, 0.6 and 6 μM), a water-soluble derivative of vitamin E with potent antioxidant properties, for 10 min at 37 °C (Dreiem and Seegal, 2007).

2.4. Synaptosomal reactive species formation

Reactive oxygen species were assayed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA), which is de-esterified within synaptosomes to the ionized free acid, dichlorofluorescein, DCFH. This is trapped within cells and thus accumulated. DCFH is capable of being oxidized to the fluorescent 2′,7′-dichlorofluorescein diacetate by reactive oxygen. The utility of this probe in isolated subcellular cerebral systems has been described (Bondy et al., 1998). Assays were performed in a final volume of 2000 μl in a standard incubation medium composed of Krebs' buffer, 100 μl synaptosomes (1 mg/ml). The synaptosomes were loaded with 5 μM DCFH-DA for 15 min at 37 °C. This incubation was stopped by centrifugation (16,000 × g for 5 min). The synaptosomal pellet was resuspended in Krebs' buffer in a final volume of 2000 μl. In this solution was added GA 10 nM and was read a long time for 1, 3, 5, 10, 15 and 30 min at 37 °C. Controls did not contain GA. The fluorescence was determined on a spectrofluorometer, with excitation wavelength at 488 nm, and emission wavelength 525 nm.

Other experiment was done in the presence of trolox (0.3, 0.6 and 6 μM) for 10 min at 37 °C (Dreiem and Seegal, 2007), to evaluate whether this antioxidant could decrease GA-induced synaptosomal reactive species formation.

The synaptosomal reactive species formation was also determinate in the presence of NMDA glutamate receptor antagonist, dizocilpine (MK-801; 5 μM) (Tavares et al., 2000) and non-NMDA glutamate receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 100 μM) (Barnes et al., 1994), to investigate the involvement of glutamate receptors on the GA-induced DCFH oxidation increase in striatal synaptosomes.

2.5. Lactate dehydrogenase (LDH) assay

Synaptosomes were incubated at 37 °C for 30 min in Krebs' buffer in the presence or absence of 10 nM GA. Viability was assessed by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH). LDH measurement was carried out in 25 μl aliquots using the LDH kit from Labtest reagents, Brazil. The LDH activity in the incubation medium was assayed spectrophotometrically at a wavelength of 340 nm. The results were expressed as percentage of total LDH release. Total LDH release (100% release) was achieved with 1% Triton X-100 in the incubation medium. Under the experimental conditions used, no changes in LDH were observed.

2.6. Chemiluminescence generated in cell-free systems

The assays were conducted in a standard medium composed of phosphate buffered saline (PBS, 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.4) and luminol (50 mM, prepared daily in PBS) mixture. In this medium was added H_2O_2 (3.5 mM) or GA (10 nM). Controls contained only phosphate buffered saline and luminol mixture. Chemiluminescence generated was measured continuously for 3 min (Yildiz et al., 1998).

2.7. Protein determination

Protein content was measured colorimetrically by the method of Bradford (1976) using bovine serum albumin (1 mg/ml) as a standard.

2.8. Statistics

The synaptosomal L-[^{3}H]glutamate uptake and DCFH oxidation were analyzed by one- or two-way ANOVA, with time of measures treated as within subject factor, depending on the experimental design, followed by a Student–Newman–Keuls test. Data from kinetic analysis of Na^+-dependent transport of synaptosomal L-[^{3}H]glutamate uptake in the presence and absence the GA were analyzed by a student's *t*-test for paired and independent samples. Effect of trolox in the synaptosomal L-[^{3}H]glutamate uptake and DCFH oxidation were analyzed by a 2 (presence or absence of trolox) × 2 (presence or absence of GA) factorial ANOVA. Effect of PDC in the synaptosomal L-[^{3}H]glutamate uptake was analyzed by a 2 (presence or absence of PDC) × 2 (presence or absence of GA) factorial ANOVA. Effect of glutamate receptor antagonists, MK-801 and CNQX, in the DCFH oxidation was analyzed by a 2 (presence or absence of MK-801/CNQX) × 2 (presence or absence of GA) factorial ANOVA. *Post hoc* analyses were carried out by the *F* test for simple effect or the Student–Newman–Keuls test, when appropriate. All data are expressed as mean ± S.E.M., $P < 0.05$ was considered significant.

