UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA

PROGRAMA DE PÓS – GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

EFEITOS DOS ÁCIDOS GLUTÁRICO E 3-HIDROXIGLUTÁRICO SOBRE O SISTEMA GLUTAMATÉRGICO EM CÉREBRO DE RATOS DURANTE O DESENVOLVIMENTO

RAFAEL BORBA ROSA

ORIENTADOR: PROF. DR. MOACIR WAJNER

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, como requisito parcial para a obtenção do título de Doutor em Bioquímica.

Porto Alegre, Março de 2008.

“O essencial é invisível para os olhos”

Saint-Exupéry

AGRADECIMENTOS

A todos os funcionários e professores do Departamento de Bioquímica e à UFRGS por me proporcionar uma formação de excelência e totalmente gratuita.

Aos colegas do grande grupo de Erros Inatos do Metabolismo pela ajuda e excelente convivência.

Aos funcionários do Departamento de Genética Médica - HCPA pela cooperação e paciência na cessão de espaço físico, equipamentos e companheirismo.

Aos Professores Clóvis Wannmacher, Carlos Severo Dutra-Filho e Angela Wyse pelos ensinamentos, companheirismo e amizade.

Às Professoras Christianne Salbego e Suzana Wofchuk e à Daniéli Gerhardt pela importante ajuda na elaboração deste trabalho.

Aos Professores Diogo de Souza e Lisiane Porciúncula por sua imensa colaboração em todos os momentos do desenvolvimento deste trabalho.

A todos os bolsistas de iniciação científica, “velhos e novos”, que me auxiliaram ou apenas fizeram meus dias de trabalho mais agradáveis e divertidos.

Às minhas colegas e amigas Carol e Ângela que me acompanharam desde o início dessa caminhada, por sua amizade e carinho.

Aos colegas de pós-graduação Patrícia (Lorpinha), César, Guilhian, Carol, Gustavo, Fabrício, Dênis, Letícia, Giórgia, Ana Paula e Lucila pela amizade, companheirismo, ajuda e alegre convivência.

À Professora Alexandra Latini pelo seu exemplo na busca do conhecimento e da felicidade.

À Karina Dalcin e Anna Laura Schmidt por sua amizade, dedicação e parceria em todos os momentos da realização deste trabalho.

Ao Professor Moacir Wajner por sua imensa dedicação na formação e no futuro de seus alunos, pelos infindáveis ensinamentos e amizade.

À minha família, base e exemplo de caráter em todos os âmbitos da minha formação.

À Sandra, Vó Iracy e Dani, por me acolherem como um filho, neto e irmão, fazendo da sua casa minha casa e me dedicando muito amor e carinho.

Às minhas irmãs, exemplos de perseverança e caráter, por seu amor, carinho e amizade incondicionais.

À Michele, meu amor, que me acompanha todos os momentos, me ensinando todos os dias que o real sentido da vida é amar, independentemente de qualquer circunstância.

À minha mãe, por toda sua paciência, perseverança e luta, no exemplo e orientação do meu caráter e pelo seu amor infinito.

A Deus, guia dos meus passos e atitudes em todos os momentos da minha vida.

SUMÁRIO

PARTE I

Resumo 2

Abstract 3

Lista de Abreviaturas 4

I.1 INTRODUÇÃO 5

I.1.1. Erros Inatos do Metabolismo 5

I.1.2. Acidemias Orgânicas 6

I.1.3. Acidemia Glutárica Tipo I 7

I.1.3.1. Achados Clínicos 9

I.1.3.2. Diagnóstico 10

I.1.3.3. Achados Neuropatológicos 11

I.1.3.4. Tratamento 11

I.1.3.5. Modelos Animais de Acidemia Glutárica Tipo I 12

I.1.3.6. Fisiopatologia 13

I.1.4. Sistema Glutamatérgico 18

I.1.4.1. Ontogenia dos Receptores Glutamatérgicos Ionotrópicos em Córtex Cerebral e Estriado 21

I.1.4.2. Ontogenia dos Transportadores de Glutamato no Sistema Nervoso Central 22

I.1.4.3. Excitotoxicidade Glutamatérgica 23

I.2 OBJETIVOS 25

I.2.1. Objetivo Geral 25

I.2.2. Objetivos Específicos 25

PARTE II

Capítulo I – *Evidence that 3-hydroxyglutaric acid interacts with NMDA receptors in synaptic plasma membranes from cerebral cortex of Young rats* 28

Capítulo II – *Age and brain structural related effects of glutaric and 3-hydroxyglutaric acids on glutamate binding to plasma membranes during rat brain development* 37

Capítulo III – *Evidence that glutaric acid reduces glutamate uptake by cerebral cortex of infant rats* 52

PARTE III

III.1 DISCUSSÃO 63

III.2 CONCLUSÕES 77

REFERÊNCIAS BIBLIOGRÁFICAS 81

ANEXOS – *trabalhos publicados relacionados à tese* 105

3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats 106

3-Hydroxyglutaric acid enhances glutamate uptake into astrocytes from cerebral cortex of young rats 113

3-Hydroxyglutaric acid moderately impairs energy metabolism in brain of young rats 122

PARTE I

Introdução e Objetivos

RESUMO

Diversos trabalhos têm relacionado a fisiopatogenia da acidemia glutárica tipo I (GA I) com a excitotoxicidade glutamatérgica. Ainda que sob intenso debate, a maioria desses trabalhos se baseia na semelhança estrutural existente entre o glutamato e os principais ácidos orgânicos acumulados na GA I, os ácidos glutárico (GA) e 3-hidroxiglutárico (3-OHGA). Tendo em vista a janela de vulnerabilidade cerebral dos pacientes afetados pela GA I durante os primeiros anos de vida e o distinto padrão de expressão e propriedades de receptores e transportadores glutamatérgicos ao longo do desenvolvimento, o presente trabalho teve por objetivo investigar os efeitos dos ácidos GA e 3-OHGA sobre a ligação de glutamato a seus receptores e transportadores de membrana plasmática e sobre a captação de glutamato por fatias e preparações sinaptossomais de córtex cerebral e estriado de ratos de 7 a 60 dias de vida. Nossos resultados demonstraram que o 3-OHGA inibiu a ligação de glutamato a seus receptores e transportadores de membrana em córtex cerebral de ratos de 30 dias de vida. Observamos ainda que esse ácido parece interagir com receptores do tipo NMDA, agindo como um fraco agonista desses receptores por aumentar a ligação de MK-801 em receptores de membrana e o influxo de Ca^{2+} em fatias de córtex cerebral de ratos. Através do estudo ontogenético da ligação de glutamato a seus receptores e transportadores de membrana plasmática de ratos na presença de GA e 3-OHGA, verificamos que o GA inibe a ligação de glutamato a seus receptores de membrana em córtex cerebral e estriado de ratos de 7 e 15 dias de vida. A utilização de antagonistas glutamatérgicos juntamente com a inibição da ligação de cainato a seus receptores na presença de GA indicaram que os efeitos desse ácido sobre a ligação de glutamato a seus receptores parecem envolver receptores do tipo não-NMDA, possivelmente do tipo cainato. O ácido 3-OHGA inibiu a ligação de glutamato somente a transportadores de membrana de estriado de ratos de 7 dias de vida. Os estudos de captação de glutamato mostraram que o GA inibe o transporte desse neurotransmissor apenas em fatias de córtex cerebral de ratos de 7 dias de vida. Esse efeito não foi devido à morte celular e foi prevenido por pré-administração de *N*-acetilcisteína sugerindo o envolvimento de dano oxidativo na presença do GA. Além disso, o efeito do GA parece envolver os transportadores glutamatérgicos do tipo GLAST, expressos nos estágios iniciais de desenvolvimento cerebral. Em contraste, o ácido 3-OHGA não afetou a captação de glutamato por fatias de córtex cerebral e estriado de ratos. Observamos também que os ácidos GA e 3-OHGA não afetaram a captação de glutamato por preparações sinaptossomais de cérebro de ratos. Os efeitos detectados no presente estudo pelos ácidos GA e 3-OHGA em períodos específicos do desenvolvimento e também nas distintas estruturas cerebrais estudadas podem estar relacionados com a expressão diferenciada de receptores e transportadores glutamatérgicos no cérebro de ratos. Apesar de não explicarem a janela de vulnerabilidade estriatal, tais resultados podem auxiliar na elucidação dos mecanismos fisiopatogênicos envolvidos no dano neurológico ocorrido durante os primeiros anos de vida dos pacientes com GA I.

ABSTRACT

The role of excitotoxicity in the cerebral damage of glutaric acidemia type I (GA I) is under intense debate. Several studies relate the excitotoxic actions of the major metabolites accumulating in GA I, glutaric (GA) and 3-hydroxyglutaric acids (3-OHGA), based on the chemical structural similarity between these organic acids and glutamate. Considering that GA I patients present a window of cerebral vulnerability during the first years of life and the variable ontogenetic pattern of glutamate receptors and transporters expression and properties, the objective of the present work was to investigate the effects of GA and 3-OHGA on glutamate binding to receptors and transporters from synaptic plasma membranes and on glutamate uptake by slices and synaptosomal preparations from cerebral cortex and striatum during rat brain development. Our results demonstrated that 3-OHGA inhibited glutamate binding to receptors and transporters from plasma membranes of cerebral cortex of 30-day-old rats. Moreover, its effect on glutamate receptors seems to be directed towards NMDA receptors and suggests that 3-OHGA acts as a weak agonist of these receptors by increasing the MK-801 binding to membrane receptors and Ca^{2+} influx into cerebral cortex slices of rats. The ontogenetic study of glutamate binding to receptors and transporters in the presence of GA and 3-OHGA revealed that GA decreased the glutamate binding to receptors from plasma membranes of cerebral cortex and striatum of 7- and 15-day-old rats. In addition, by using glutamate antagonists and kainate binding assay, we verified that this effect involved non-NMDA receptors, probably kainate receptors. On the other hand, 3-OHGA inhibited glutamate binding to transporters from plasma membranes of striatum of 7-day-old rats. The glutamate uptake studies showed that GA decreased glutamate transport only in cerebral cortex slices of 7-day-old rats. Furthermore, this effect was not due to cellular death and was prevented by *N*-acetylcysteine preadministration suggesting the involvement of oxidative damage. Moreover, immunoblot analysis and glutamate uptake assays in the presence of GA and dihydrokainate (DHK) revealed that GA effect seems to involve GLAST transporters, which are expressed in early stages of rat brain development. In contrast, 3-OHGA did not affect glutamate uptake by brain slices. We also observed that both GA and 3-OHGA did not affect glutamate uptake by synaptosomal preparations from rat brain. Concluding, the effects provoked by GA and 3-OHGA at specific ages of development and also in distinct cerebral structures may possibly be explained by the differential ontogenetic and regional specific expression of the glutamate receptors and transporters in rat brain. Although these results can not explain the window of striatal vulnerability, they may contribute to elucidate the mechanisms of the neurological damage occurred during the first years of life in GA I patients.

LISTA DE ABREVIATURAS

EIM – erros inatos do metabolismo

GA I – acidemia glutárica tipo I

GCDH – glutaril-CoA desidrogenase

GA – ácido glutárico

3-OHGA – ácido 3-hidroxiglutárico

ATP – trifosfato de adenosina

GABA – ácido γ-aminobutírico

NMDA – ácido *N*-metil-D-aspártico

DNQX – 6,7-dinitroquinoxalina-2,3-diona

SNC – sistema nervoso central

AMPA – α-amino-3-hidróxi-5-metil-4-isoxazol propiônico

EAAT – transportador de aminoácidos excitatórios

GLAST – transportador de glutamato/aspartato de ratos

GLT1 – transportador de glutamato de ratos 1

EAAC1 – carreador de aminoácidos excitatórios 1

MK-801 – dizocilpina

CNQX – 6-ciano-7-nitroquinoxalina-2,3-diona

AP5 – ácido DL-2-amino-fosfonovalérico

PDC – L-*trans*-pirrolidina-2,4-dicarboxilato

MTT – azul de tiazolil

LDH – lactato desidrogenase

DHK - dihidrocainato

I.1 INTRODUÇÃO

I.1.1. Erros Inatos do Metabolismo

Em 1908, Sir Archibald E. Garrod criou o termo erros inatos do metabolismo (EIM) para designar doenças como a alcaptonúria, em que os indivíduos afetados excretam grandes quantidades de ácido homogentísico na urina. Garrod observou uma maior freqüência desta doença em indivíduos de uma mesma família e maior incidência de consangüinidade 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. Com o surgimento de novos distúrbios relacionados a alterações genéticas e que envolviam o acúmulo de outras substâncias nos líquidos biológicos dos pacientes, postulou-se que estas doenças resultavam da síntese qualitativa ou quantitativamente anormal de uma proteína, enzimática ou não, pertencente ao metabolismo (Scriver et al., 2001). Presumiu-se então, que em conseqüência deste bloqueio metabólico, pode ocorrer o acúmulo de precursores da reação catalisada pela enzima envolvida, com a formação de rotas metabólicas alternativas e a deficiência de produtos essenciais ao organismo (Bickel, 1987).

Até o momento, foram descritos mais de 500 EIM, a maioria deles envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (Scriver et al., 2001). Embora individualmente raras,

essas doenças afetam aproximadamente 1 a cada 500/2000 recém nascidos vivos (Baric, Furnic e Hoffmann, 2001).

I.1.2. Acidemias Orgânicas

As acidemias ou acidúrias orgânicas constituem um grupo de EIM e são caracterizadas pelo acúmulo de um ou mais ácidos orgânicos nos líquidos biológicos e tecidos dos pacientes afetados devido à deficiência da atividade de enzimas do metabolismo de aminoácidos, lipídeos ou carboidratos (Chalmers e Lawson, 1982). A freqüência destas doenças na população em geral é pouco conhecida, o que pode ser creditado à falta de laboratórios especializados para o seu diagnóstico e ao desconhecimento médico sobre essas enfermidades. Na Holanda, país considerado referência para o diagnóstico de erros inatos do metabolismo, a incidência destas doenças é estimada em 1: 2.200 recém-nascidos, enquanto que, na Alemanha, Israel e Inglaterra é de aproximadamente 1: 6.000 - 1: 9.000 recém-nascidos (Hoffmann et al., 2004). Na Arábia Saudita, onde a taxa de consangüinidade é elevada, a freqüência é de 1: 740 nascidos vivos (Rashed et al., 1994). Chalmers e colaboradores (1980) demonstraram que as acidemias orgânicas eram os EIM mais freqüentes em crianças hospitalizadas motivando diversos estudos clínicos, laboratoriais e epidemiológicos nos anos seguintes.

Clinicamente os pacientes afetados apresentam predominantemente disfunção neurológica 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, etc. As mais freqüentes manifestações laboratoriais são cheiro peculiar na urina e/ou suor, 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). Recentemente, com o uso da tomografia computadorizada, foram encontradas na maioria dos pacientes pacientes afetados por essas doenças, alterações de substância branca (hipomielização e/ou desmielização), atrofia cerebral generalizada ou dos gânglios da base (necrose ou calcificação), megaencefalia, atrofia frontotemporal e atrofia cerebelar (Mayatepek et al., 1996).

I.1.3. Acidemia Glutárica Tipo I

A acidemia glutárica tipo I (GA I, OMIM # 231670) é uma desordem neurometabólica autossômica recessiva primeiramente descrita por Goodman e colaboradores (1975). Esse EIM dos aminoácidos lisina, hidroxilisina e triptofano é causado pela deficiência na atividade da enzima mitocondrial glutaril-CoA desidrogenase (GCDH, EC 1.3.99.7) (Goodman e Frerman, 2001). A GCDH catalisa a descarboxilação oxidativa da glutaril-CoA formando crotonil-CoA e CO_2, transferindo os elétrons para a cadeia respiratória via flavoproteína transferidora de elétrons (ETF) (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 a 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 mais freqüente mutação em caucasianos (R402W) (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, 1999; Kölker et al., 2006a). Com o bloqueio da atividade enzimática, formam-se rotas metabólicas alternativas que culminam na presença de concentrações elevadas de ácido glutárico (GA), 3-hidroxiglutárico (3-OHGA) e, algumas vezes, glutacônico nos tecidos e líquidos biológicos (plasma, urina e líquor) dos indivíduos afetados (Goodman et al., 1975; Goodman e Frerman, 2001) (Figura 1).

As concentrações plasmáticas destes ácidos variam entre 5 e 400 μmol/L (Hoffmann et al., 1991, 1996; Merinero et al., 1995) mas as concentrações cerebrais podem atingir 500–5000 μmol/L para o GA e 40–200 μmol/L para o 3-OHGA (Sauer et al., 2006). Tais diferenças podem ser explicadas pelo fato de que o GA e o 3-OHGA são produzidos nas células neurais e que a barreira hematoencefálica é pouco permeável a esses ácidos orgânicos, ocasionando o acúmulo dessas substâncias no sistema nervoso central o que se constitui em um fator de risco na neurodegeneração característica dos pacientes afetados (Hoffmann et al., 1993; Sauer et al. 2006; Kölker et al., 2006a,b).

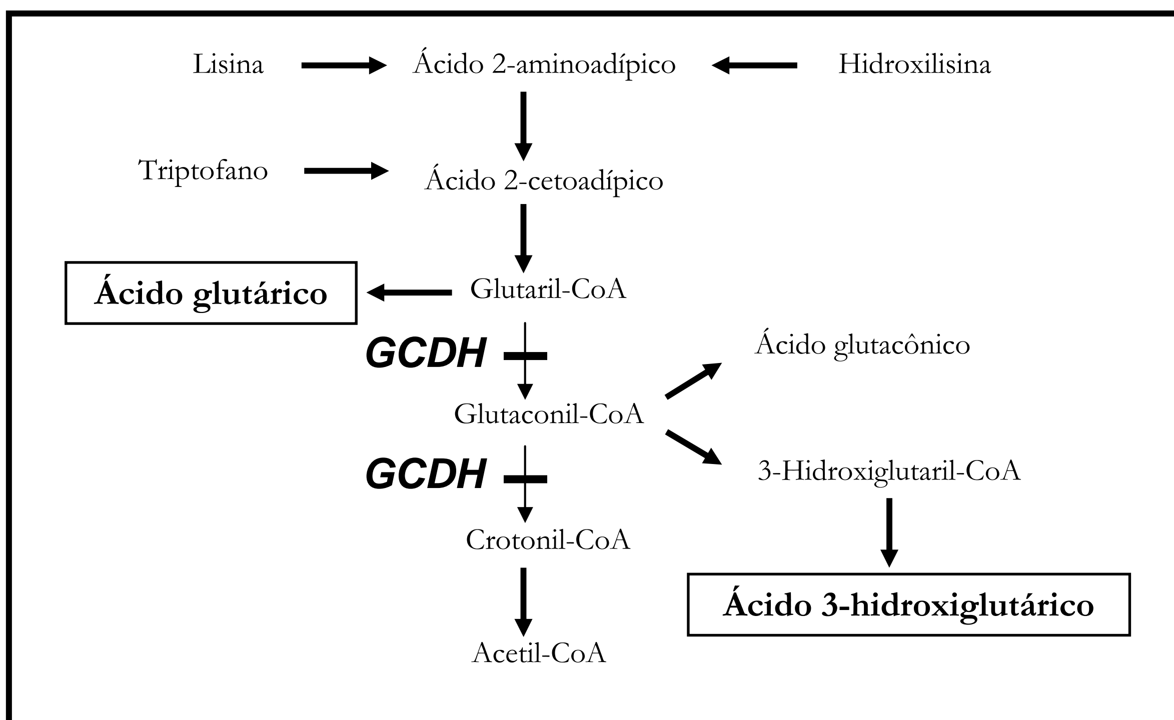

Figura 1. Deficiência da enzima glutaril-CoA desidrogenase (GCDH).

A prevalência da GA I é estimada em 1: 30.000 nascidos vivos (Goodman e Frerman, 2001) podendo ser bastante aumentada (até de 1: 300 nascidos vivos, Kölker et al., 2004a,b, 2006a) em algumas comunidades fechadas como na Ordem Amish da Pensilvânia (Biery et al., 1996) e nos índios Salteaux/Ojibway do Canadá (Greenberg et al., 1995).

I.1.3.1. Achados Clínicos

Entre os achados clínicos mais comuns está a macrocefalia presente ao nascimento. A sintomatologia inicial é geralmente branda com alguns pacientes desenvolvendo-se normalmente até o aparecimento das crises encefalopáticas (Hoffmann et al., 1995). Após as crises agudas surgem sintomas relacionados à destruição estriatal como distonia e discinesia, hipotonia, convulsões, rigidez

muscular e espasticidade (Hoffmann e Zschocke, 1999; Strauss et al., 2003; Kölker et al., 2004a). Ataxia, irritabilidade, retardo mental e demência também estão entre os achados clínicos da GA I (Külkens et al., 2005).

I.1.3.2. Diagnóstico

Apesar do desenvolvimento de diversas estratégias terapêuticas para o tratamento da GA I, o diagnóstico precoce continua sendo determinante para um melhor prognóstico para os pacientes afetados. Usualmente, o marcador bioquímico da GA I é a presença de quantidades elevadas de GA e 3-OHGA nos líquidos biológicos (especialmente urina) dos pacientes (Goodman et al., 1977; Funk et al., 2005; Kölker et al., 2006a). 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., 2006a). O perfil de acilcarnitinas e a diminuição de carnitinas livres nos líquidos biológicos determinados por espectrometria de massa podem ser usados como métodos auxiliares no diagnóstico (Ziadeh et al., 1995). A análise mutacional não é muito utilizada para fins de diagnóstico devido ao grande número de mutações conhecidas, apresentando maior valor em estudos de comunidades onde a consangüinidade é elevada e para fins de pesquisa (Busquets et al., 2000; Kölker et al., 2006a).

Alguns pacientes apresentam excreção pouco elevada, intermitente, ausente ou normal de GA (Merinero et al., 1995; Hoffmann et al., 1996; Baric et al., 1998) e nesses casos 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).

I.1.3.3. Achados Neuropatológicos

Os achados neuropatológicos da deficiência da GCDH incluem atrofia frontotemporal cortical ao nascimento, formação espongiforme e diminuição de substância branca (leucoencefalopatia) e uma característica degeneração bilateral aguda do estriado que é geralmente precipitada por infecções ou vacinações (situações onde o paciente se encontra em catabolismo elevado) nos primeiros 36 meses de idade (Amir et al., 1987; Chow et al., 1988; Brismar e Ozand, 1995; Hoffmann e Zschocke, 1999). Freqüentemente, os pacientes apresentam um alargamento dos espaços subaracnóideos que, devido à alta irrigação sangüínea, os tornam suscetíveis a hemorragias agudas (Drigo et al., 1996; Hoffmann e Zschocke, 1999).

I.1.3.4. Tratamento

Restrição dietética dos aminoácidos lisina, hidroxilisina e triptofano, com ênfase maior para lisina, precursores diretos dos ácidos orgânicos acumulados, é essencial para o bom prognóstico dos indivíduos afetados (Goodman e Frerman, 2001; Kölker et al., 2006a). Além disso, suplementação com dieta hipercalórica, L-carnitina e riboflavina têm mostrado resultados positivos na diminuição da toxicidade dessas substâncias e na prevenção das crises encefalopáticas dos pacientes (Hoffmann et al., 1996; Külkens et al., 2005; Chalmers, Bain e Zschocke, 2006).

Diversos medicamentos têm sido testados na terapia da GA I, sendo que anticolinérgicos e toxina botulínica (Burlina et al., 2004), anticonvulsivantes (Yamaguchi et al., 1987; Hoffmann et al., 1996), suplementação com creatina e antioxidantes (Hoffmann e Zschocke, 1999) mostraram alguns resultados satisfatórios. Mais recentemente, Zinnanti e colaboradores (2007), baseados em estudos prévios em um modelo animal de GA I (Zinnanti el al., 2006a), propuseram a utilização da suplementação com glicose e homoarginina para reduzir o acúmulo cerebral dos metabólitos tóxicos gerados pela deficiência da GCDH.

Novas estratégias terapêuticas devem ser intensamente pesquisadas, porém a utilização dessas terapias requer muito estudo e cautela quando de seu uso para os pacientes.

I.1.3.5. Modelos Animais de Acidemia Glutárica Tipo I

O desenvolvimento de modelos animais que mimetizem as características metabólicas e neuropatológicas apresentadas pelos pacientes com GA I também se constitui num desafio. Koeller e colaboradores (2002) desenvolveram um modelo *knockout* do gene da GCDH em camundongos. Apesar dos animais apresentarem um fenótipo bioquímico similar ao dos pacientes, com elevados níveis de GA, 3-OHGA 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 (2006a) com a administração via oral de uma sobrecarga de lisina aos animais. Neste particular, foi verificado que as concentrações de ácido

glutárico no cérebro dos camundongos *knockout* 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.

I.1.3.6. Fisiopatologia

A GA I é considerada uma acidemia orgânica "cerebral" pois os indivíduos afetados apresentam essencialmente sintomatologia neurológica. Na última década, distintos mecanismos têm sido propostos para explicar a fisiopatogenia do dano cerebral da GA I, considerando o 3-OHGA a principal neurotoxina acumulada nessa doença (Ullrich et al., 1999; Kölker et al, 2002a).

Distúrbios de bioenergética estão entre esses mecanismos. Estudos realizados em culturas de neurônios de ratos mostraram que o 3-OHGA inibe os complexos II e V da cadeia respiratória e diminui os níveis de fosfocreatina (Ullrich et al., 1999; Das, Luche e Ullrich, 2003). Latini e colaboradores (2005a) também encontraram uma inibição do complexo II da cadeia respiratória em homogeneizados de córtex cerebral e células C6 de glioma de ratos na presença desse metabólito. Além disso, o mesmo estudo demonstrou que o 3-OHGA 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. 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 de neurônios de telencéfalos de embriões de pinto, concordando em parte com um estudo realizado em partículas submitocondriais de coração bovino que não mostrou nenhum efeito desse ácido sobre os complexos enzimáticos da cadeia transportadora de elétrons (Sauer et al., 2005). Foi também demonstrado que o GA 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). Outros trabalhos mostraram uma inibição dos complexos I-III, II, II-III e da enzima creatina quinase em músculo esquelético e cérebro médio de ratos tratados cronicamente com GA (Ferreira et al., 2005; Ferreira et al., 2007). Resultados semelhantes foram descritos também *in vitro* (da C. Ferreira et al., 2005).

Por outro lado, vários trabalhos demonstraram a produção de radicais livres e a diminuição das defesas antioxidantes no cérebro de ratos na presença de GA e 3-OHGA. Latini e colaboradores (2002, 2005b) mostraram que o 3-OHGA aumenta a lipoperoxidação, a produção de óxido nítrico e de peróxido de hidrogênio, além de diminuir as defesas antioxidantes e os níveis de glutationa reduzida em córtex cerebral e estriado de ratos. A produção de espécies reativas de oxigênio na presença de 3-OHGA também foi evidenciada em culturas de neurônios de telencéfalos de embriões de pinto (Kölker et al., 2001). Com respeito

ao GA, foi demonstrado um aumento na lipoperoxidação e uma diminuição das defesas antioxidantes, incluindo a atividade da enzima glutationa peroxidase em cérebro de ratos (de Oliveira Marques et al., 2003). Além disso, Latini e colaboradores (2007) mostraram que a administração aguda e crônica de GA aumenta a lipoperoxidação e diminui as defesas antioxidantes em diferentes estruturas cerebrais, fígado e eritrócitos de ratos.

A neurotransmissão GABAérgica também está relacionada com a fisiopatogenia da GA I (Wajner et al., 2004). O primeiro trabalho evidenciando um efeito dos metabólitos acumulados na deficiência da GCDH sobre o metabolismo do ácido γ-aminobutírico (GABA) foi realizado por Stokke, Goodman e Moe (1976). Esse trabalho mostrou uma inibição competitiva da enzima glutamato descarboxilase (enzima que converte o glutamato em GABA) na presença de GA, 3-OHGA e ácido glutacônico. Neste particular, concentrações estriatais (caudato e putamen) reduzidas de GABA foram encontradas em um paciente com GA I (Leibel et al., 1980). Outros estudos mostraram que as convulsões decorrentes da administração intraestriatal de GA (Lima et al., 1998) e 3-OHGA (de Mello et al., 2001) em ratos foram prevenidas por muscimol, um agonista de receptores $GABA_A$. Ullrich e colaboradores (1999) contudo, não encontraram efeitos do 3-OHGA sobre receptores de GABA.

Outros trabalhos sugerem que a disfunção endotelial com perda da integridade da barreira hematoencefálica (Strauss e Morton 2003; Zinnanti et al., 2006a; Mühlhausen et al., 2006) e metabólitos da via das quinureninas, uma das rotas de catabolismo do triptofano, juntamente com outras substâncias

acumuladas na GA I podem estar envolvidos na neurodegeneração dessa doença (Heyes, 1987; Varadkar e Surtees, 2004; Lehnert e Sass, 2005).

Diversos autores relacionam as vacuolizações encontradas em estudos *postmortem* de cérebro de pacientes afetados pela deficiência da GCDH com a toxicidade glutamatérgica (Goodman et al., 1977; Forstner et al., 1999; Hoffmann e Zschocke, 1999). Esta hipótese se constitui no foco deste trabalho e está fortemente baseada na similaridade estrutural existente entre o glutamato e os principais ácidos orgânicos acumulados na GA I (GA e 3-OHGA) (Flott-Rahmel et al., 1997; Lima et al., 1998; Hoffmann e Zschocke, 1999; Wajner et al., 2004; Goodman, 2004) (Figura 2).

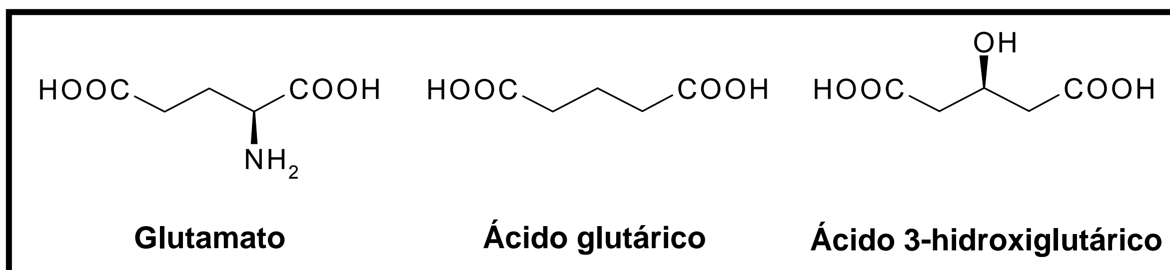

Figura 2. Similaridade estrutural entre glutamato, ácido glutárico e 3-hidroxiglutárico.

Assim, vários trabalhos explicam a neurotoxicidade da GA I pela interação desses ácidos orgânicos com receptores e transportadores glutamatérgicos. Kölker e colaboradores (2000, 2002a) propuseram que o 3-OHGA ativa seletivamente receptores do tipo NMDA compostos pelas subunidades NR1/NR2A e NR1/NR2B em culturas neuronais de telencéfalos de embriões de pinto. Corroborando este achado, Bjugstad e colaboradores (2001) mostraram que culturas de neurônios de cérebro de ratos não se mostram sensíveis a esse ácido

antes da expressão de receptores do tipo NMDA. Em adição, 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-OHGA (Kölker et al., 2000; de Mello et al., 2001). Entretanto, Ullrich e colaboradores (1999) utilizando estudos eletrofisiológicos em diferentes sistemas celulares não encontraram evidências de que o 3-OHGA liga-se diretamente a receptores NMDA, sugerindo que um déficit energético possa explicar de modo indireto a ativação desses receptores. Considerando-se que os receptores NMDA NR1/NR2B são predominantemente expressos em cérebro imaturo (McDonald, Silverstein e Johnston, 1988) e que o dano neuronal muda conforme os diferentes modelos de estudo, sugere-se que exista uma dependência da distribuição regional e do período de desenvolvimento na suscetibilidade dos neurônios à toxicidade do 3-OHGA (Ullrich et al., 1999; Kölker et al., 2004b; Goodman, 2004). Para o GA, foi demonstrada uma inibição da ligação de glutamato a seus transportadores e da captação dessa substância por preparações sinaptossomais de cérebro de ratos (Porciúncula et al., 2000). O GA também interferiu com a captação de glutamato por vesículas e parece interagir com receptores glutamatérgicos do tipo não-NMDA em cérebro de ratos (Porciúncula et al., 2004). Neste contexto, convulsões em ratos provocadas por administração intraestriatal de GA foram prevenidas por DNQX, um conhecido antagonista de receptores 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 com o sistema glutamatérgico, recentes trabalhos não confirmam essa hipótese (Lund et al., 2004; Freudenberg, Lukacs e Ullrich, 2004), fazendo com que esta questão continue sob intenso debate.

Apesar da intensa investigação, as causas da suscetibilidade frontotemporal cortical durante a gestação e da janela de vulnerabilidade estriatal durante os primeiros anos de vida permanecem obscuras, constituindo-se nos principais desafios da pesquisa da patogênese da GA I (Goodman, 2004).

I.1.4. Sistema Glutamatérgico

O glutamato é considerado o maior mediador de sinais excitatórios no sistema nervoso central (SNC) e está envolvido em diversos aspectos do funcionamento normal do cérebro (Fonnum, 1984; Erecinska e Silver, 1990; Ozawa, Kamiya e Tsuzuki, 1998; Danbolt, 2001). A neurotransmissão glutamatérgica desempenha um importante papel na cognição, formação e evocação da memória, aprendizado, manutenção da consciência, movimento, sensações (como dor e sabor), plasticidade celular, diferenciação e morte (Fonnum, 1984; Lipton e Rosenberg, 1994; Ozawa, Kamiya e Tsuzuki, 1998; Daikhin e Yudkoff, 2000; Danbolt, 2001). A ação do glutamato no cérebro de mamíferos é mediada pela ativação de canais iônicos permeáveis ao Ca^{2+} e Na^{+} chamados receptores ionotrópicos e de receptores ligados a proteínas G chamados metabotrópicos que também culminam no aumento dos níveis de Ca^{2+} intracelulares via cascata de segundos mensageiros (Nakanishi, 1992; Hollmann e Heinemann, 1994; Ozawa, Kamiya e Tsuzuki, 1998). Os receptores ionotrópicos podem ser divididos em receptores ligantes de *N*-metil-D-aspartato (NMDA:

NR1/NR2A-D) e não-NMDA que incluem os receptores ligantes dos ácidos α-amino-3-hidróxi-5-metil-4-isoxazol propiônico (AMPA: GluR1-4) e cainato (GluR5-7 and KA1-2). Os receptors metabotrópicos (mGluRs) são divididos nos grupos I, II e III (Conn e Pin, 1997; Ozawa, Kamiya e Tsuzuki, 1998). Esses receptores apresentam inúmeras diferenças nas suas propriedades farmacológicas apresentando um padrão distinto de afinidade por diferentes ligantes, variáveis seletividade iônica e cinética (Lipton e Rosenberg, 1994; Ozawa, Kamiya e Tsuzuki, 1998; Danbolt, 2001).

Após sua liberação pelo neurônio pré-sináptico, o glutamato extracelular é removido majoritariamente por um sistema de transportadores astrocitários de alta afinidade que utiliza o gradiente transmembrana do Na^+ como força motriz (Danbolt, 2001; Schlüter et al., 2002). Até hoje, cinco subtipos de transportadores glutamatérgicos foram descritos: GLAST (EAAT1), GLT1 (EAAT2), EAAC1 (EAAT3), EAAT4 and EAAT5 (Danbolt, 2001). GLAST e GLT1 são predominantemente localizados em astrócitos e considerados os principais responsáveis pela captação de glutamato, enquanto EAAC1, EAAT4 e EAAT5 parecem ser mais abundantes em neurônios (Rothstein et al., 1996; Berger e Hediger, 1998; Kugler e Schmitt, 1999; Danbolt, 2001; Amara e Fontana, 2002).

As concentrações cerebrais de glutamato são altas (5-15 mmol/Kg de peso úmido dependendo da região), porém somente uma pequeníssima fração está presente no espaço extracelular (Danbolt, 2001). Concentrações citoplasmáticas chegam a 10 mM enquanto os níveis extracelulares variam de 2-5 µM podendo atingir até 1 mM após a liberação vesicular na fenda sináptica frente a um estímulo

dependente de Ca^{2+} (Danbolt, 1994; Daikhin e Yudkoff, 2000; Yudkoff et al., 2005). Os níveis extracelulares de glutamato devem permanecer baixos para garantir uma resposta adequada dos receptores às pequenas concentrações liberadas na fenda sináptica, bem como para evitar as ações tóxicas desse neurotransmissor (Danbolt, 1994; Daikhin e Yudkoff, 2000; Danbolt, 2001). A compreensão do ciclo glutamato-glutamina é fundamental para o entendimento do metabolismo cerebral do glutamato (Daikhin e Yudkoff, 2000) (Figura 3).