3. Results

We first evaluated the effect GA (10 nM) on L-[^{3}H]glutamate uptake by striatum synaptosomes of rats. Fig. 1 shows that GA significantly decreased L-[^{3}H]glutamate uptake into striatum synaptosomes as compared to controls [$F(5, 60) = 23.56$; $P < 0.001$]. In order to verify whether cellular death could be responsible for the GA-induced glutamate uptake reduction, we evaluated the viability of striatum synaptosomes measured by LDH release assays. Statistical analysis revealed that synaptosomes incubated for 30 min with GA (10 nM) showed no significant leakage of the cytosolic marker LDH as compared to controls. Percentages of the total LDH content achieved by synaptosomal disruption with Triton X-100 were: 25.71 ± 2.51 (control); 25.06 ± 2.04 (GA 10 nM, $n = 4$ experiments).

Next experiments, we evaluated the involvement of inhibitor of glutamate transporters PDC on the Na^+-dependent L-[^{3}H]gluta-

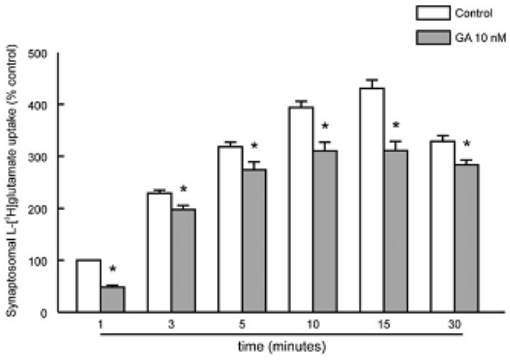

Fig. 1. Effect of glutaric acid (10 nM) on L-[^{3}H]glutamate uptake by synaptosome from striatum of rats. Data are mean ± S.E.M. for $n = 6$ in each group. *$P < 0.001$ as compared to control (Student–Newman–Keuls test).

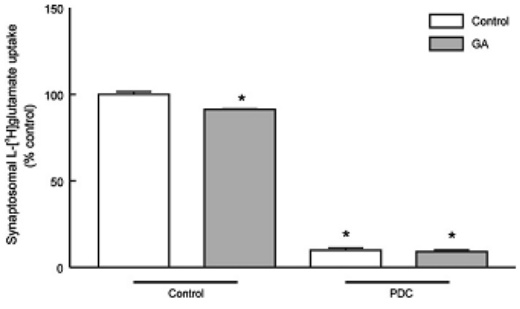

Fig. 2. Effect of PDC (in the absence and presence glutaric acid) on L-[^{3}H]glutamate uptake by synaptosome from striatum of rats. Data are mean ± S.E.M. for $n = 5$ in each group. *$P < 0.005$ compared with control (Student–Newman–Keuls test).

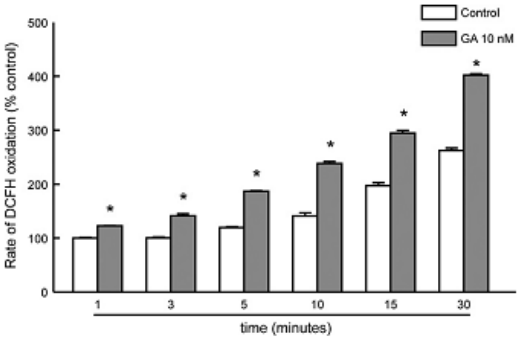

Fig. 4. Effect of glutaric acid on DCFH oxidation by synaptosome from striatum of rats. The DCFH oxidation increase initiated 1 min after GA administration and persisted up to the end of the incubation period (30 min). Data are the means ± S.E.M. $n = 4$ animals in each group. *$P < 0.001$ compared with control (Student–Newman–Keuls test).

mate uptake in the presence or absence of GA in striatal synaptosomes. We observed that both GA and PDC inhibited Na^+-dependent L-[^{3}H]glutamate uptake. Furthermore, GA did not change the inhibitory effect of PDC [$F(1, 16) = 12.7$; $P < 0.005$] (Fig. 2).