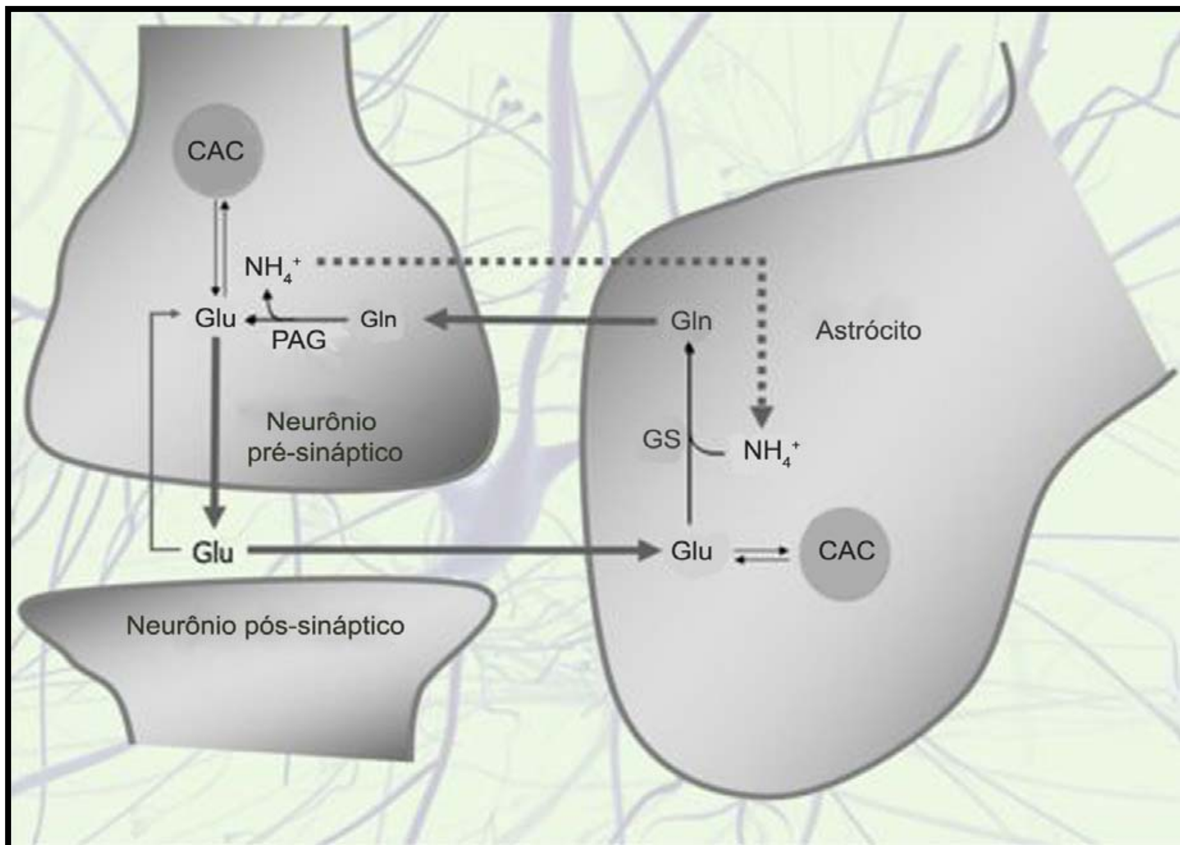

Figura 3. Ciclo glutamato-glutamina. Glu, glutamato; Gln, glutamina; GS, glutamina sintetase; PAG; glutaminase ativada por fosfato; CAC, ciclo do ácido cítrico (Fonte: adaptado da página da internet da Faculdade de Ciências Farmacêuticas da Universidade de Copenhague, Dinamarca).

O ciclo glutamato-glutamina desempenha importantes funções no sistema glutamatérgico: 1) permite a rápida remoção de glutamato do espaço extracelular; 2) efetua a conversão astrocitária de glutamato a glutamina, um aminoácido não-excitatório capaz de "transportar" o glutamato de volta aos neurônios; 3) importante mecanismo para regeneração de glutamato neuronal que pode ser utilizado como neurotransmissor (captação vesicular por uma bomba de prótons dependente de ATP) ou como substrato energético; e 4) mecanismo de tamponamento de NH_3 (Danbolt, 1994; Daikhin e Yudkoff, 2000).

I.1.4.1. Ontogenia dos Receptores Glutamatérgicos Ionotrópicos no Sistema Nervoso Central de Ratos

Os tipos e subunidades de receptores de glutamato são diferentemente distribuídos ao longo do desenvolvimento e nas diferentes regiões do SNC (McDonald e Johnston, 1990). No córtex cerebral de ratos, a expressão e a maturação dos receptores NMDA ocorre no início da vida pós-natal (Insel, Miller e Gelhard, 1990; Nansen et al., 2000). As diferentes associações NR1/NR2A e NR1/NR2B já estão presentes ao nascimento, atingindo um pico de expressão após os 21 dias de vida (Wenzel et al., 1997). Portera-Cailliau, Price e Martin (1996) encontraram resultados similares também em cérebro de ratos, porém não encontraram a expressão de receptores contendo a subunidade NR2A durante a primeira semana pós-natal. A expressão de receptores contendo a subunidade NR2B parece estar mais restrita ao cérebro imaturo, enquanto que a subunidade NR2A se apresenta como preponderante no cérebro adulto (McDonald, Silverstein e Johnston, 1988; Wenzel et al., 1997). Por sua vez, receptores não-NMDA

aparecem mais tardiamente no córtex cerebral, atingindo um pico de expressão entre a segunda e terceira semana de vida (Insel, Miller e Gelhard, 1990; Nansen et al., 2000). O estriado apresenta um padrão oposto ao córtex na expressão dos receptores ionotrópicos (Colwell et al., 1998; Nansen et al., 2000). Receptores AMPA e cainato parecem mediar as respostas sinápticas glutamatérgicas no início da vida pós-natal (Hurst, Villablanca e Levine, 1998). A maturação de receptores NMDA no estriado é mais tardia, especificamente da subunidade NR2A que apresenta um pico de densidade somente a partir da terceira semana de vida pós-natal em ratos (Portera-Cailliau, Price e Martin, 1996).

I.1.4.2. Ontogenia dos Transportadores de Glutamato no Sistema Nervoso Central de Ratos

A expressão dos diferentes tipos de transportadores de glutamato também varia ao longo do desenvolvimento e nas diferentes estruturas cerebrais (Ullensvang et al., 1997; Furuta, Rothstein e Martin, 1997; Schlüter et al., 2002). Em ratos, GLAST é o principal transportador glutamatérgico ao nascimento, enquanto GLT1 aumenta sua expressão a partir da segunda semana de vida (Ullensvang et al., 1997). GLT1 é o transportador predominante no cérebro adulto enquanto GLAST desempenha um importante papel no transporte de glutamato especificamente no cerebelo (Ullensvang et al., 1997; Danbolt, 2001). No período embrionário e durante o período inicial da vida pós-natal o transporte neuronal, principalmente via EAAC1, pode predominar em relação ao transporte astroglial. No estriado imaturo, a expressão de EAAC1 é superior a todos os transportadores

gliais podendo ser considerada a principal forma de transporte de glutamato (Furuta, Rothstein e Martin, 1997).

I.1.4.3. Excitotoxicidade Glutamatérgica

Lucas e Newhouse (1957) foram os primeiros pesquisadores a descrever as potencialidades do glutamato como neurotoxina. Cerca de uma década depois, Olney (1969) utilizou o termo excitotoxicidade para definir o processo de estimulação excessiva de receptores glutamatérgicos e conseqüente dano celular (Danbolt, 2001). A estimulação exacerbada de receptores glutamatérgicos implica em quantidades excessivas de Ca^{2+} no meio citoplasmático e ativação descontrolada de enzimas envolvidas no desenvolvimento e função neuronal normais (Lipton e Rosenberg, 1994; Sattler e Tymianski, 2000). Além da ativação de lipases, proteases, fosfatases e outras enzimas, o influxo excessivo de Ca^{2+} e Na^{+} gera um inchamento celular que, para ser freado, consome altas quantidades de energia levando a um colapso energético. O déficit energético pode comprometer a captação de glutamato pelos astrócitos potencializando o efeito excitotóxico (Lipton e Rosenberg, 1994; Danbolt, 2001). A produção de espécies reativas de oxigênio e nitrogênio também está ligada com a ativação de receptores glutamatérgicos e pode culminar no aumento das concentrações extracelulares de glutamato por inibir a função dos transportadores gliais de glutamato (Volterra et al., 1994; Trotti et al., 1996; Trotti, Danbolt e Volterra, 1998; Danbolt, 2001). A direta estimulação de receptores glutamatérgicos bem como processos que prejudicam o sistema de transporte desse aminoácido excitatório estão relacionados a diversas neuropatologias que afetam o SNC adulto e imaturo

(Lipton e Rosenberg, 1994; Furuta, Rothstein e Martin, 1997; Maragakis e Rothstein, 2001; Danbolt, 2001). Neste contexto, uma variável expressão de diferentes tipos de receptores e transportadores, com distintas propriedades, ao longo do desenvolvimento e em regiões específicas pode conferir uma janela de suscetibilidade a muitas neurotoxinas e doenças (Ullensvang et al., 1997; Danbolt, 2001; Haberny et al., 2002; Jensen, 2002).

I.2 OBJETIVOS

I.2.1. Objetivo Geral

A acidemia glutárica tipo I (GA I) apresenta um padrão de neurodegeneração dependente do período de desenvolvimento do SNC. As duas principais estruturas cerebrais afetadas na doença (córtex cerebral e estriado) sofrem danos severos em períodos bem determinados, com o córtex cerebral sendo afetado ainda na vida intra-uterina e ao nascimento e o estriado durante os primeiros 36 meses de idade. Considerando os achados fisiopatológicos responsáveis pela neurodegeneração característica dos pacientes com GA I e a expressão diferenciada de receptores e transportadores de glutamato nas distintas estruturas ao longo do desenvolvimento do SNC, o objetivo geral deste trabalho foi investigar o efeito dos principais metabólitos acumulados na GA I sobre a ligação de glutamato a seus receptores e transportadores de membrana e sobre a captação de glutamato em córtex cerebral e estriado de ratos ao longo do desenvolvimento visando uma melhor compreensão dos mecanismos neurotóxicos desses ácidos orgânicos.

I.2.2. Objetivos Específicos

- Investigar a interação do ácido 3-hidroxiglutárico (3-OHGA) com receptores do tipo NMDA em córtex cerebral de ratos de 30 dias de vida.
- Investigar o efeito *in vitro* dos ácidos glutárico (GA) e 3-hidroxiglutárico (3-OHGA) sobre a ligação de L-[^{3}H]glutamato a seus receptores e

transportadores de membranas plasmáticas sinápticas de córtex cerebral e estriado de ratos de 7, 15 e 60 dias de vida.

- Investigar o efeito *in vitro* dos ácidos GA e 3-OHGA sobre a captação de L-[^{3}H]glutamato por fatias de córtex cerebral e estriado de ratos de 7, 15 e 30 dias de vida.

- Investigar o efeito dos ácidos GA e 3-OHGA sobre a captação de L-[^{3}H]glutamato por preparações sinaptossomais de córtex cerebral e estriado de ratos de 7, 15 e 30 dias de vida.

- Investigar os mecanismos das alterações provocadas pelos ácidos GA e 3-OHGA sobre os parâmetros avaliados do sistema glutamatérgico.

PARTE II

Artigos Científicos

Capítulo I

Evidence that 3-hydroxyglutaric acid interacts with NMDA receptors in synaptic plasma membranes from cerebral cortex of young rats

Rafael B. Rosa, Carolina Schwarzbold, Karina B. Dalcin, Gabrielle C. Ghisleni, César A. J. Ribeiro, Maria B. Moretto, Marcos E. S. Frizzo, Georg F. Hoffmann, Diogo O. Souza, Moacir Wajner

Neurochemistry International 45: 1087-1094, 2004

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Neurochemistry International 45 (2004) 1087–1094
NEUROCHEMISTRY International
www.elsevier.com/locate/neuint

Evidence that 3-hydroxyglutaric acid interacts with NMDA receptors in synaptic plasma membranes from cerebral cortex of young rats

Rafael B. Rosa [a], Carolina Schwarzbold [a], Karina B. Dalcin [a], Gabrielle C. Ghisleni [a], César A.J. Ribeiro [a], Maria B. Moretto, Marcos E.S. Frizzo [a,b], Georg F. Hoffmann [c], Diogo O. Souza [a], Moacir Wajner [a,d,*]

[a] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS, Rua Ramiro Barcellos, 2600 Anexo, CEP 90035-003, Porto Alegre, RS, Brazil*
[b] *Departamento de Ciências Morfológicas, Instituto de Ciências Básicas da Saúde, UFRGS, Porto Alegre, RS, Brazil*
[c] *University Children's Hospital, Division of Metabolic and Endocrine Diseases, Im Neuenheimer Feld 150, D-69120 Heidelberg, Germany*
[d] *Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, RS, Brazil*

Received 29 November 2003; received in revised form 3 May 2004; accepted 10 May 2004

Available online 1 July 2004

Abstract

Neurological symptoms are common in patients with glutaric acidemia type I (GA-I). Although the pathophysiology of this disorder is not yet fully established, 3-hydroxyglutaric acid (3-HGA), which accumulates in affected patients, has recently been demonstrated to be excitotoxic to embryonic chick and neonatal rat neurons probably via NMDA glutamate receptors. In the present study, we investigated the in vitro effects of 3-HGA on the [^{3}H]glutamate and [^{3}H]MK-801 (dizocilpine) binding to rat synaptic plasma membranes from cerebral cortex of young rats in order to elucidate the interactions of 3-HGA with glutamate receptors and its possible contribution to the in vitro excitotoxic properties of 3-HGA. 3-HGA (10–100 μM) significantly decreased Na^+-dependent (up to 62%) and Na^+-independent (up to 30%) [^{3}H]glutamate binding to synaptic membranes, reflecting a possible competition between glutamate and 3-HGA for the glutamate transporter and receptor sites, respectively. Since a decrease in Na^+-independent glutamate binding might represent an interaction of 3-HGA with glutamate receptors, we next investigated whether 3-HGA interacts with NMDA receptors by adding NMDA alone or combined with 3-HGA and measuring Na^+-independent [^{3}H]glutamate binding to synaptic membranes (binding to receptors). We verified that 3-HGA and NMDA, at 10 and 100 μM concentrations, decreased glutamate binding by up to 20 and 45%, respectively, and that the simultaneous addition of both substances did not provoke an additive effect, implying that they bind to NMDA receptors at the same site. Furthermore, the binding of the NMDA-channel blocker [^{3}H]MK-801 was significantly increased (approximately 32–40%) by 10 and 100 μM 3-HGA, implying that 3-HGA was able to open the NMDA channel allowing MK-801 binding, which is a characteristic of NMDA agonists. On the other hand, glutamate had a much higher stimulatory effect on this binding (180% increase), reflecting its strong NMDA agonist property. Furthermore, the simultaneous addition of 3-HGA and glutamate provoked an additive stimulatory effect on [^{3}H]MK-801 binding to the NMDA receptor. These data indicate that, relatively to glutamate, 3-HGA is a weak agonist of NMDA receptors. Finally, we demonstrated that 3-HGA provoked a significant increase of extracellular calcium uptake by cerebral cortex slices, strengthening therefore, the view that 3-HGA activates NMDA receptors. The present study therefore, demonstrates at the molecular level that 3-HGA modulates glutamatergic neurotransmission and may explain previous findings relating the neurotoxic actions of this organic acid with excitotoxicity.
© 2004 Elsevier Ltd. All rights reserved.

Keywords: 3-Hydroxyglutaric acid; Glutaric acidemia type I; Glutaryl-CoA dehydrogenase; Excitotoxicity; Glutamate; NMDA receptors

1. Introduction

Glutaryl-CoA dehydrogenase deficiency (GDD, OMIM # 231670), also known as glutaric acidemia type I (GA I), is an autosomal recessive inherited neurometabolic disorder biochemically characterized by the accumulation of glutaric (GA), 3-hydroxyglutaric (3-HGA) and to a lesser extend of glutaconic acids in tissues and body fluids of affected patients due to a blockage in the L-lysine, hydroxy-L-lysine and tryptophan catabolic pathway (Goodman and Frerman, 2001). GA I is included in a subgroup of organic acidurias called "cerebral" since neurological manifestations without clear metabolic derangement are the predominant symptoms

* Corresponding author. Tel.: +55 51 33165571; fax: +55 51 33168010.
E-mail address: mwajner@ufrgs.br (M. Wajner).

0197-0186/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2004.05.001

(Hoffmann et al., 1994; Hoffmann and Zschocke, 1999). Clinically, the disease is characterized by macrocephaly, progressive dystonia and dyskinesia, which usually is apparent within the first 3 years of life. Symptoms may have a gradual rate of onset and progression, or occur suddenly after an acute metabolic crisis. During these crises the accumulating metabolites can reach millimolar concentrations (Goodman et al., 1977; Hoffmann and Zschocke, 1999). Thereafter, degeneration of the caudate and putamen of the basal ganglia and fronto-temporal atrophy are commonly demonstrable in these patients by radiographic imaging studies of the brain (Chow et al., 1988; Brismar and Ozand, 1995; Forstner et al., 1999). Frontotemporal atrophy can be identified at birth, indicating that the impaired development of the central nervous system already begins in uterus and that the accumulating metabolites act very early during development (Forstner et al., 1999). Pathologically, the most characteristic feature of GA I is a loss of neurons in the caudate and putamen, but severe spongiform change in the white matter is also usually observed (Chow et al., 1988; Soffer et al., 1992; Brismar and Ozand, 1995).

At present, the exact pathomechanisms of the neurological sequelae of the affected patients are still in debate. Evidence has emerged from several recent studies showing toxicity of GA and/or 3-HGA towards rat corticostriatal and chick telencephalic neuronal cultures (Flott-Rahmel et al., 1997; Kölker et al., 1999, 2000a,b; Ullrich et al., 1999). Excitotoxicity attributed mainly to 3-HGA has been considered as a potential explanation for the neurological dysfunction of GA I (Flott-Rahmel et al., 1997; Kölker et al, 1999, 2000a,c, 2002a, b; Ullrich et al., 1999; Mello et al., 2001). In this context, substantial evidence relates the specific time course and localization of the neurological disease in GA I with a selective stimulation of the NR2B subtype of NMDA receptors by 3-HGA (Kölker et al, 1999, 2000a,c). Post mortem examination of basal ganglia and cortex of GDD patients has revealed postsynaptic vacuolization similar to that of glutamate-mediated damage (Forstner et al., 1999).

It has been also hypothesized that the maturation-dependent and regional vulnerability in this disease is determined by a variety of factors, e.g. expression of NMDA receptors (in particular of the NR1/NR2B subtype), high excitotoxic susceptibility of striatal medium spiny neurons, the high energy demand of the basal ganglia, and the glutamatergic input via the corticostriatal pathway.

Glutamate receptors mediate most of the excitatory neurotransmission in mammalian central nervous system, participating in cerebral plasticity, memory and learning, and in the formation of neural networks during development (Ozawa et al., 1998). The appropriate activation of these receptors is essential for normal brain development and function, whereas their overstimulation potentially leads to excitotoxicity, as found in neurodegenerative disorders and brain injuries (Olney, 1980; Meldrum and Garthwaite, 1990). Glutamatergic neurotransmission is mediated through the activation of ionotropic (ligand-gated ion channels) or metabotropic (G-protein coupled receptors) receptors. The most studied ionotropic receptor is the NMDA-subtype, which is a receptor–ionophore complex containing separated binding sites for the endogenous agonists glutamate (NMDA site) and glycine (strychnine-insensitive site), both of which must be occupied for receptor activation. The ion channel is permeable to Ca^{2+}, Na^{+} and K^{+} and contains binding sites for Mg^{2+} and MK-801 (dizocilpine), which induce channel blockage (Ozawa et al., 1998).

An overstimulation of glutamatergic system may be triggered by a direct stimulation of glutamate receptors and/or by an impairment of the glutamate uptake system leading to an increase of the glutamate levels in synaptic cleft. Despite the behavioral evidence for an interaction of 3-HGA with the glutamatergic system (Mello et al., 2001), few studies have been conducted in vitro to determine the molecular mechanisms that could be involved in such interaction. The only available study indicated that 3-HGA stimulates Na^{+}-dependent glutamate uptake into astrocytes, without interfering with basal and potassium-induced release of $[^3H]$glutamate by synaptosomes, Na^{+}-dependent synaptosomal glutamate uptake and Na^{+}-independent glutamate uptake by synaptic vesicles from 30-day-old Wistar rats (Frizzo et al., 2004). However, there is no data on the possible interaction of 3-HGA with glutamatergic receptors, which seems to be of relevance to the excitotoxicity. Therefore, in the present study, we have investigated the effects of 3-HGA, considered the main neurotoxin in GA-I, on $[^3H]$glutamate and $[^3H]$MK-801 binding in synaptic membranes from cerebral cortex of young rats in order to elucidate the interaction of 3-HGA with the NMDA receptors. We also evaluated whether 3-HGA could affect calcium uptake by rat cerebral cortex slices.

2. Experimental procedures

2.1. Materials

$[^3H]$glutamate (48 Ci/mmol) and $^{45}Ca^{2+}$ (50 mCi/mg) were purchased from Amersham International, UK and $[^3H]$MK-801 (24.2 Ci/mmol) was from Perkin-Elmer Life Sciences, Ltd, USA. All other chemicals were of analytical reagent grade and were obtained from Sigma Chemical Co. (St Louis, Mo, USA).

2.2. Animals

Thirty-day-old Wistar rats were used in the experiments. They were maintained at approximately 25 °C, on a 12 h light/12 h dark cycle with free access to food and water. Animals were killed by decapitation without anesthesia, the brain was rapidly removed and the cerebral cortices were dissected. The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre and followed the

"Principles of Laboratory Animal Care" (NIH publication 85–23, revised 1985).

2.3. Membrane preparation

Synaptic membranes were isolated from the cerebral cortex of rats, as described by Jones and Matus (1974), and stored frozen at −70 °C for no more than 2 months. On the day of assay, membranes were thawed at 37 °C, for 30 min, and washed three times in hypotonic solution consisting of 5 mM Tris–HCl, pH 7.4, by centrifugation at 45,000 × *g* at 4 °C for 15 min. We have previously observed that this procedure avoids membrane sealing, in agreement to the literature (Danbolt, 1994).

2.4. [^{3}H]glutamate binding

[^{3}H]Glutamate binding experiments were carried out in the presence of high sodium concentrations, which favor glutamate binding to transporters, and in the absence of sodium, which reflects glutamate binding to receptors (Emanuelli et al., 1998).

Experiments for sodium-dependent glutamate binding were carried out in polycarbonated tubes containing 50 mM Tris–acetate buffer, pH 7.4, 100 mM NaCl, 100 nM [^{3}H]glutamate, and 3-HGA (10–100 μM). Controls did not contain 3-HGA. Incubation was started by adding 100–200 μg of protein membrane, and run at 30 °C for 30 min. The reaction was stopped by centrifugation at 16,800 × *g* for 15 min at 4 °C. The pellet was carefully washed with ice-cold distilled water and solubilised with 0.1% sodium dodecyl sulfate (w/v) overnight. Radioactivity was determined with a Wallac scintillation spectrometer. In order to determine specific binding of [^{3}H]glutamate, each experiment was processed with parallel control tubes containing [^{3}H]glutamate in the presence of 1000 times of non-radioactive glutamate. Specific binding was considered as the difference of [^{3}H]glutamate binding between tubes without (total binding) and with (non-specific binding) unlabeled glutamate in excess concentration. Non-specific binding was about 10–20% of the total binding. The sodium-independent glutamate binding experiments were carried out under identical conditions as those performed for sodium-dependent binding, except that sodium chloride was not present in the medium.

In order to determine the effects of the NMDA ligand, which recognize the specific glutamate receptors NMDA, membranes were incubated with 40 μM NMDA. With this approach [^{3}H]glutamate does not bind to NMDA receptors already occupied by the excess of the non-labeled specific ligand NMDA. The difference between the remaining binding (with excess of non-labeled NMDA) and the total [^{3}H]glutamate binding (in the absence of NMDA) was taken as a measure of binding to the NMDA receptors. The effect of 3-HGA on NMDA receptor subtype binding was evaluated by incubating [^{3}H]glutamate along 3-HGA and/or NMDA.

2.5. [^{3}H]MK-801 binding

Incubations were started by the addition of membrane preparation (80–200 μg of protein) to a medium containing 5 mM Tris–HCl, pH 7.4, 4 nM [^{3}H]MK-801, 1 μM glutamate and 3-HGA 10 and 100 μM. Following 60 min of incubation at 37 °C, bound and free [^{3}H]MK-801 were separated by filtration through GF/B Whatman filters presoaked in 0.03% polyethylenimique. Radioactivity was determined using a Wallac 1409 liquid scintillation counter. Specific binding was calculated as the difference between total binding and non-specific binding, which was measured in the presence of 4 μM MK-801. Non-specific binding typically amounted to 20–30% of the total binding. 3-HGA solutions used in [^{3}H]MK-801 binding assays had their pH adjusted to 7.4 with Tris–HCl buffer and were prepared on the day of the experiment.

2.6. $^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ uptake was carried out essentially as described by Eason and Aronstam (1984), with some modifications. Two salt solutions were used in these studies: (1) Krebs buffer containing 127 mM NaCl, 1.2 mM Na_2HPO_4, 5.36 mM KCl, 0,44 mM KH_2PO_4, 0.95 mM $MgCl_2$, 0.70 mM $CaCl_2$, 10 mM glucose, and 0.50 mM Hepes, pH 7.4; (2) Lanthanium solution containing 127 mM NaCl, 5.0 mM KCl, 0.95 mM $MgCl_2$, 10 mM $La(NO_3)$, 10 mM glucose, and 0.60 mM Hepes, pH 7.4. To measure $^{45}Ca^{2+}$ uptake, rats were killed by decapitation, the cerebral cortex was dissected, isolated and the parietal cortex was cut into 400 μm slices, which were washed with Krebs buffer (solution 1). The slices (0.8–1.3 mg protein) were preincubated in 24 well-polycarbonate plates for 22 min at 32 °C in the absence (control group) or presence of 100 μM 3-HGA. 0.68 μCi $^{45}Ca^{2+}$ was added to the incubation medium and the uptake was carried out for 8 min at 32 °C, after which the reaction was stopped by two washes with 1 mL iced-cold lanthanium solution (solution 2). Immediately after washing, 0.3 mL of 0.5 M NaOH was added to the wells and the slices were digested overnight. Aliquots of the lysates were taken for protein content measurement by the Lowry et al.'s (1951) method and for determination of the intracellular amount of calcium by liquid scintillation counting. Nonspecific calcium uptake (20–30% of the total uptake) was determined by carrying out the same experiment using solution 2, which contains the nonspecific voltage-dependent calcium blocker lanthanium. Results were expressed as nmol $^{45}Ca^{2+}$/mg protein. Specific uptake was considered as the difference between total uptake and nonspecific uptake.

2.7. Protein measurement

The protein content of the synaptic membrane preparations and of cortical slices was determined by the method

of Lowry et al. (1951), using bovine serum albumin as standard.

2.8. Statistical analysis

All experiments were performed at least in triplicate. Statistical significance of three or more groups was assessed by one-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test. The Student's t test was also used when indicated. A value of $P < 0.05$ was considered to be significant. Data were expressed as mean ± S.E.M. All analyses were carried out in an IBM-compatible PC using the statistical package for the social sciences (SPSS) software.

3. Results

In order to search for a direct interaction of 3-HGA with glutamate transporters and receptors we investigated the effects of 3-HGA (10–100 μM) on Na^+-dependent and Na^+-independent [^{3}H]glutamate binding, respectively, to cerebral cortical synaptic membranes. In the presence of high sodium (100 mM), 3-HGA (10–100 μM) inhibited [^{3}H]glutamate binding by maximally 62% [$F(2,15) = 9.353$, $P < 0.01$] (Fig. 1A). In parallel, 3-HGA induced a significant decrease of [^{3}H]glutamate binding to synaptic plasma membranes at 10–100 μM by up to 30% in sodium-free media [$F(2,6) = 10.26$, $P < 0.01$] (Fig. 1B).

As regards to the possibility that 3-HGA may induce release of glutamate from these membrane preparations that may reseal and contain high levels of glutamate and interfere with our data, this is unlikely because the methodology we used in our experiments avoids at maximum the sealing of our membrane preparations. We used through the preparation and incubation for binding studies hypotonic solutions with low sodium concentrations, a condition that avoids the sealing of brain membrane fragments (Danbolt, 1994).

It is well established that NMDA receptors play a crucial role in the convulsions elicited by glutamate or other NMDA agonists (Chapman, 1988, Rothman and Olney, 1987). On the other hand, it has been suggested that NMDA receptors are involved in the neurotoxicity caused by 3-HGA (Flott-Rahmel et al., 1997; Kölker et al., 1999, 2000a,c, 2002a,b; Ullrich et al., 1999; Mello et al., 2001). Therefore, in order to test for a possible interaction between 3-HGA and these receptors, we studied Na^+-independent [^{3}H]glutamate binding (receptors), in the presence of the specific ligand NMDA (10–100 μM). We found that, despite the fact that NMDA (around 45%) was more efficient in displacing glutamate than 3-HGA (around 20%) at 10 μM concentration, when 10 μM of both compounds were co-incubated, the percentage of glutamate binding remained practically the same and there was no sum of the individual effects (42% binding decrease), indicating that the displacement of glutamate binding provoked by both drugs occurred probably through the same site at the NMDA receptor [$F(3,20) = 20.37$, $P < 0.0001$] (Fig. 2). The use of 100 μM 3-HGA and 100 μM NMDA alone or combined in the incubation medium gave rise to similar results.

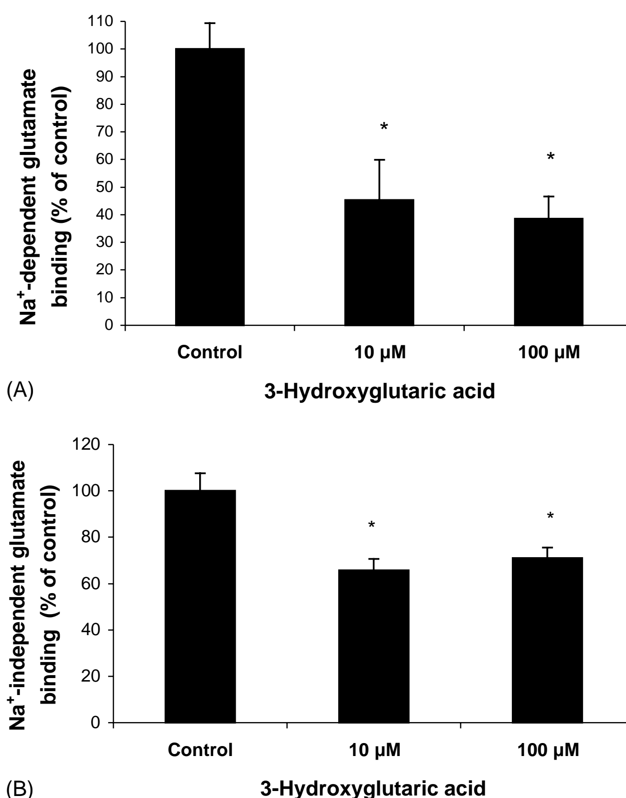

Fig. 1. Effect of 3-hydroxyglutaric acid (3-HGA, 10–100 μM) on [^{3}H]glutamate binding to synaptic plasma membranes from rat cerebral cortex in the presence (A) or absence (B) of sodium. Results are presented as mean ± S.E.M. and represent three to six separate experiments from different synaptic membrane preparations (animals) performed in triplicate and are expressed as percentage of control (Na^+-dependent binding: 17.3 pmol/mg protein; Na^+-independent binding: 4.06 pmol/mg protein). $^*P < 0.05$, $^{**}P < 0.01$ vs control, one-way analysis of variance (ANOVA) with post hoc Duncan's multiple range test.

We also investigated the effect of 3-HGA (10 and 100 μM) on the binding of the NMDA channel blocker [^{3}H]MK-801 to rat cortical synaptic membranes (Fig. 3). The concentrations of glutamate included in MK-801 binding assay were sub-maximal (Euler and Liu, 1993) in order to look for a possible agonist effect of 3-HGA on NMDA receptors. 3-HGA (10 μM and 100 μM) significantly stimulated [^{3}H]MK-801 binding by approximately 32–40%, whereas glutamate (1 μM) had a similar but a much stronger effect on this binding (180% increase). Furthermore, when 3-HGA and glutamate were co-incubated, an additive stimulatory effect on [^{3}H]MK-801 binding (230–270% increase) could be observed [$F(5,35) = 40.76$, $P < 0.0001$]. These results are strongly indicative that 3-HGA is an agonist to NMDA receptors.

Next, we performed a set of experiments testing whether 3-HGA could alter calcium uptake by cortical slices. 3-HGA

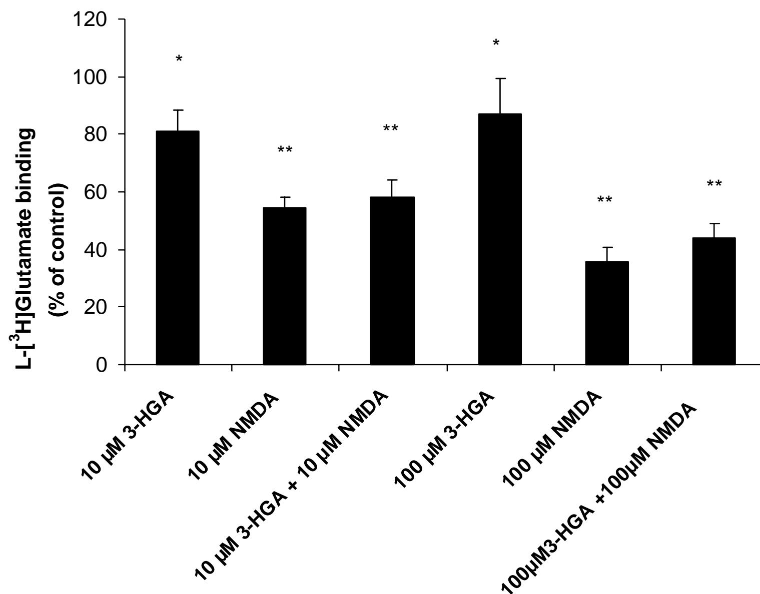

Fig. 2. Effect of 3-hydroxyglutaric acid (3-HGA, 10–100 μM) and NMDA (10–100 μM) on Na^+-independent [^{3}H]glutamate binding to synaptic plasma membranes from rat cerebral cortex. Results are presented as mean ± S.E.M. and represent five to six separate experiments from different synaptic membrane preparations (animals) performed in triplicate and are expressed as percentage of control (Na^+-independent binding: 4.86 pmol/mg protein). $*P < 0.05$, $**P < 0.01$ vs control, one-way analysis of variance (ANOVA) with post hoc Duncan's multiple range test.

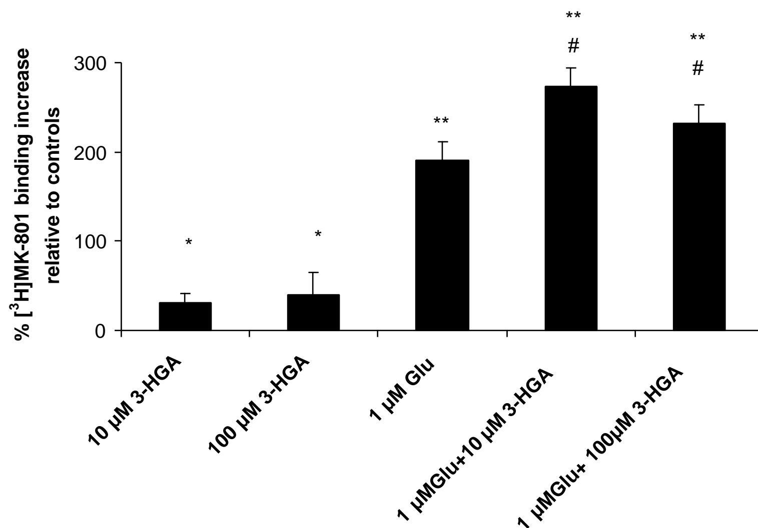

Fig. 3. Effect of 3-hydroxyglutaric acid (3-HGA) on Na^+-independent [^{3}H]MK-801 binding to rat cerebral cortical membranes. Rat cerebral cortical membranes were incubated with 4 nM [^{3}H]MK-801 in the presence of 3-HGA (10–100 μM) and glutamate (Glu, 1 μM). Data are mean ± S.E.M. from six independent experiments. $*P < 0.05$, $**P < 0.01$ vs control, # $P < 0.01$ vs 1 μM Glu, one-way analysis of variance (ANOVA) with post hoc Duncan's multiple range test.

(100 μM) significantly increased extracellular calcium influx into these slices [$t(5) = 3.791$, $P < 0.05$] (Fig. 4).

4. Discussion

In the present study, we have initially shown that 3-HGA at low concentrations (10–100 μM), similar to those described in brain of a glutaric acidemic patient (Kölker et al., 2003) significantly reduced Na^+-independent and Na^+-dependent [^{3}H]glutamate binding to synaptic plasma membranes from cerebral cortex of young rats by approximately 62 and 30%, respectively. It is feasible that the displacement of glutamate from its transporters (Na^+-dependent binding) and receptors (Na^+-independent binding) caused by 3-HGA may be due to the structural similarity between glutamate and 3-HGA, possibly reflecting a competition between glutamate and 3-HGA at the same protein sites.

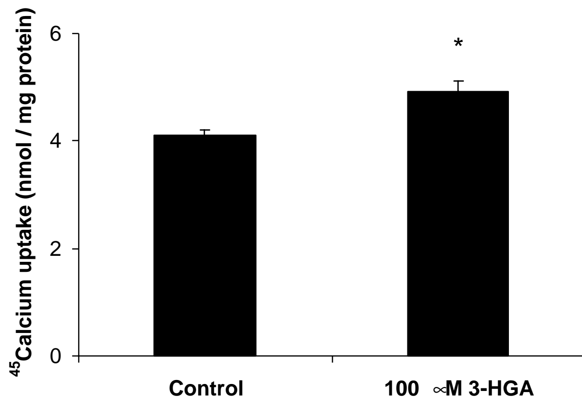

Fig. 4. Effect of 3-hydroxyglutaric acid (3-HGA) on calcium uptake into rat cerebral cortex slices. Rat cerebral cortical slices (0.8–1.3 mg protein) were incubated with 0.7 mM $^{45}Ca^{2+}$ (0.68 μCi) in the presence of 3-HGA (100 μM). Data are mean ± S.E.M. from six independent experiments. $^{*}P < 0.05$ compared to control (Student's t test for paired samples).