Fig. 3 shows alterations on kinetic parameters of the glutamate uptake induced by the low concentration of GA. The hyperbolic glutamate concentration–velocity curve demonstrates typical substrate saturation kinetics expected of Na^+-dependent glutamate transport in synaptosomes, with K_D value of 29.8 ± 4.4 μM and V_{MAX} of 77.5 ± 3.5 pmoles/(mg protein min). Statistical analysis revealed that GA significantly decreased [$t = 2.35$; $P < 0.05$] V_{MAX} (to 66.6 ± 2.8 pmoles/(mg protein min)) and did not changed [$t = 0.91$; $P > 0.05$] K_D value (23.6 ± 3.4 μM) of glutamate uptake as compared to controls.

Considering that glutamate uptake transporters can also be reduced by indirect mechanisms, including reactive species formation, we decided to verify the effect of GA on the synaptosomal oxidative stress. Statistical analysis that GA did not show oxidant activity *per se* as compared to H_2O_2 (data not shown), but increased [$F(5, 30) = 80.06$; $P < 0.001$] DCFH oxidation in striatal synaptosomes at all times (1, 3, 5, 10, 15 and 30 min), as compared to control (Fig. 4). In addition, we observed that trolox (0.3, 0.6 and 6 μM) decreased [significant treatment (control or trolox) by treatment (control or GA) interaction: $F(3, 24) = 8.37$; $P < 0.001$] GA-induced oxidative stress production by synaptosomal preparations from striatum (Fig. 5). However, statistical analysis revealed that trolox (0.3, 0.6 and 6 μM) did not decrease inhibition of glutamate uptake [$F(3, 56) = 0.76$; $P > 0.05$] induced by GA (Fig. 6).

Since GA increased DCFH oxidation in striatal synaptosomes, we decided to investigate whether ionotropic glutamate receptors were involved in the currently described increase of oxidative stress by GA. Statistical analysis showed that the non-NMDA glutamate receptor antagonist CNQX decreased [$F(2, 24) = 6.49$; $P < 0.01$] GA-induced DCFH oxidation increase (Fig. 7). In contrast, the addition of the NMDA glutamate receptor antagonist MK-801 in medium of incubation did not protect against GA-induced oxidative stress. These results agree with previous studies that demonstrated the lack of effect of NMDA glutamate antagonists against GA-induced decrease of Na^+-independent glutamate binding to synaptic membranes and convulsions (Lima et al., 1998; Dalcin et al., 2007).

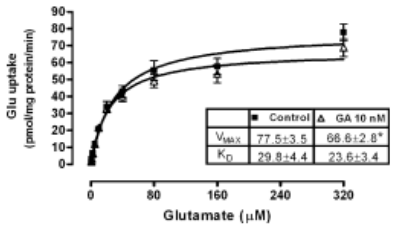

Fig. 3. Effect of glutaric acid (10 nM) on kinetic analysis of high affinity, Na^+-dependent transport L-[^{3}H]glutamate uptake by synaptosome from striatum of rats. Data are mean μmol/(kg protein min) ± S.E.M. for $n = 6$ in each group. Corresponding kinetic analysis (V_{MAX} = μmol/kg protein; K_D = μM) are provided and insert in table. *$P < 0.05$ as compared to control (Student's t-test).

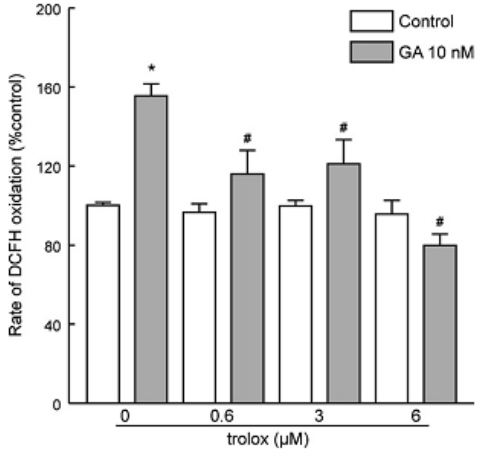

Fig. 5. Effect of trolox (in the absence and presence glutaric acid) on DCFH oxidation by synaptosome from striatum of rats. Data are mean ± S.E.M. for $n = 6$ in each group. *$P < 0.001$ compared with control; #$P < 0.001$ as compared to GA group (Student–Newman–Keuls test).

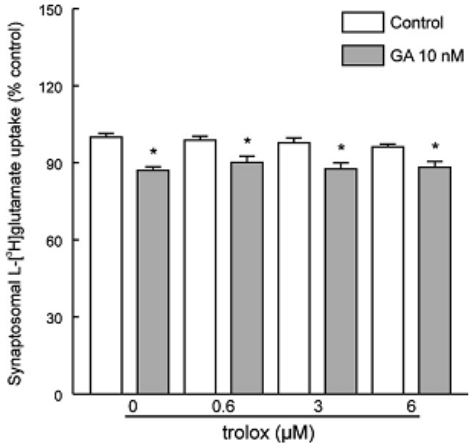

Fig. 6. Effect of trolox (in the absence and presence glutaric acid) on L-[^{3}H]glutamate uptake by synaptosome from striatum of rats. Data are mean ± S.E.M. for $n = 6$ in each group. *$P < 0.05$ compared with control (Student–Newman–Keuls test).

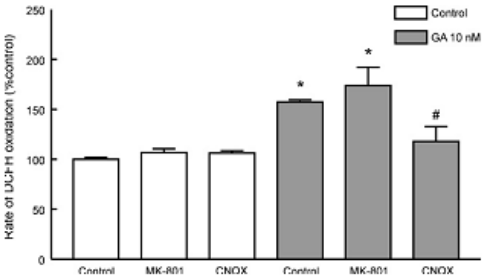

Fig. 7. Effect of NMDA (5 μM) and CNQX (100 μM) (in the absence and presence glutaric acid) on DCFH oxidation by synaptosome from striatum of rats. Data are mean ± S.E.M. for $n = 5$ in each group. *$P < 0.01$ compared with control; #$P < 0.001$ as compared to GA group (Student–Newman–Keuls test).

4. Discussion

Although the pathophysiology of GA-I is not yet fully established, the excitotoxicity has been proposed as an important neurotoxic mechanism in GA-I, especially due to the structural similarity between glutamate, GA and 3-OH-GA (Flott-Rahmel et al., 1997; Lima et al., 1998; Kölker et al., 1999, 2004b; Wajner et al., 2004; Rosa et al., 2004). It is conceivable that some conflicting results about GA-induced excitotoxicity could be due to the ontogenetic and organ specific differences of glutamate receptor and transporter expression (McDonald and Johnston, 1990; Ullensvang et al., 1997; Furuta et al., 1997; Ozawa et al., 1998). Therefore, the present investigation was undertaken to evaluate the effect of GA, at the lower concentration present during initial development of GA-I, on L-[^{3}H]glutamate uptake and reactive species formation from striatum synaptosomal of rats. Furthermore, we evaluated, for first time, what kinetic parameters of glutamane uptake could be altered by GA.

In the present study, we have initially shown a time curve where the GA decreased L-[^{3}H]glutamate uptake from striatal synaptosomes in all times tested. However, this effect was not due to cellular death, as evidenced by LDH viability test, suggesting that specific glutamate transporters localized in striatum could be responsible for that effect. Considering that the glial GLAST and GLT1 glutamate transporters and the neuronal glutamate transporter EAAC1 can be present in synaptosomes preparations (Tanaka et al., 1997; Danbolt, 2001), it is plausible to propose that one or more of these transporters could serve as a target for GA inhibitory effect. In agreement with this view, previous studies have demonstrated that glutamate uptake in synaptosomal preparations is mediated by GLT1 transporters (Robinson et al., 1993; Bridges et al., 1999). Moreover, it has been demonstrated that synaptosomes prepared from GLT1 deficient mutant mice have very low uptake activities (Tanaka et al., 1997; Danbolt, 2001). Furthermore, Western blotting analysis revealed that EAAC1 and GLAST transporters are also present on the striatum of rats at later ages (Furuta et al., 1997).