Since there is a great deal of evidence relating 3-HGA and excitotoxicity, particularly through NMDA glutamate receptors (Kölker et al., 1999, 2000a,b, 2001a, 2002a,b), we decided to investigate whether 3-HGA could behave as a ligand and an agonist to NMDA receptors. We first verified that 3-HGA and NMDA, at 10 μM and 100 μM concentrations, significantly inhibited [^{3}H]glutamate binding to synaptic plasma membranes, and that co-incubation of both substances at the same concentrations did not increase [^{3}H]glutamate displacement from its receptors. These results provide evidence that 3-HGA interacts with NMDA receptors probably at the same site of NMDA. Thus, it is suggested that 3-HGA inhibits glutamate binding by selectively affecting the NMDA subtype of glutamate receptors.

We have also shown here that 3-HGA stimulated [^{3}H]MK-801 binding to rat cortical membranes. The access of MK-801 into its site within the NMDA ion channel is spatially restricted unless the NMDA receptor is activated by an agonist (Euler and Liu, 1993). Thus, [^{3}H]MK-801 binding is generally used as an indicator of NMDA channel opening. Therefore, our results using sub-maximal concentrations of glutamate indicate that 3-HGA has a direct action on a regulatory site of NMDA receptors, since it increased [^{3}H]MK-801 binding.

We also demonstrated that 3-HGA significantly increased calcium influx into cortical slices, a property of NMDA glutamate agonists (Ozawa et al., 1998; Meldrum, 2002), strengthening therefore, the view that 3-HGA activates these receptors. These results are in agreement with those demonstrating that 3-HGA provoked an increase of calcium influx in cultured neurons from chick embryo telencephalons via NMDA receptors and that there was an inverse correlation between the intracellular concentration of calcium and neuronal viability, attesting therefore, the 3-HGA-induced cytotoxicity (Kölker et al., 2002a). However, our data was obtained with cortical slices, which contain all neural cells and is thought to be a more physiological system. The same group of investigators also showed by electrophysiological studies that 3-HGA was able to activate currents in the NR1/NR2A, but not in the NR1/NR2B NMDA glutamate channels in HEK293 cells expressing the NMDA subtype receptors NR1/NR2A and NR1/NR2B. Furthermore, they observed that 3-HGA-activated currents were abolished by the competitive NMDA receptor antagonist D-AP5 (Kölker et al., 2002a).

To our mind the present report is the first to demonstrate a direct interaction of 3-HGA with NMDA receptors at the molecular level and, besides, that 3-HGA is able to open the NMDA channel/receptor, providing therefore, unequivocal evidence of an agonistic action for this organic acid (Planells-Cases et al., 2002; Bolshakov et al., 2003). Previous publications suggested that 3-HGA was excitotoxic via NMDA receptors by indirect means, i.e., mainly by the use of the non-competitive and competitive NMDA antagonists MK-801 and D-AP5, respectively, and also by using specific NR2B antagonists and antibodies that blocked the neurotoxicity induced by 3-HGA towards neuronal cells in culture (Flott-Rahmel et al., 1997; Ullrich et al., 1999; Kölker et al. 1999, 2000a,b,c, 2002a). It should be however, emphasized that the NMDA antagonists are also able to block secondary excitotoxicity that is mediated via NMDA receptors due to a compromised brain energy production (secondary excitotoxicity). Furthermore, it has been previously shown that 3-HGA impairs energy production in neural cells in culture, as identified through reduction of phosphocreatine levels and inhibition of the respiratory chain (Ullrich et al., 1999) and impaired energy metabolism has been suggested as a relevant factor to explain the striatal damage of glutaric acidemic patients (Strauss and Morton, 2003).

Therefore, our present findings strongly suggest that 3-HGA causes primary excitotoxicity via direct stimulation of NMDA receptors, whereas previous findings could be due to primary or secondary excitotoxicity. The present study therefore, demonstrates at the molecular level that 3-HGA modulates glutamatergic neurotransmission and may explain previous findings relating the neurotoxic actions of this organic acid with excitotoxicity.

On the other hand, although the concentrations of 3-HGA found in the brain of GAI patients are not yet absolutely established, it appears that 3-HGA brain levels (within the micromollar range) are similar to those that produced the significant effects of 3-HGA on the glutamatergic parameters evaluated in the present study (Kölker et al., 2003). Therefore, it is conceivable that our findings may be of pathophysiological significance and related to the human condition.

In conclusion, the modulation of glutamatergic neurotransmission by 3-HGA, as shown here, may explain, at least in part, the neurotoxicity attributed to this neurotoxin and more particularly previous in vitro and in vivo results indicating that 3-HGA is excitotoxic via NMDA receptors. We have however, to emphasize that, although 3-HGA is a neurotoxin, its neurotoxic properties are quite weak compared to other compounds inducing primary or secondary

excitotoxic cell damage, such as glutamate, NMDA, malonate, or 3-nitropropionate. Therefore, it has been suggested that 3-HGA can only exert relevant neurotoxicity under pathophysiological conditions if additional trigger factors are present. It has been shown that hyperthermia as well as cytokine-induced stimulation of astrocytic iNOS expression strongly enhanced 3-HGA neuronal cell damage (Kölker et al. 2000c, 2001b). This is in agreement with our findings demonstrating that the increase of [^{3}H]MK-801 binding provoked by 3-HGA (approximately 30%) was much lower than that caused by glutamate itself (180%), reflecting therefore, the weaker agonistic effect of 3-HGA. The weak agonistic effect of 3-HGA was also suggested by electrophysiological studies performed in HEK293 cultured cells, in which this organic acid induced weak currents as compared to glutamate (Kölker et al., 2002a).

It has been also shown that 3-HGA-induced neurodegeneration also involves generation of ROS, as well as inhibition of the respiratory chain (complexes II and V) and creatine phosphate depletion (Kölker et al., 1999, 2000b, 2001a, 2002a,b; Ullrich et al., 1999; Mello et al., 2001; Latini et al., 2002). However, recent results have not confirmed 3-HGA-induced inhibition of respiratory chain complexes I–V in submitochondrial particles from bovine heart (Kölker et al., 2002a). Most likely, excitotoxicity and the above mentioned single mechanisms cooperate in a synergistic way to result in the dramatic neuropathological findings and more particularly in the acute striatal neurodegeneration found in GA-I patients occurring during acute encephalopathic crises.

Acknowledgements

This study was supported by grants from the Brazilian National Research Council (CNPq), Financing Agency for Research of Rio Grande do Sul (FAPERGS) and The Excellence Nuclei Program (PRONEX).

References

Bolshakov, K.V., Gmiro, V.E., Tikhonov, D.B., Magazanik, L.G., 2003. Determinants of trapping block of *N*-methyl-D-aspartate receptor channels. J. Neurochem. 87, 56–65.

Brismar, J., Ozand, P.T., 1995. CT, MR of the brain in glutaric acidemia type I: a review of 59 published cases and a report of 5 new patients. Am. J. Neuroradiol. 16, 675–683.

Chapman, A.G., 1988. Glutamate receptors in epilepsy. Prog. Brain Res. 116, 371–383.

Chow, C.W., Haan, E.A., Goodman, S.I., Anderson, R.M., Evans, W.A., Kleinschmidt-DeMasters, B.K., Wise, G., McGill, J.J., Danks, D.M., 1988. Neuropathology in glutaric acidaemia type 1. Acta Neuropathol. (Berl.) 76, 590–594.

Danbolt, N.C., 1994. Glutamate uptake (1994). The high affinity uptake system for excitatory amino acids in the brain. Prog. Neurobiol. 44, 377–396.

Eason, K.E., Aronstam, R.S., 1984. Influence of sulfhydryl reagents on potassium-stimulated calcium uptake by rat brain synaptosomes. Res. Commun. Chem. Pathol. Pharmacol. 44, 503–506.

Emanuelli, T., Antunes, V., Souza, D.O.G., 1998. Characterization of L-[^{3}H]glutamate binding to fresh and frozen crude plasma membranes isolated from cerebral cortex of adult rats. Biochem. Mol. Biol. Int. 44, 1265–1272.

Euler, G.V., Liu, Y., 1993. Glutamate and glycine decrease the affinity of [^{3}H]MK-801 binding in the presence of Mg^{2+}. Eur. J. Pharmacol. 245, 233–239.

Flott-Rahmel, B., Falter, C., Schluff, P., Fingerhut, R., Christensen, E., Jakobs, C., Musshoff, U., Fautek, J.D., Ludolph, A., Ullrich, K., 1997. Nerve cell lesions caused by 3-hydroxyglutaric acid: a possible mechanism for neurodegeneration in glutaric acidaemia. I. J. Inherit. Metab. Dis. 20, 387–390.

Forstner, R., Hoffmann, G.F., Gassner, I., Heideman, P., De Klerk, J.B., Lawrenz-Wolf, B., Doringer, E., Weiss-Wichert, P., Troger, J., Colombo, J.P., Plochl, E., 1999. Glutaric aciduria type I: ultrasonographic demonstration of early signs. Pediatr. Radiol. 29, 138–143.

Frizzo, M.E.S., Schwarzbold, C., Porciúncula, L.O., Dalcin, K.B., Rosa, R.B., Ribeiro, C.A.J., Souza, D.O., Wajner, M., 2004. 3-Hydroxyglutaric acid enhances glutamate uptake into astrocytes from cerebral cortex of young rats. Neurochem. Int. 44, 345–353.

Goodman, S.I., Frerman, F.E., 2001. Organic acidemias due to defects in lysine oxidation: 2-ketoadipic academia and glutaric acidemia. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Walle, D., (Eds.), The Metabolic and Molecular Bases of Inherited Disease, eighth ed. McGraw-Hill, New York, pp. 2195–2204.

Goodman, S.I., Norenberg, M.D., Shikes, R.H., Breslich, D.J., Moe, P.G., 1977. Glutaric aciduria type 1: biochemical and morphological considerations. J. Pediatr. 90, 746–750.

Hoffmann, G.F., Zschocke, J., 1999. Glutaric aciduria type I: From clinical, biochemical and molecular diversity to successful therapy. J. Inherit. Metab. Dis. 22, 381–391.

Hoffmann, G.F., Gibson, K.M., Trekz, F.K., Nyhan, W.L., Bremer, H.J., Rating, D., 1994. Neurological manifestations of organic acid disorders. Eur. J. Pediatr. 153, S94–S100.

Jones, D.H., Matus, A.I., 1974. Isolation of synaptic plasma membrane from brain by combined flotation-sedmentation density gradient centrifugation. Biochim. Biophys. Acta 356, 276–287.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 1999. 3-Hydroxyglutaric and glutaric acids are neurotoxic through NMDA receptors in vitro. J. Inherit. Metab. Dis. 22, 259–262.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2000a. Cerebral organic acid disorders induce neuronal damage via excitotoxic organic acids in vitro. Amino Acids 18, 31–40.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2000b. Maturation-dependent neurotoxicity of 3-hydroxyglutaric and glutaric acids in vitro: A new pathophysiologic approach to glutaryl-CoA dehydrogenase deficiency. Pediatr. Res. 47, 495–503.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2000c. Evaluation of trigger factors of acute encephalopathy in glutaric aciduria type I: fever and tumour necrosis factor-alpha. J. Inherit. Metab. Dis. 23, 359–362.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2001a. Contribution of reactive oxygen species to 3-hydroxyglutarate neurotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr. Res. 50, 76–82.

Kölker, S., Ahlemeyer, B., Hühne, R., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., 2001b. Potentiation of 3-hydroxyglutarate neurotoxicity following induction of astrocytic iNOS in neonatal rat hippocampal cultures. Eur. J. Neurosci. 13, 2115–2122.

Kölker, S., Köhr, G., Ahlemeyer, B., Okun, J.G., Pawlak, V., Horster, F., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., 2002a. Ca^{2+} and Na^{+} dependence of 3-hydroxyglutarate-induced excitotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr. Res. 52, 199–206.

Kölker, S., Okun, J.G., Ahlemeyer, B., Wyse, A.T., Horster, F., Wajner, M., Kohlmuller, D., Mayatepek, E., Krieglstein, J., Hoffmann, G.F.,

2002b. Chronic treatment with glutaric acid induces partial tolerance to excitotoxicity in neuronal cultures from chick embryo telencephalons. J. Neurosci. Res. 685, 424–431.

Kölker, S., Hoffmann, G.F., Schor, D.S.M., Feyh, P., Wagner, L., Jeffrey, I., Pourfarzam, M., Okum, J.G., Zschocke, J., Baric, I., Bain, M.D., Jakobs, C., Chalmers, R.A., 2003. Gluratyl-CoA dehydrogenase deficiency: Region-specific analysis of organic acids and acylcarnitines in post mortem brain predicts vulterability of the putamen. Neuropediatrics 34, 253–260.

Latini, A., Rosa, R.B., Scussiato, K., Llesuy, S., Belló-Klein, A., Wajner, M., 2002. 3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats. Brain Res. 956, 367–373.

Lowry, O.H., Roseborough, N.R., Farr, A.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Meldrum, B., Garthwaite, J., 1990. Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends Pharmacol. Sci. 11, 379–387.

Meldrum, B., 2002. Implications for neuroprotective treatments. Prog. Brain Res. 135, 487–495.

Mello, C.F., de Kölker, S., Ahlemeyer, B., de Souza, F.R., Fighera, M.R., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., Wajner, M., 2001. Intrastriatal administration of 3-hydroxyglutaric acid induces convulsions and striatal lesions in rats. Brain Res. 916, 70–75.

Olney, J.W., 1980. Excitotoxic mechanisms of neurotoxicity. In: Spencer P.S. Schaumburg H.H. (Eds), Clinical and Experimental Neurotoxicology, Williams & Wilkins, Baltimore, MD, pp. 272–294.

Ozawa, S., Kamyiar, K., Tsuzuki, K., 1998. Glutamate receptors in the mammalian cerebral nervous system. Prog. Neurobiol. 54, 581–618.

Planells-Cases, R., Montoliu, C., Humet, M., Fernandez, A.M., Garcia-Martinez, C., Valera, E., Merino, J.M., Perez-Paya, E., Messeguer, A., Felipo, V., Ferrer-Montiel, A., 2002. A novel *N*-methyl-D-aspartate receptor open channel blocker with in vivo neuroprotectant activity. J. Pharmacol. Exp. Ther. 302, 163–173.

Rothman, S.M., Olney, J.W., 1987. Excitotoxicity and the NMDA receptors. Trends Neurosci. 10, 299–302.

Strauss, K.A., Morton, H., 2003. Type I glutaric aciduria, Part 2: A model of acute striatal necrosis. Am. J. Med. Gen. 121, 53–70.

Ullrich, K., Flott-Rahmel, B., Schluff, P., Musshoff, U., Das, A., Lücke, T., Steinfeld, R., Christensen, E., Jacobs, C., Ludolph, A., Neu, A., Röper, R., 1999. Glutaric aciduria type I: pathomechanisms of neurodegeneration. J. Inherit. Metab. Dis. 22, 392–403.

Capítulo II

Age and brain structural related effects of glutaric and 3-hydroxyglutaric acids on glutamate binding to plasma membranes during rat brain development

Karina B. Dalcin, Rafael B. Rosa, Anna L. Schmidt, Juliana S. Winter, Guilhian Leipnitz, Carlos S. Dutra-Filho, Clóvis M. D. Wannmacher, Lisiane O. Porciúncula, Diogo O. Souza, Moacir Wajner

Cellular and Molecular Neurobiology 27: 805-818, 2007

Cell Mol Neurobiol (2007) 27:805–818
DOI 10.1007/s10571-007-9197-2

ORIGINAL PAPER

Age and Brain Structural Related Effects of Glutaric and 3-Hydroxyglutaric Acids on Glutamate Binding to Plasma Membranes During Rat Brain Development

Karina B. Dalcin · Rafael B. Rosa · Anna L. Schmidt · Juliana S. Winter · Guilhian Leipnitz · Carlos S. Dutra-Filho · Clóvis M. D. Wannmacher · Lisiane O. Porciúncula · Diogo O. Souza · Moacir Wajner

Received: 24 May 2007 / Accepted: 7 August 2007 / Published online: 5 September 2007
© Springer Science+Business Media, LLC 2007

Abstract (1) In the present study we determined the effects of glutaric (GA, 0.01–1 mM) and 3-hydroxyglutaric (3-OHGA, 1.0–100 μM) acids, the major metabolites accumulating in glutaric acidemia type I (GA I), on Na^+-independent and Na^+-dependent [^{3}H]glutamate binding to synaptic plasma membranes from cerebral cortex and striatum of rats aged 7, 15 and 60 days. (2) GA selectively inhibited Na^+-independent [^{3}H]glutamate binding (binding to receptors) in cerebral cortex and striatum of rats aged 7 and 15 days, but not aged 60 days. In contrast, GA did not alter Na^+-dependent glutamate binding (binding to transporters) to synaptic membranes from brain structures of rats at all studied ages. Furthermore, experiments using the glutamatergic antagonist CNQX indicated that GA probably binds to non-NMDA receptors. In addition, GA markedly inhibited [^{3}H]kainate binding to synaptic plasma membranes in cerebral cortex of 15-day-old rats, indicating that this effect was probably directed towards kainate receptors. On the other hand, experiments performed with 3-OHGA revealed that this organic acid did not change Na^+-independent [^{3}H]glutamate binding to synaptic membranes from cerebral cortex and striatum of rats from all ages, but inhibited Na^+-dependent [^{3}H]glutamate binding to membranes in striatum of 7-day-old rats, but not in striatum of 15- and 60-day-old rats and in cerebral cortex of rats from all studied ages. We also provided some evidence that 3-OHGA competes with the glutamate transporter inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate, suggesting a possible interaction of 3-OHGA with glutamate transporters on synaptic membranes. (3) These results indicate that glutamate binding to receptors and transporters can be

K. B. Dalcin · R. B. Rosa · A. L. Schmidt · J. S. Winter · G. Leipnitz · C. S. Dutra-Filho · C. M. D. Wannmacher · L. O. Porciúncula · D. O. Souza · M. Wajner (✉)
Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil
e-mail: mwajner@ufrgs.br

M. Wajner
Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

M. Wajner
Universidade Luterana do Brasil, Canoas, RS, Brazil

inhibited by GA and 3-OHGA in cerebral cortex and striatum in a developmentally regulated manner. It is postulated that a disturbance of glutamatergic neurotransmission caused by the major metabolites accumulating in GA I at early development may possibly explain, at least in part, the window of vulnerability of striatum and cerebral cortex to injury in patients affected by this disorder.

Keywords Glutaric acidemia type I · Glutaric acid · 3-Hydroxyglutaric acid · Glutamatergic system

Introduction

Glutaric acidemia type I (GA I, McKusick 23167; OMIM # 231670) 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), which is involved in the catabolic pathway of lysine, hydroxylysine and tryptophan (Goodman et al. 1975). Increased concentrations of glutaric acid (GA, 500–5000 μmol/l), as well as of 3-hydroxyglutaric acid (3-OHGA) in lower amounts (40–200 μmol/l), are found in the body fluids and in the brain of GA I patients (Goodman and Frerman 2001; Strauss and Morton 2003; Strauss et al. 2003; Sauer et al. 2006). Clinical manifestations of GA I are predominantly neurological and appear especially after encephalopathic crises, which occur between 6 and 36 months of age and are accompanied by bilateral destruction of caudate and putamen (Hoffmann and Zschocke 1999; Morton et al. 1991). Frontotemporal atrophy, frequently detected at birth, is a distinctive radiological appearance that may be patho-gnomonic in GA I (Strauss et al. 2003). *Post mortem* examination of the basal ganglia and cerebral cortex of patients with GA I revealed postsynaptic vacuolization characteristic of glutamate-mediated brain injury (Goodman et al. 1977).