An important result of the present investigation was that a low concentration of GA (10 nM) significantly reduced the efficacy (V_{MAX}), but not the affinity (K_D) of glutamate uptake in striatal synaptosomes. These findings suggest that glutamate uptake reduction induced by low concentration of GA is due non-competitive inhibition. Our results are in agreement with previous findings demonstrating that a high concentration of GA (1 mM) may reduce glutamate uptake in synaptosomes of whole brain (Porciúncula et al., 2000). Moreover, it was shown that 1 mM, but not 1 or 10 nM, of GA reduced the sodium-dependent glutamate binding in plasma membranes of whole brain, indicating that GA directly interacts with glutamate transporters (Porciúncula et al., 2000). In accordance with that idea that low concentration of GA did not directly interact with glutamate transporter, our kinetic data revelled that GA (10 nM) did not altered K_D value for glutamate uptake in striatal synaptosomes. Furthermore, the reduction in glutamate uptake produced by low concentrations of GA is mediated by a decrease in V_{Max}, which is consistent with a non-competitive inhibition of glutamate transporters. Thus, GA could reduce glutamate uptake directly by an interaction with an alosteric site in glutamate transporters (as acts reactive species). In fact, our experiments showed that the simultaneous addition of the glutamate transporter inhibitor PDC (50 μM) and GA (10 nM) did not alter the inhibitory effect on the glutamate uptake in striatal synaptosomes compared to the effect elicited by PDC alone, indicating that GA can be binding to glutamate transporters and that this disturbance of glutamatergic neurotransmission may explain, at least in part, cerebral damage observed in GA-I.

Other point to be considered is that glutamate transporter activity can be inhibited by oxidation (Volterra et al., 1994; Trotti et al., 1996, 1998; Nanitsos et al., 2004). Thus, we evaluated whether GA-induced glutamate uptake reduction can be due reactive species formation on the striatum synaptosomes of rats. Although trolox protects against GA-induced DCFH oxidation increase in all concentrations, it did not protect against GA-induced glutamate uptake reduction on the synaptosomes of cerebral structure studied, suggesting that ROS formation is a late event in the GA-induced neurotoxicity. Our results are in agreement with previous findings demonstrating that even though trolox may reduce DCFH increase to control levels (Dreiem and Seegal, 2007), it provides no protection against inhibition of aspartate uptake induced by methylmercury (MeHg) (Allen et al., 2001). Taken together these observations, our results suggest that the excitotoxicity induced by low GA concentration can be the initial mechanism of striatal damage and that free radical generation can occur secondarily to glutamatergic overstimulation, a fact that may be related to striatum degeneration observed in GA-I patients. Furthermore, since that a previous study showed

that GA inhibited synaptosomal glutamate uptake at 1 mM concentration (Porciúncula et al., 2000), it may be presumed that much lower intracellular concentrations of GA (10 nM) are sufficient to inhibit glutamate transport and increase oxidative stress. In line of this view, it has been reported that slight reduction on the excitatory neurotransmitter uptake can account an excitotoxic response (Allen et al., 2001).

Besides, we cannot rule out the possibility of that GA may also stimulate glutamate receptors since we found that DCFH oxidation increase provoked by GA was significantly attenuated by the non-NMDA receptor antagonist, but not by MK-801, suggesting that these receptors contributed, at least partly, to the GA-induced oxidative stress. Our present findings may possibly explain a previous *in vivo* report showing that the behavioral alterations and convulsions provoked by intrastriatal administration of GA, in the same dose that caused oxidative stress (Fighera et al., 2006), were prevented by the non-NMDA antagonist DNQX, but not by the NMDA antagonist MK-801 in adult rats (Lima et al., 1998). Reinforcing this point, a recent report also showed that GA ca bind to non-NMDA receptors in brain from rats (Porciúncula et al., 2004; Dalcin et al., 2007).

In addition, it has been demonstrated that glutamate transporters are mainly responsible for the maintenance of low extracellular glutamate concentrations (Rothstein et al., 1996; Danbolt, 2001; Amara and Fontana, 2002). Moreover, the transporter system present in synaptosomes has been considered as an important step for the modulation of the glutamatergic system by controlling the glutamate–glutamine cycle (Otis, 2001). Thus, inhibiting glutamate uptake, GA probably alters the synaptic turnover of glutamate, possibly leading to an increased cytosolic pool of the excitatory neurotransmitter, which in turn may result stimulation of glutamate receptors, causing intracellular Ca^{2+} increase, and leading to ROS generation (Volterra et al., 1994; Nanitsos et al., 2004). We hypothesize that this sequence of events is responsible for the GA-induced neurochemical alterations reported here. Taken together these observations and previous reports demonstrating that low GA concentration inhibits glutamate uptake (Porciúncula et al., 2004) and markedly reduces viability of neurons in culture via glutamate receptors (Kölker et al., 2000a,b), it is conceivable that our results may be related to these findings.

In this context, these mechanisms may explain the involvement of the glutamatergic system in the neuronal toxicity, convulsions and oxidative damage elicited by GA in rats (Kölker et al., 2001; Fighera et al., 2006; Magni et al., 2007; Rosa et al., 2007). Furthermore, the results presented in this report may be related to some of the pathological changes observed in patients with GA-I such as the post-synaptic vacuolization characteristic of excitotoxic neuronal death (Olney, 1980), which has been described in post-mortem examination of the brain of patients with GA-I (Amir et al., 1987). In fact, previous studies have demonstrated the presence of glutamate receptors in the basal ganglia and the characteristic lesions of these cerebral structures in GA-I (Amir et al., 1987; Goodman et al., 1995).

To determine precisely whether the induction of free radicals by GA occurs indirectly via its inhibitory activity on cellular metabolism, or whether it is a direct source of free radicals because of its chemical reactivity, we verified whether GA (10 nM) could increase chemiluminescence in cell-free system. We observed that GA did not show oxidant activity *per se* (data not shown), indicating that chemical reactivity of GA is not direct source of free radicals. In addition, these results reinforce the assumption that ROS generation elicited by this organic acid occur indirectly (Wajner et al., 2004) and that, in low concentration, some degree of cellular intactness is required to GA induce oxidative damage. In this context, it has been proposed that GA and 3-OHGA induce striatal degeneration by disrupting mitochondrial energy metabolism (Ullrich et al., 1999; Das et al., 2003; Ferreira et al., 2005), increasing glutamatergic neurotransmission (Wajner et al., 2004) and promoting oxidative stress (de Oliveira Marques et al., 2003; Latini et al., 2002, 2005b), which ultimately causes secondary excitotoxicity (Kölker et al., 1999, 2000a,b, 2002a,b; Porciúncula et al., 2004; Rosa et al., 2004).

Mechanisms other than glutamatergic facilitation have been claimed to account for the neurotoxic actions of GA, such as Na^+,K^+-ATPase activity and GABAergic mechanisms inhibition induced by GA (Fighera et al., 2006), inhibition of α-ketoglutarate dehydrogenase complex by glutaryl-CoA (Sauer et al., 2005) and bioenergetic impairment (Sauer et al., 2005). Therefore, we cannot rule out that the neurotoxic effects of GA on the glutamate uptake and free radicals production may be mediated, at least in part, by one of these mechanisms (Fighera et al., 2006; Sauer et al., 2006). However, specific studies are necessary to determine the involvement of these alternative mechanisms in the currently described excitotoxic effect of GA-induced on glutamate uptake and oxidative stress increase, and if these events are closely linked.

As regards to the physiological significance of our findings, although we cannot establish with certainty whether our *in vitro* data is related to the neurotoxicity observed in GA-I *in vivo*, it should be emphasized that the effects provoked by GA were observed with concentrations similar to those encountered in brain of glutaric acidemic patients (Goodman et al., 1977; Kölker et al., 2003; Külkens et al., 2005; Sauer et al., 2006). Furthermore, the degree of alterations of the glutamatergic system detected in our study is accepted to cause excitotoxicity in systems testing the effect of potential excitotoxins (Ozawa et al., 1998; Allen et al., 2001; Danbolt, 2001; Meldrum, 2002).

In conclusion, to our knowledge this is the first report showing a kinetic study to glutamate uptake in the presence GA in striatum synaptosomes of rats. Furthermore, taken together our results suggest that the inhibition of glutamate uptake on synaptosomes by the metabolite could result in elevated concentrations of the excitatory neurotransmitter in the synaptic cleft and secondary stimulation of glutamate receptors by GA (a glutamate structurally similar molecule). This glutamatergic neurotransmission increase can lead to reactive species formation and potentially causing excitotoxicity to neural cells, a fact that may be related to the brain damage characteristic of glutaric acidemia type I. The present data may explain, at least partly, initial striatal damage at birth, as evidenced by acute bilateral destruction of caudate and putamen observed in children with GA-I (Goodman, 2004). However, further investigation should be carried out to define initial mechanisms that can be involved on striatum degeneration in this disorder.

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