Glutamate is the main excitatory neurotransmitter in the mammalian brain and its interactions with specific membrane receptors are responsible for many CNS functions such as cognition, memory and movement (Ozawa et al. 1998; Danbolt 2001). The role of glutamate in mammalian brain is mediated by activation of glutamate-gated cation channels termed ionotropic receptors and of GTP-binding protein (G-protein)-linked receptors termed 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 α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA: GluR1-4) and kainate (GluR5-7 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). On the other hand, NMDA receptor maturation occurs later than kainate and AMPA receptor expression in the neonatal rat

striatum (Colwell et al. 1998; Nansen et al. 2000). 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). In the rat, GLAST is expressed at birth, whereas GLT1 is mainly expressed during the second to third postnatal week. Both transporters are fully expressed by postnatal week 5, but GLT1 is the predominant glutamate astroglial transporter in the adult brain (Ullensvang et al. 1997).

Glutamate is also a potent neurotoxin when present at high amounts in the synaptic cleft leading to excitotoxicity by over-stimulation of glutamate receptors, a process related to the neuropathology of acute and chronic brain disorders (Olney 1969; Lipton and Rosenberg 1994; Maragakis and Rothstein 2001).

A considerable body of evidence has emerged from recent studies indicating excitotoxic actions for GA and 3-OHGA, two organic acids with similar chemical structure to that of 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; Mello et al. 2001; Rosa et al. 2004). However, the exact underlying mechanisms by which GA and 3-OHGA lead to excitotoxicity are to date poorly known. Therefore, in the present work we studied the influence of GA and 3-OHGA on Na^+-independent and Na^+-dependent $[^3H]$glutamate binding in synaptic plasma membranes from cerebral cortex and striatum of rats from different ages (7, 15 and 60 days) in order to better characterize the role of these organic acids on glutamate receptors and transporters during rat brain development. We choose these rat ages because children with GA I are more vulnerable to the effects of an acute metabolic stress during the first 3 years of age (Bjugstad et al. 2000), which correspond approximately to 7–15 days of life in rats, whereas 60-day-old rats match to young adult humans (Haberny et al. 2002).

Materials and Methods

Materials

Chemicals of analytical reagent grade were mainly purchased from Sigma (St Louis, MO, USA) and Tocris Cookson Inc. (Ellisville, MO, USA), whereas 3-OHGA was prepared with 99% purity by Dr Ernesto Brunet (Universidad Autonoma de Madrid, Spain). The labeled product L-$[^3H]$glutamate (52 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA), whereas $[^3H]$kainic acid (58 Ci/mmol) was obtained from New England Nuclear (Germany) .

Animals

Wistar rats of 7, 15 and 60 days of age were obtained from the Central Animal House of the Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. They were maintained at 25°C, on a 12:12 h light/dark cycle, with free access to food and water. The ‘‘Principles of laboratory animal care’’ (NIH publications

No. 80-23, revised 1996) were followed in all experiments. All efforts were made to minimize the number of animals used and their suffering.

Membrane Preparation

Animals were killed by decapitation without anesthesia, the brain rapidly removed and the cerebral cortex and striatum immediately dissected on a Petri dish on ice. Synaptic membranes were isolated from the cerebral cortex and striatum of rats, as described by Jones and Matus (1974) with slight modifications (Rosa et al. 2004), and stored frozen at –70°C for no more than 2 weeks. On the day of assay, membranes were thawed at 37°C for 30 min, and washed three times in hypotonic solution consisting of 5 mM Tris–acetate (glutamate binding) or Tris–HCl (kainate binding), pH 7.4, by centrifugation at 45,000*g* at 4°C for 15 min. We have previously observed that this procedure avoids membrane sealing, in agreement to the literature (Danbolt 1994).

[^{3}H]Glutamate Binding

Glutamate binding experiments were carried out in the absence of sodium, aiming glutamate binding to receptors, and in the presence of sodium, aiming glutamate binding to transporters, which depends on sodium for their activity. The incubations were carried out in triplicate in polycarbonated tubes (total volume 500 μl) containing 50 mM Tris–acetate (Na^+-independent binding) or 50 mM Tris–acetate/120 mM NaCl (Na^+-dependent glutamate binding), pH 7.4, 40 nM [^{3}H]glutamate (0.3 μCi) and GA ranging from 0.01 to 1 mM, or 3-OHGA ranging from 1 to 100 μM. Some experiments (Na^+-independent glutamate binding) were performed in the presence of 100 μM of the ionotropic non-NMDA and NMDA receptors antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and DL-2-amino-phosphonovaleric acid (AP5), respectively. Na^+-dependent [^{3}H]glutamate binding was also evaluated in the presence of 50 μM L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC), which is a substrate inhibitor of glutamate transporters. Controls did not contain GA or 3-OHGA. Incubation was started by adding 50–100 μg of protein membrane, and run at 30°C for 30 min. The reaction was stopped by centrifugation at 16,800*g* for 15 min at 4°C. The pellet was carefully washed with ice-cold distilled water and resuspended with 0.1 M NaOH and 0.01% sodium dodecyl sulfate (w/v) overnight. Radioactivity was determined using a Wallac 1409 liquid scintillation counter. Non-specific binding (10–20% of the total binding) was determined by adding 40 μM non-radioactive glutamate to the medium in parallel assays. Specific binding was considered as the difference between total binding and non-specific binding. Results were calculated as pmol [^{3}H]glutamate/mg protein and expressed as percentage of control.

[^{3}H]Kainate Binding

[^{3}H]Kainate binding assays were carried out in small polycarbonate tubes (total volume 500 μl) using synaptic plasma membranes from cerebral cortex of 15-day-old rats. Incubations were started by the addition of membrane preparation (100–150 μg of protein) to a medium containing 50 mM Tris–HCl, pH 7.4, 10 nM [^{3}H]kainate (0.29 μCi), in the absence or presence of 400 μM non-radioactive kainate (non-specific binding). In some experiments GA (1.0 mM) was also added to the medium, whereas

controls did not contain this acid. Following 90 min of incubation at 0°C, bound and free [^{3}H]kainate were separated by centrifugation at 16,800*g* for 15 min at 4°C. The pellet was washed with ice-cold distilled water and dissolved overnight with 0.1 M NaOH and 0.01% sodium dodecyl sulfate (w/v). Radioactivity was determined using a Wallac 1409 liquid scintillation counter. Specific binding was calculated as the difference between total binding and non-specific binding. Results were calculated as pmol [^{3}H]kainate/mg protein and expressed as percentage of control.

Protein Determination

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

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan's multiple range test when appropriate. The Student's *t*-test for paired samples was also used for comparison between two means. Only significant values are shown in the text. Analyses were performed using the SPSS (Statistical Package for the Social Sciences) software in a PC-compatible computer. A value of $P < 0.05$ was considered to be significant.

Results

Figure 1 shows the effect of GA on glutamate binding to synaptic plasma membranes from cerebral cortex and striatum of rats aged 7 (A), 15 (B) and 60 (C) days in the absence of sodium, which reflects glutamate binding to receptors. GA significantly decreased Na^{+}-independent [^{3}H]glutamate binding (up to 25%) at concentrations as low as 0.01 mM in cerebral cortex and at 1 mM concentration in striatum from rats of 7 (cerebral cortex: [$F(3, 26) = 3.50$; $P < 0.05$]; striatum: [$F(3, 20) = 7.96$; $P < 0.01$]) and 15 (cerebral cortex: [$F(3, 26) = 8.73$; $P < 0.001$]; striatum: [$F(3, 19) = 4.88$; $P < 0.05$]) days of life. In contrast, Na^{+}-independent [^{3}H]glutamate binding to synaptic membranes from rats aged 60 days was not changed by GA.

The next set of experiments tested the role of glutamate antagonists on GA-induced Na^{+}-independent [^{3}H]glutamate binding decrease in synaptic membranes from cerebral cortex and striatum of 15-day-old rats. GA (1 mM) and the ionotropic non-NMDA antagonist CNQX (100 μM) were incubated alone or combined. We observed that GA did not change the decrease provoked by CNQX on Na^{+}-independent [^{3}H]glutamate binding both in cerebral cortex [$F(5, 36) = 14.09$; $P < 0.001$] (Fig. 2A) and striatum [$F(5, 36) = 5.56$; $P < 0.01$] (Fig. 2B). Furthermore, when the NMDA antagonist AP5 (100 μM) was co-incubated with GA (1 mM), we observed a slight higher decrease of Na^{+}-independent [^{3}H]glutamate binding relatively to that of AP5 alone.

We then investigated the effect of GA on [^{3}H]kainate binding to synaptic membranes from cerebral cortex of 15-day-old rats in order to clarify which non-NMDA receptors were involved in GA effect. We observed that [^{3}H]kainate binding was markedly decreased (60% decrease) by 1 mM GA [$t(4) = 4.13$; $P < 0.05$] (Fig. 3).

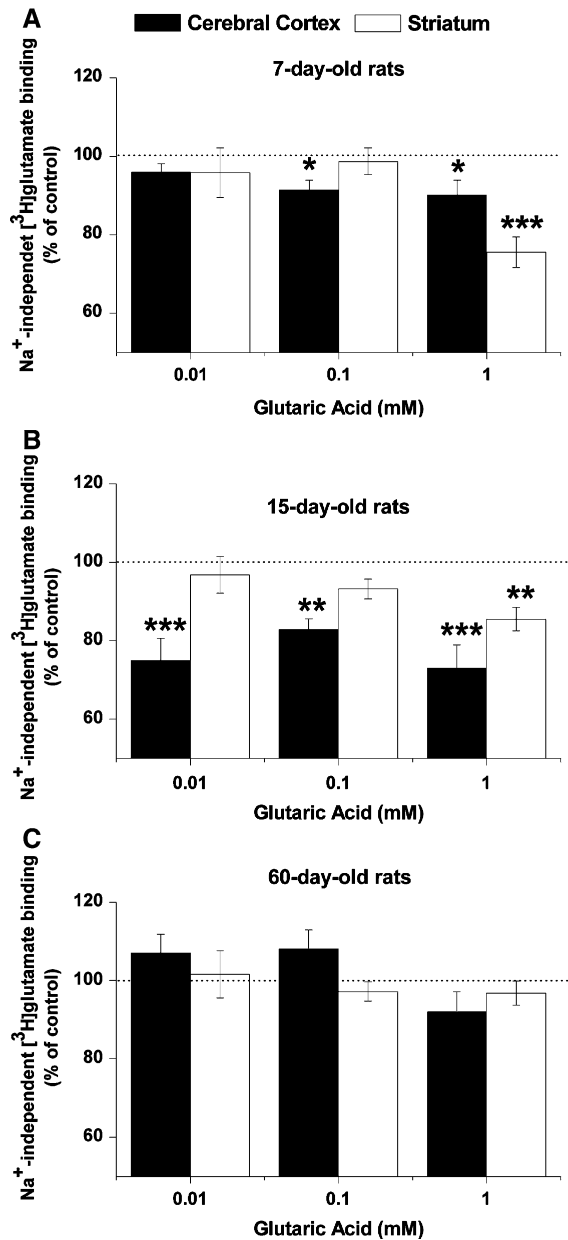

Fig. 1 Effect of glutaric acid (GA, 0.01–1 mM) exposure on Na^+-independent [^{3}H]glutamate binding (binding to receptors) to synaptic plasma membranes from cerebral cortex and striatum of rats aged 7 (**A**), 15 (**B**) and 60 (**C**) days. Results are presented as mean ± SEM from five to eight independent experiments performed in triplicate and are expressed as percentage of controls. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with controls (Duncan multiple range test)

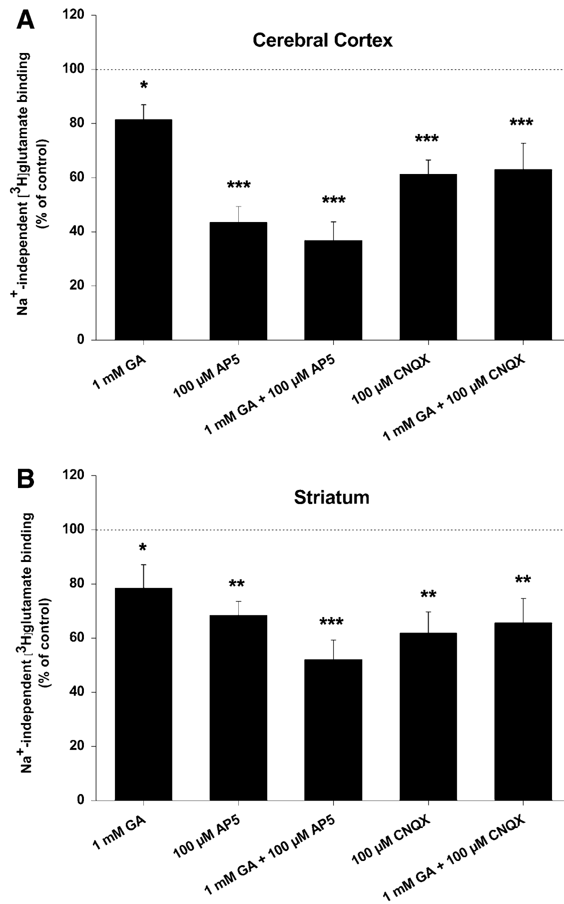

Fig. 2 Effect of glutaric acid (GA, 1 mM), AP5 (100 μM) and CNQX (100 μM) on Na^+-independent [^{3}H]glutamate binding to synaptic plasma membranes from cerebral cortex (**A**) and striatum (**B**) of 15-day-old rats. Results are presented as mean ± SEM from seven independent experiments performed in triplicate and are expressed as percentages of controls. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with controls (Duncan multiple range test)

We also verified that GA (0.01–1 mM) did not modify Na^+-dependent [^{3}H]glutamate binding to synaptic membranes in cerebral cortex and striatum from rats of 7, 15 and 60 days of life (data not shown).

The next set of experiments were carried out in order to test the influence of 3-OHGA on Na^+-independent [^{3}H]glutamate binding. We observed that 3-OHGA did not change Na^+-independent [^{3}H]glutamate binding in cerebral cortex and striatum from rats of 7, 15 and 60 days of life (data not shown). In contrast, 3-OHGA (at 100 μM concentration) significantly inhibited (20%) Na^+-dependent [^{3}H]glutamate binding to membranes in striatum of 7-day-old rats [$F(3, 16) = 3.58$; $P < 0.05$] (Fig. 4A), but not in striatum of 15- (Fig. 4B) and 60-day-old rats (Fig. 4C) and in cerebral cortex from all rat ages. Next we tested the action of 3-OHGA on Na^+-dependent [^{3}H]glutamate binding in synaptic membranes from striatum of 7-day-old rats in the presence of 50 μM of the competitive inhibitor (PDC) of glutamate transporters . We observed that both

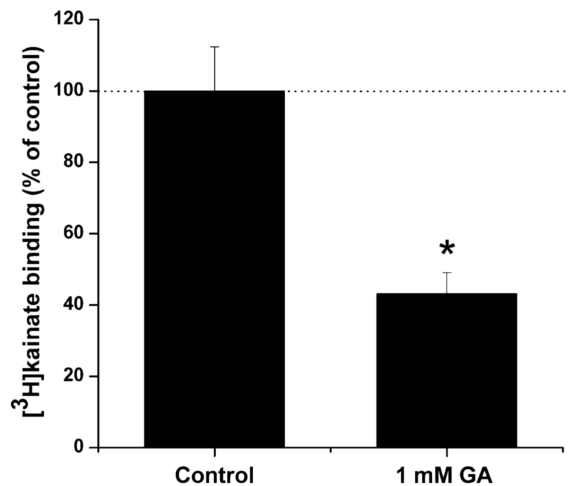

Fig. 3 Effect of glutaric acid (GA, 1 mM) on [^{3}H]kainate binding to synaptic plasma membranes from cerebral cortex of 15-day-old rats. Results are means ± SEM of five independent experiments performed in triplicate and are expressed as percentage of control. *$P < 0.05$, compared with control (Student's *t*-test for paired samples)

3-OHGA and PDC inhibited Na^+-dependent [^{3}H]glutamate binding to synaptic membranes from striatum. Furthermore, 3-OHGA did not change the inhibitory effect of PDC [$F(3, 12) = 209.12$; $P < 0.001$] (Fig. 5).

Discussion

The cause of the frontal and temporal cortex vulnerability during gestation and at early development and of the striatum degeneration during the first three years of life in GA I is still obscure despite intense investigation performed in cultivated neurons from chick and rats, in fresh rat neural tissue and also in tissues from mice deficient in GCDH. However, in the last decade alterations of energy metabolism (Ullrich et al. 1999; Silva et al. 2000; Kölker et al. 2002b, 2004; Das et al. 2003; Latini et al. 2005a; da Costa Ferreira et al. 2005a, b; Sauer et al. 2005), oxidative stress (de Oliveira Marques et al. 2003; Latini et al. 2002, 2005b) and particularly disturbance of the glutamatergic system due to the structural similarity between glutamate, GA and 3-OHGA (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; Mello et al. 2001; Rosa et al. 2004; Frizzo et al. 2004) have been considered important pathomechanisms underlying neural damage in GA I. On the other hand, recent works performed on neurons in culture did not confirm excitotoxic actions for 3-OHGA (Lund et al. 2004; Freudenberg et al. 2004), suggesting that more work is necessary to clarify this matter.

The results of the present investigation demonstrate that glutamate binding to receptors is decreased by GA in cerebral cortex and striatum from rats aged 7 and 15 days and that 3-OHGA decreased glutamate binding to transporters in striatum of 7-day-old rats, indicating developmentally regulated and tissue-specific effects of these metabolites.

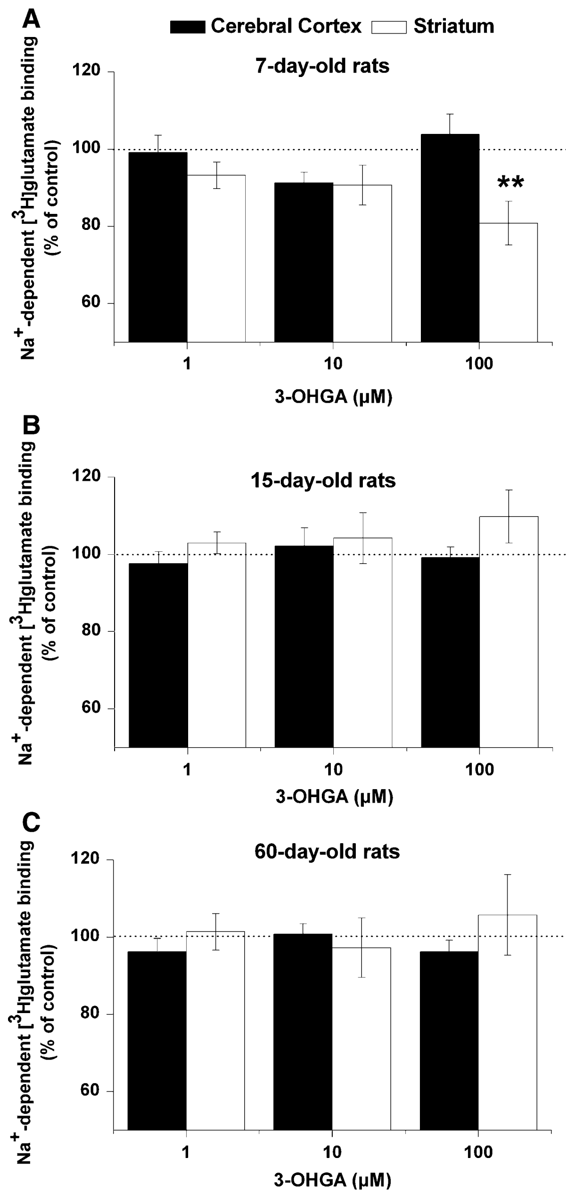

Fig. 4 Effect of 3-hydroxyglutaric acid (3-OHGA, 1–100 μM) exposure on Na^+-dependent [^{3}H]glutamate binding (binding to transporters) to synaptic plasma membranes from cerebral cortex and striatum of rats aged 7 (**A**), 15 (**B**) and 60 (**C**) days. Results are presented as mean ± SEM from five to six independent experiments performed in triplicate and are expressed as percentage of controls. $^{**}P < 0.01$, compared with controls (Duncan multiple range test)

We first observed that GA (0.01–1 mM) induced a significant decrease of Na^+-independent glutamate binding to synaptic membranes at the concentrations found in *post mortem* brain examination of GA I patients (Kölker et al. 2003; Sauer et al. 2006). Furthermore, this effect was mainly directed to glutamate receptors given that the

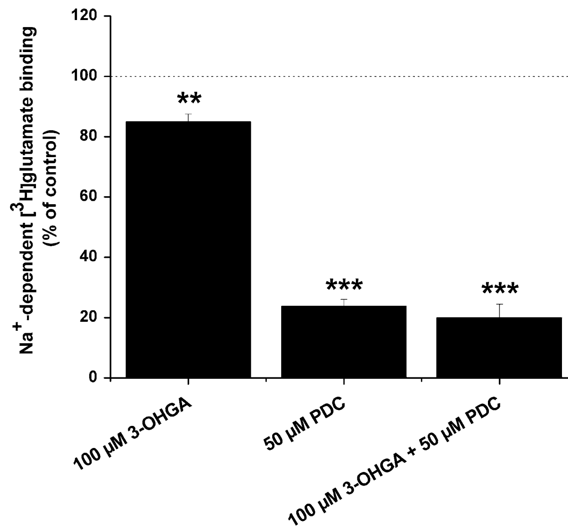

Fig. 5 Effect of 3-hydroxyglutaric acid (3-OHGA, 100 µM) and L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC, 50 µM) exposure on Na^+-dependent [^{3}H]glutamate binding (binding to transporters) to synaptic plasma membranes from striatum of 7-day-old rats. Results are presented as mean ± SEM from four independent experiments performed in triplicate and are expressed as percentage of controls. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with controls (Duncan multiple range test)

incubation medium did not contain sodium, which is necessary for glutamate to bind to its transporters. The inhibitory effect provoked by GA was probably directed to non-NMDA glutamate receptors and possibly to kainate receptors since co-incubation of GA with CNQX (a non-NMDA receptor antagonist) resulted in an identical reduction of glutamate binding as that found for CNQX alone, and GA markedly displaced kainate (60% decrease) from its receptors. Our present findings may possibly explain previous in vivo findings showing that the behavioral alterations and clonic convulsions provoked by intrastriatal administration of GA to adult rats were prevented by the non-NMDA antagonist DNQX, but not by the NMDA antagonist MK-801 (Lima et al. 1998). A recent report also showed that GA binds to non-NMDA receptors in brain from 30-day-old rats (Porciúncula et al. 2004). Furthermore, patch clamp studies failed to find any effect of GA on AMPA receptors (Ullrich et al. 1999; Kölker et al. 2002b; Lund et al. 2004), which is in agreement with our present findings indicating that GA binds preferentially to kainate receptors. In contrast, previous in vitro studies demonstrated the inefficacy of CNQX to prevent death of neonatal cultured neurons induced by GA and 3-OHGA (Kölker et al. 2000a). It should be stressed that our present findings and those of Lima and collaborators (1998) were carried out with postnatal striatum and cerebral cortex of rats aged 7–60 days rats, whereas Kölker and colleagues (2000a) used chick embryonic telencephalons and mixed neuronal and glial cell cultures from neonatal rat hippocampus in their experiments. Therefore, it is feasible that the effects provoked by GA are age-, species- and regional-dependent, being probably related to the differential ontogenetic expression and properties of glutamate receptor subtypes (Luo et al. 1996; Anderson et al. 1999; Ritter et al. 2002). This agrees with the fact that the variable subunits pattern of these proteins expressed ontogenetically usually confer different pharmacological and physiological responses (McDonald and Johnston 1990; Ozawa et al. 1998). One fact that may contribute to the

interaction of GA with glutamate receptors is that glutamate and GA are structurally chemical similar molecules so that a competition for the same receptor sites may have occurred. Furthermore, our present in vitro results showing that GA inhibits glutamate binding to cortical and striatal receptors at an early postnatal age (7- and 15-day-old rats), but not in older animals (60-day-old rats), may be possibly related to the amount of glutamate receptors that are expressed at higher levels during early postnatal development, a fact that may explain the higher vulnerability of neonatal brain to excitotoxicity (Ritter et al. 2002). This is in line with the observations that AMPA and kainate receptors in rat brain reach the density peaks around the second to third postnatal weeks, decreasing thereafter (Insel et al. 1990; Miller et al. 1990; Kossut et al. 1993; Brennan et al. 1997).

On the other hand, we also observed that GA (0.01–1 mM) did not inhibit Na^+-dependent glutamate binding to synaptic membranes obtained from cerebral cortex and striatum of rats from all ages, implying that GA, at concentrations found in the brain of glutaric acidemic patients, does not alter glutamate binding to these transporters.

With respect to 3-OHGA, we found that this organic acid was unable to alter glutamate binding to receptors (Na^+-independent glutamate binding) in cerebral cortex and striatum of rats from all ages. In contrast, 3-OHGA significantly decreased Na^+-dependent glutamate binding (binding to transporters) only in striatum from 7-day-old rats, but not in striatum from older animals and in cerebral cortex from rats aged 7–60 days. We further observed that 3-OHGA did not alter the inhibition provoked by the competitive glutamate transporter inhibitor PDC on Na^+-dependent glutamate binding to synaptic membranes from 7-day-old rat striatum, suggesting that 3-OHGA in fact binds to glutamate transporters rather than to unspecific sites on the synaptic membranes. Considering that our synaptic membrane preparations contain glial cell membranes (astrocytic glutamate transporters) and that at this age GLAST transporters are more prevalent, it could be presumed that 3-OHGA interfered with glutamate binding to these Na^+-dependent high affinity transporters. Although the mechanism by which 3-OHGA, but not GA, inhibited glutamate binding to membrane transporters is unknown, it has been suggested that some substances with the hydroxyl group attached to carbon 3 have more affinity to glutamate transporters and can competitively inhibit glutamate binding (Balcar et al. 1977).

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 and 3-OHGA were observed with concentrations similar to those encountered in brain of glutaric acidemic patients (Goodman et al. 1977; Kölker et al. 2003; Kulkens et al. 2005, Sauer et al. 2006). In this context, untreated GA I patients present brain GA concentrations of 500–5000 μmol/l and 3-OHGA concentrations of 40–200 μmol/l (Sauer et al. 2006). Furthermore, the degree of alterations of the glutamatergic system detected in our study is widely accepted to cause excitotoxicity in systems testing the effect of potential excitotoxins (Ozawa et al. 1998; Danbolt 2001; Meldrum 2002). Taken together these observations and previous reports demonstrating that GA and 3-OHGA markedly reduce viability of neurons in culture via glutamate receptors (Kölker et al. 2000a, b), it is conceivable that our findings may be related to these findings.

In summary, it is likely that the effects demonstrated here for GA and 3-OHGA at specific ages of rat development and also in distinct cerebral structures are probably associated to the differential ontogenetic and regional-specific expression of these proteins in rat brain synaptic membranes. The present findings suggest a disturbance of

glutamatergic neurotransmission caused by the major metabolites accumulating in GA I at early development. They may be possibly related to the regional- and age-specific window of susceptibility responsible for the neuropathology of GA I characterized by frontotemporal subcortical atrophy and striatum degeneration that occur during the first three years of life in the affected patients.

Acknowledgements Financial support: CNPq, FAPERGS, PRONEX and the FINEP research grant Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00.

References

Amara SG, Fontana AC (2002) Excitatory amino acid transporters: keeping up with glutamate. Neurochem Int 41:313–318

Anderson KJ, Mason KL, McGraw TS, Theophilopoulos DT, Sapper MS, Burchfield DJ (1999) The ontogeny of glutamate receptors and D-aspartate binding sites in the CNS. Brain Res Dev Brain Res 118:69–77

Balcar VJ, Johnston GA, Twitchin B (1977) Stereospecificity of the inhibition of L-glutamate and L-aspartate high affinity uptake in rat brain slices by threo-3-hydroxyaspartate. J Neurochem 28:1145–1146

Bjugstad KB, Goodman SI, Freed CR (2000) Age of symptom onset predicts severity of motor impairment and clinical outcome of glutaric academia type 1. J Pediatr 137:681–686

Brennan EM, Martin LJ, Johnston MV, Blue ME (1997) Ontogeny of non-NMDA glutamate receptors in rat barrel field cortex: II. α-AMPA and kainate receptors. J Comp Neurol 386:29–45

Colwell CS, Cepeda C, Crawford C, Levine MS (1998) Postnatal development of glutamate receptor-mediated responses in the neostriatum. Dev Neurosci 20:154–163

Conn JP, Pin JP (1997) Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 37:205–237

da Costa Ferreira G, Viegas CM, Schuck PF, Latini A, Dutra-Filho CS, Wyse AT, Wannmacher CM, Vargas CR, Wajner M (2005a) Glutaric acid moderately compromises energy metabolism in rat brain. Int J Dev Neurosci 23:687–693

da Costa Ferreira G, Viegas CM, Schuck PF, Tonin A, Ribeiro CA, Coelho D de M, Dalla-Costa T, Latini A, Wyse AT, Wannmacher CM, Vargas CR, Wajner M (2005b) Glutaric acid administration impairs energy metabolism in midbrain and skeletal muscle of young rats. Neurochem Res 30:1123–1131

Danbolt NC (1994) The high affinity uptake system for excitatory amino acids in the brain. Prog Neurobiol 44:377–396

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1–105

Das AM, Lücke T, Ullrich K (2003) Glutaric aciduria I: creatine supplementation restores creatine phosphate levels in mixed cortex cells from rat incubated with 3-hydroxyglutarate. Mol Genet Metab 78:108–111

De Oliveira Marques F, Hagen ME, Pederzolli CD, Sgaravatti AM, Durigon K, Testa CG, Wannmacher CMD, de Souza D, Wyse ATS, Wajner M, Dutra-Filho CS (2003) Glutaric acid induces oxidative stress in brain of young rats. Brain Res 964:153–158

Flott-Rahmel B, Falter C, Schluff P, Fingerhut R, Christensen E, Jakobs C, Musshoff U, Fautek JD, Ludolph A, Ullrich K (1997) Nerve cell lesions caused by 3-hydroxyglutaric acid: a possible mechanism for neurodegeneration in glutaric acidaemia I. J Inherit Metab Dis 20:387–390

Freudenberg F, Lukacs Z, Ullrich K (2004) 3-Hydroxyglutaric acid fails to affect the viability of primary neuronal rat cells. Neurobiol Dis 16:581–584

Frizzo ME, Schwarzbold C, Porciuncula LO, Dalcin KB, Rosa RB, Ribeiro CA, Souza DO, Wajner M (2004) 3-Hydroxyglutaric acid enhances glutamate uptake into astrocytes from cerebral cortex of young rats. Neurochem Int 44:345–353

Goodman SI, Frerman FE (2001) Organic acidemias due to defects in lysine oxidation: 2-ketoadipic acidemia and glutaric acidemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease, 8th edn. McGraw-Hill, New York, pp 2195–2204

Goodman SI, Markey SD, Moer PG, Miles BS, Teng CC (1975) Glutaric aciduria: a ‘‘new’’ disorder of amino acid metabolism. Biochem Med 12:12–21

Goodman SI, Norenberg MD, Shikes RH, Breslich DJ, Moe PG (1977) Glutaric aciduria type I: biochemical and morphological considerations. J Pediatr 90:746–750

Haberny KA, Paule MG, Scallet AC, Sistare FD, Lester DS, Hanig JP, Slikker W Jr (2002) Ontogeny of the N-methyl-D-aspartate (NMDA) receptor system and susceptibility to neurotoxicity. Toxicol Sci 68:9–17

Hoffmann GF, Zschocke J (1999) Glutaric aciduria type I: from clinical, biochemical and molecular diversity to successful therapy. J Inherit Metab Dis 22:381–391

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annu Rev Neurosci 17:31–108

Insel TR, Miller LP, Gelhard RE (1990) The ontogeny of excitatory amino acid receptors in the rat forebrain I: *N*-methyl-D-aspartate and quisqualate receptors. Neuroscience 35:31–43

Jensen FE (2002) The role of glutamate receptor maturation in perinatal seizures and brain injury. Int J Dev Neurosci 20:339–347

Jones DH, Matus AI (1974) Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. Biochim Biophys Acta 356:276–287

Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF (1999) 3-Hydroxyglutaric and glutaric acids are neurotoxic through NMDA receptors *in vitro*. J Inherit Metab Dis 22:259–262

Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF (2000a) Maturation-dependent neurotoxicity of 3-hydroxyglutaric and glutaric acids in vitro: a new pathophysiologic approach to glutaryl-CoA dehydrogenase deficiency. Pediatr Res 47:495–503

Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF (2000b) Cerebral organic acid disorders induce neuronal damage via excitotoxic organic acids in vitro. Amino Acids 18:31–40

Kölker S, Kohr G, Ahlemeyer B, Okun JG, Pawlak V, Horster F, Mayatepek E, Krieglstein J, Hoffmann GF (2002a) Ca^{2+} and Na^{+} dependence of 3-hydroxyglutarate-induced excitotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr Res 52:199–206

Kölker S, Okun JG, Ahlemeyer B, Wyse AT, Horster F, Wajner M, Kohlmuller D, Mayatepek E, Krieglstein J, Hoffmann GF (2002b) Chronic treatment with glutaric acid induces partial tolerance to excitotoxicity in neuronal cultures from chick embryo telencephalons. J Neurosci Res 68:424–431

Kölker S, Hoffmann GF, Schor DS, Feyh P, Wagner L, Jeffrey I, Pourfarzam M, Okun JG, Zschocke J, Baric I, Bain MD, Jakobs C, Chalmers RA (2003) Glutaryl-CoA dehydrogenase deficiency: region-specific analysis of organic acids and acylcarnitines in post mortem brain predicts vulnerability of the putamen. Neuropediatrics 34:253–260

Kölker S, Koeller DM, Sauer S, Horster F, Schwab MA, Hoffmann GF, Ullrich K, Okun JG (2004) Excitotoxicity and bioenergetics in glutaryl-CoA dehydrogenase deficiency. J Inherit Metab Dis 27:805–812

Kossut M, Glazewski S, Siucinska E, Skangiel KJ (1993) Functional plasticity and neurotransmitter receptor binding in the vibrissal barrel cortex. Acta Neurobiol Exp (Wars) 53:161–173

Kulkens S, Harting I, Sauer S, Zschocke J, Hoffmann GF, Gruber S, Bodamer OA, Kolker S (2005) Late-onset neurologic disease in glutaryl-CoA dehydrogenase deficiency. Neurology 64:2142–2144

Latini A, Borba Rosa R, Scussiato K, Llesuy S, Bello-Klein A, Wajner M (2002) 3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats. Brain Res 956:367–373

Latini A, Rodriguez M, Borba Rosa R, Scussiato K, Leipnitz G, Reis de Assis D, da Costa Ferreira G, Funchal C, Jacques-Silva MC, Buzin L, Giugliani R, Cassina A, Radi R, Wajner M (2005a) 3-Hydroxyglutaric acid moderately impairs energy metabolism in brain of young rats. Neuroscience 135:111–120

Latini A, Scussiato K, Leipnitz G, Dutra-Filho CS, Wajner M (2005b) Promotion of oxidative stress by 3-hydroxyglutaric acid in rat striatum. J Inherit Metab Dis 28:57–67

Lima TTF, Begnini J, Bastiani J, Fialho DB, Jurach A, Ribeiro MCP, Wajner M, Mello CF (1998) Pharmacological evidence for GABAergic and glutamatergic involvement in the convulsant and behavioral effects of glutaric acid. Brain Res 802:55–60

Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 330:613–622

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Lund TM, Christensen E, Kristensen AS, Schousboe A, Lund AM (2004) On the neurotoxicity of glutaric, 3-hydroxyglutaric, and trans-glutaconic acids in glutaric acidemia type 1. J Neurosci Res 77:143–147

Luo J, Bosy TZ, Wang Y, Yasuda R, Wolfe BB (1996) Ontogeny of NMDA R1 subunit protein expression in five regions of rat brain. Brain Res Dev Brain Res 92:10–17

Maragakis NJ, Rothstein JD (2001) Glutamate transporters in neurologic disease. Arch Neurol 58:365–370

McDonald JW, Johnston MV (1990) Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res Rev 15:41–70

Meldrum BS (2002) Implications for neuroprotective treatments. Prog Brain Res 135:487–495

Mello CF, Kölker S, Ahlemeyer B, Souza FR, Fighera MR, Mayatepek E, Krieglstein J, Hoffmann GF, Wajner M (2001) Intrastriatal administration of 3-hydroxyglutaric acid induces convulsions and striatal lesions in rats. Brain Res 916:70–75

Miller LP, Johnson AE, Gelhard RE, Insel TR (1990) The ontogeny of excitatory amino acid receptors in the rat forebrain II. Kainic acid receptors. Neuroscience 35:45–51

Morton DH, Bennet MJ, Seargeant LE, Nichter CA, Kelley RI (1991) Glutaric aciduria type I: A common cause of episodic encephalopathy and spastic paralysis in the Amish of Lancaster Country, Pennsylvania. Am J Med Genet 41:89–95

Nakanishi S (1992) Molecular diversity of glutamate receptors and implications for brain function. Science 258:597–603

Nansen EA, Jokel ES, Lobo MK, Micevych PE, Ariano MA, Levine MS (2000) Striatal ionotropic glutamate receptor ontogeny in the rat. Dev Neurosci 22:329–340

Olney JW (1969) Brain lesions, obesity, and others disturbances in mice treated with monosodium glutamate. Science 164:719–721

Ozawa S, Kamiya H, Tsuzuki K (1998) Glutamate receptors in the mammalian central nervous system. Prog Neurobiol 54:581–618

Petralia RS, Esteban JA, Wang YX, Partridge JG, Zhao HM, Wenthold RJ, Malinow R (1999) Selective acquisition of AMPA receptors over postnatal development suggests a molecular basis for silent synapses. Nat Neurosci 2:31–36

Porciúncula LO, Dal-Pizzol A Jr, Coitinho AS, Emanuelli T, Souza DO, Wajner M (2000) Inhibition of synaptosomal [^{3}H]glutamate uptake and [^{3}H]glutamate binding to plasma membranes from brain of young rats by glutaric acid in vitro. J Neurol Sci 173:93–96

Porciúncula LO, Emanuelli T, Tavares RG, Schwarzbold C, Frizzo ME, Souza DO, Wajner M (2004) Glutaric acid stimulates glutamate binding and astrocytic uptake and inhibits vesicular glutamate uptake in forebrain from young rats. Neurochem Int 45:1075–1086

Ritter LM, Vazquez DM, Meador-Woodruff JH (2002) Ontogeny of ionotropic glutamate receptor subunit expression in the rat hippocampus. Brain Res Dev Brain Res 139:227–236

Rosa RB, Schwarzbold C, Dalcin KB, Ghisleni GC, Ribeiro CA, Moretto MB, Frizzo ME, Hoffmann GF, Souza DO, Wajner M (2004) Evidence that 3-hydroxyglutaric acid interacts with NMDA receptors in synaptic plasma membranes from cerebral cortex of young rats. Neurochem Int 45:1087–1094

Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger M, Wang Y, Schielke JP, Welty DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16:675–686

Sauer SW, Okun JG, Schwab MA, Crnic LR, Hoffmann GF, Goodman SI, Koeller DM, Kolker S (2005) Bioenergetics in glutaryl-coenzyme A dehydrogenase deficiency: a role for glutaryl-coenzyme A. J Biol Chem 280:21830–21836

Sauer SW, Okun JG, Fricker G, Mahringer A, Müller I, Crnic LR, Mühlhausen C, Hoffmann GF, Hörster F, Goodman SI, Harding CO, Koeller DM, Kölker S (2006) Intracerebral accumulation of glutaric and 3-hydroxyglutaric acids secondary to limited flux across the blood–brain barrier constitute a biochemical risk factor for neurodegeneration in glutaryl-CoA dehydrogenase deficiency. J Neurochem 97:899–910

Silva CG, Silva AR, Ruschel C, Helegda C, Wyse AT, Wannmacher CM, Dutra-Filho CS, Wajner M (2000) Inhibition of energy production in vitro by glutaric acid in cerebral cortex of young rats. Metab Brain Dis 15:123–131

Strauss KA, Morton H (2003) Type I glutaric aciduria, Part 2: A model of acute striatal necrosis. Am J Med Genet C Semin Med Genet 121:53–57

Strauss KA, Puffenberger EG, Robinson DL, Morton H (2003) Type I glutaric aciduria, Part 1: Natural history of 77 patients. Am J Med Genet C Semin Med Genet 121:38–52

Ullensvang K, Lehre KP, Storm-Mathisen J, Danbolt NC (1997) Differential development expression of the two rat brain glutamate transporter proteins GLAST and GLT. Eur J Neurosci 9:1646–1655

Ullrich K, Flott-Rahmel B, Schluff P, Musshoff U, Das A, Lucke T, Steinfeld R, Christensen E, Jakobs C, Ludolph A, Neu A, Roper R (1999) Glutaric aciduria type I: pathomechanisms of neurodegeneration. J Inherit Metab Dis 22:392–403

Capítulo III

Evidence that glutaric acid reduces glutamate uptake by cerebral cortex of infant rats

Rafael B. Rosa, Karina B. Dalcin, Anna L. Schmidt, Daniéli Gerhardt, César A. J. Ribeiro, Gustavo C. Ferreira, Patricia F. Schuck, Angela T. S. Wyse, Lisiane O. Porciúncula, Susana Wofchuk, Christianne G. Salbego, Diogo O. Souza, Moacir Wajner

Life Sciences 81: 1668-1676, 2007

Available online at www.sciencedirect.com
ScienceDirect
Life Sciences 81 (2007) 1668–1676
Life Sciences
www.elsevier.com/locate/lifescie

Evidence that glutaric acid reduces glutamate uptake by cerebral cortex of infant rats

Rafael B. Rosa [a], Karina B. Dalcin [a], Anna L. Schmidt [a], Daniéli Gerhardt [a], César A.J. Ribeiro [a], Gustavo C. Ferreira [a], Patricia F. Schuck [a], Angela T.S. Wyse [a], Lisiane O. Porciúncula [a], Susana Wofchuk [a], Christianne G. Salbego [a], Diogo O. Souza [a], Moacir Wajner [a,b,c,*]

[a] Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil
[b] Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
[c] Universidade Luterana do Brasil, Canoas, RS, Brazil

Received 13 June 2007; accepted 28 September 2007

Abstract

The role of excitotoxicity in the cerebral damage of glutaryl-CoA dehydrogenase deficiency (GDD) is under intense debate. We therefore investigated the in vitro effect of glutaric (GA) and 3-hydroxyglutaric (3-OHGA) acids, which accumulate in GDD, on [^{3}H]glutamate uptake by slices and synaptosomal preparations from cerebral cortex and striatum of rats aged 7, 15 and 30 days. Glutamate uptake was significantly decreased by high concentrations of GA in cortical slices of 7-day-old rats, but not in cerebral cortex from 15- and 30-day-old rats and in striatum from all studied ages. Furthermore, this effect was not due to cellular death and was prevented by *N*-acetylcysteine preadministration, suggesting the involvement of oxidative damage. In contrast, glutamate uptake by brain slices was not affected by 3-OHGA exposure. Immunoblot analysis revealed that GLAST transporters were more abundant in the cerebral cortex compared to the striatum of 7-day-old rats. Moreover, the simultaneous addition of GA and dihydrokainate (DHK), a specific inhibitor of GLT1, resulted in a significantly higher inhibition of [^{3}H]glutamate uptake by cortical slices of 7-day-old rats than that induced by the sole presence of DHK. We also observed that both GA and 3-OHGA exposure did not alter the incorporation of glutamate into synaptosomal preparations from cerebral cortex and striatum of rats aged 7, 15 and 30 days. Finally, GA in vivo administration did not alter glutamate uptake into cortical slices from 7-day-old rats. Our findings may explain at least in part why cortical neurons are more vulnerable to damage at birth as evidenced by the frontotemporal cortical atrophy observed in newborns affected by GDD.
© 2007 Elsevier Inc. All rights reserved.

Keywords: Glutaryl-CoA dehydrogenase deficiency; Glutamate transporters; Excitotoxicity

Introduction

Glutaryl-CoA dehydrogenase deficiency (GDD, McKusick 23167; OMIM # 231670) is an inherited metabolic disorder biochemically characterized by increased concentrations of glutaric (GA) and 3-hydroxyglutaric (3-OHGA) acids in the body fluids and in the brain (GA, 500–5000 μmol/L; 3-OHGA, 40–200 μmol/L) of affected individuals (Goodman et al., 1975; Goodman and Frerman, 2001; Kölker et al., 2004a; Sauer et al., 2006).

Neuropathology of this disease is characterized by frontotemporal cortical atrophy at birth, progressive spongy formation and attenuation of the white matter signal (leukoencephalopathy), as well as by acute bilateral destruction of caudate and putamen after encephalophatic crises precipitated by infections or vaccination in the first 36 months of age (Amir et al., 1987; Chow et al., 1988; Brismar and Ozand, 1995; Hoffmann and Zschocke, 1999). Thereafter, patients present severe dystonia–dyskinesia, sometimes associated with extreme hypotonia, rigidity and spasticity (Hoffmann and Zschocke, 1999; Strauss et al., 2003; Kölker et al., 2004a).

* Corresponding author. Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, Porto Alegre, RS, Brazil. Tel.: +55 51 21018011; fax +55 51 21018010.
E-mail address: mwajner@ufrgs.br (M. Wajner).

0024-3205/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2007.09.021

Although GDD is considered a "cerebral" organic acidemia because affected individuals present essentially neurological symptoms, the underlying mechanisms of brain damage in this disorder are only partly understood. In this scenario, considerable body of evidence have indicated that excitotoxicity, oxidative stress and energy impairment caused by the metabolites accumulating in GDD may be involved in the cerebral injury (Flott-Rahmel et al., 1997; Ullrich et al., 1999; Kölker et al., 1999, 2004a,b; Latini et al., 2002, 2005a,b; Sauer et al., 2005).

With regard to excitotoxicity, postmortem examination of the basal ganglia and cerebral cortex of patients with GDD revealed postsynaptic 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). This is in accordance with in vivo and in vitro studies demonstrating that GA and particularly 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, 2004a; Rosa et al., 2004; Wajner et al., 2004). However, recent works did not find excitotoxic actions for 3-OHGA (Lund et al., 2004; Freudenberg et al., 2004), so that the role of excitotoxicity in GDD pathophysiology is still under intense debate.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (Fonnum, 1984; Danbolt, 2001). Glutamate receptors are located on the surface of the neural cells. Since excessive stimulation of glutamate receptors may lead to neuronal damage, it is essential to keep the extracellular concentrations of glutamate low (Choi, 1992; Danbolt, 1994, 2001; Lipton and Rosenberg, 1994; Maragakis and Rothstein, 2001). Glutamate is removed from the synaptic cleft mainly by a glial high-affinity sodium-dependent excitatory amino acid transporter (EAAT) system (Danbolt, 2001; Schlüter et al., 2002). To date, five subtypes of glutamate transporters have been cloned: GLAST (EAAT1), GLT1 (EAAT2), EAAC1 (EAAT3), EAAT4 and EAAT5 (Danbolt, 2001). GLAST and GLT1 are predominantly localized in astrocytes and mainly responsible for the clearance of glutamate from the synaptic cleft in rat brain, while EAAC1, EAAT4 and EAAT5 appear to be mostly expressed in neurons (Rothstein et al., 1996; Berger and Hediger, 1998; Kugler and Schmitt, 1999; Danbolt, 2001; Amara and Fontana, 2002). The expression of these transporter subtypes changes along rat brain development and differs among cerebral structures (Ullensvang et al., 1997; Furuta et al., 1997; Schlüter et al., 2002). Thus, GLAST is well expressed at birth, in contrast to GLT1 whose content increases from the second to the third postnatal week in the rat brain. Both transporters are fully expressed at postnatal week 5, while GLT1 is the predominant glutamate astroglial transporter in the adult brain (Ullensvang et al., 1997).

The objective of the present investigation was to study the effects of GA and 3-OHGA, at the concentrations usually found in GDD, on glutamate transport in the CNS during rat brain development. We therefore evaluated the role of these metabolites on glutamate uptake by slices and synaptosomal preparations from cerebral cortex and striatum of rats aged 7 to 30 days. We choose these rat ages because children with GDD are more vulnerable to metabolic stress during the first 3 years (Bjugstad et al., 2000), which correspond approximately to 7–15 days of life in the rat, whereas 30-day-old rats match to young adult humans (Haberny et al., 2002). We also examined the effects of GA on the viability of cortical slices and the density of GLAST transporters in cerebral cortex and striatum at an early age.

Materials and methods

Animals and reagents

Wistar rats of 7, 15 and 30 days of life from our breeding colony were used. They were maintained at 25 °C, on a 12:12 h light/dark cycle, with free access to food and water. The "Principles of laboratory animal care" (NIH publications No. 80-23, revised 1996) were followed in all experiments and the protocols approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul. All efforts were made to minimize the number of animals used and their suffering. All chemicals, including glutaric acid free acid (GA, 99% pure), were purchased from Sigma (St Louis, MO, USA), whereas 3-hydroxyglutaric acid (3-OHGA free acid, 99% pure) was prepared by Dr Ernesto Brunet (Universidad Autonoma de Madrid, Spain) and L-[^{3}H]glutamate (52 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). GA and 3-OHGA solutions were prepared on the day of the experiments. The acids were first dissolved in the buffer solutions utilized in each assay and the pH was adjusted to 7.4 with 0.5 M NaOH when necessary. For the nonspecific uptake assays, these organic acid solutions were neutralized to pH 7.4 with *N*-methyl-D-glucamine (glutamate uptake by slices) or Trizma base solution (synaptosomal glutamate uptake). Control and experimental groups (GA or 3-OHGA) contained the same concentrations of the counter ion in the incubation medium.

Glutamate uptake by cerebral cortex and striatum slices

The animals were decapitated, the brain was immediately removed and submerged in Hank's balanced salt solution (HBSS), containing 137 mM NaCl, 0.63 mM Na_2HPO_4, 4.17 mM $NaHCO_3$, 5.36 mM KCl, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2$, 0.41 mM $MgSO_4$, 0.49 mM $MgCl_2$ and 1.11 mM glucose, adjusted to pH 7.2. Cerebral cortex and striatum were dissected and tissue slices (400 μm) were obtained using a McIlwain chopper. The slices were washed with HBSS.

Glutamate uptake was performed according to Frizzo et al. (2002). Slices from 7-, 15- and 30-day-old rats were preincubated at 35 °C for 23 min in the presence or absence of GA (1–50 mM) or 3-OHGA (0.1–1 mM). Some experiments were performed in the presence of 0.1 mM or 1 mM dihydrokainic acid (DHK), which is a specific inhibitor of GLT1 transporters (Moussa et al., 2007). Incubation was carried out at 35 °C by adding 100 μM [^{3}H]glutamate (0.1 μCi) in HBSS to the assays. The reaction was stopped after 7 min by two ice-cold washing with 1 mL HBSS, immediately followed by addition of 0.5 M NaOH. Sodium

independent uptake (nonspecific uptake) was determined by using 140 mM *N*-methyl-D-glucamine instead of sodium chloride. Sodium dependent uptake was calculated as the difference between glutamate uptake measured in the medium containing sodium and in the medium containing *N*-methyl-D-glucamine. Radioactivity incorporated was determined using a Wallac 1409 liquid scintillation counter. All experiments were performed in triplicate in 24 well culture plates. Results were calculated as nmol $[^3H]$glutamate/min/mg protein and expressed as percentage of control.

Acute GA treatment and glutamate uptake

GA in vivo treatment was carried out according to Ferreira et al. (2005). Seven-day-old rats were administered with three subcutaneous injections of GA buffered to pH 7.4 with NaOH (5 μmol/g body weight) or saline solution (control group) at a 90 min interval. This model produced brain GA concentrations of approximately 0.72–1.08 mM (0.6–0.9 μmol/g fresh brain). Animals were killed by decapitation 1 h after the last injection and cerebral cortex was dissected and cut into 400 μm slices. Glutamate uptake assay was carried out as described above.

Viability assays

Thiazolyl blue tetrazolium (MTT) assay

The viability assay was performed by the colorimetric thiazolyl blue tetrazolium bromide (MTT) method, with slight modifications (Funchal et al., 2004). Cortical slices of 7-day-old rats were preincubated at 35 °C for 30 min in HBSS in the presence or absence of 5 mM GA. Immediately after preincubation, 0.5 mg/ml of MTT was added to the medium containing the slices, followed by incubation at 35 °C for 60 min. The formazan product generated during the incubation was extracted with dimethyl sulfoxide (DMSO) and measured spectrophotometrically at 570 and 630 nm. Brain slice viability was evaluated using the A570 minus A630 values as an index of MTT reduction. Only viable slices are able to reduce MTT.

Lactate dehydrogenase (LDH) assay

Cortical slices from 7-day-old rats were incubated at 35 °C for 30 min in HBSS in the presence or absence of 5 mM 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 Doles reagents. 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.

Western blot analysis

GLAST immunocontent was measured in cerebral cortex and striatum slices from 7-day-old rats. The animals were decapitated, their brains were immediately removed and humidified with HBSS. Striatum and cerebral cortex were dissected and 400 μm slices obtained with a McIlwain tissue chopper. The slices were then homogenized in 50 mM Triz buffer, containing 4% sodium dodecylsulfate (SDS) and 2.1 mM EDTA. Aliquots were taken for protein determination and β-mercaptoethanol was added to a final concentration of 5%. Samples containing 40 μg of protein were resolved by 12% sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were electrotransferred to nitrocellulose membranes using a semi-dry blotting system (Bio-Rad Trans-Blot SD). Membranes were blocked for 60 min with 5% powdered milk in tween–Triz-buffered saline (M–T-TBS) and further incubated overnight at 4 °C with the appropriate primary antibody dissolved in M–T-TBS. The primary antibody used was anti-GLAST (1:500, Santa Cruz Biotechnology, CA, USA). After washing, the membranes were incubated for 2 h with anti-rabbit IgG peroxidase-conjugated (secondary antibody) (1:1000, Amersham plc). Immunoreactive bands were revealed by an enhanced chemiluminescence kit (ECL, Pharmacia Corp) and detected using X-ray films. The immunoblot films were scanned and the digitalized images analyzed with the Optiquant software (Packard Instrument). The same blots were re-probed with β-actin antibody (1:1000, Cell Signaling Technology) as an internal control. Results were expressed as total digital light units (DLU)/mm^2.

Synaptosomal preparation and glutamate uptake

Animals aged 7, 15 and 30 days were killed by decapitation without anesthesia and synaptosomes were prepared according to Gonçalves et al. (1997). Cerebral cortex and striatum were homogenized in 10 mL of 0.32 M sucrose solution (containing 1 mM EDTA, 5 mM HEPES and 1 mg/mL bovine serum albumin, pH 7.4) and centrifuged at 3000 ×*g* for 10 min at 4 °C. The supernatant was diluted to 10 mL with sucrose solution and centrifuged at 14000 ×*g* for 12 min at 4 °C. The pellet was resuspended in 1.8 mL of a 45% (vol/vol) Percoll® solution made up with Krebs solution (140 mM NaCl, 1 mM EDTA, 10 mM HEPES, 5 mM KCl and 5 mM glucose, pH 7.4) and centrifuged at 14000 ×*g* for 2 min at 4 °C. The top layer was washed with Krebs solution and again centrifuged at 14000 ×*g* for 2 min at 4 °C. The pellet containing the synaptosomal fraction was resuspended in a medium containing 0.32 sucrose and 20 mM Tris/acetate buffer, pH 7.4. Synaptosomal associated protein (SNAP25) was used as a marker to characterize our synaptosomal preparations. These preparations with approximately 90% purity were preincubated with 5 mM GA or 1 mM 3-OHGA for 15 min at 37 °C. Controls did not contain GA or 3-OHGA in the incubation medium. LDH assay was also performed to evaluate the synaptosomal integrity in the presence of GA and 3-OHGA.

Synaptosomal $[^3H]$glutamate uptake was measured in a medium containing 110 mM NaCl, 25 mM glucose, 5.3 mM KCl, 1 mM MgSO4, 1.8 mM CaCl2, 6 mM sucrose and 40 mM Tris/acetate, pH 7.4 and 1 μM $[^3H]$glutamate (0.5 μCi). The reaction was started by addition of 100–200 μg protein (synaptosomal preparation). After 1 min of incubation at 37 °C, the uptake was stopped by filtration through GF/B filters. Filters were washed at vacuum three times with 3 mL of ice-cold 15 mM Triz/acetate containing 155 mM ammonium acetate, pH 7.4. Radioactivity was determined using a Wallac 1409 liquid scintillation counter. Sodium independent

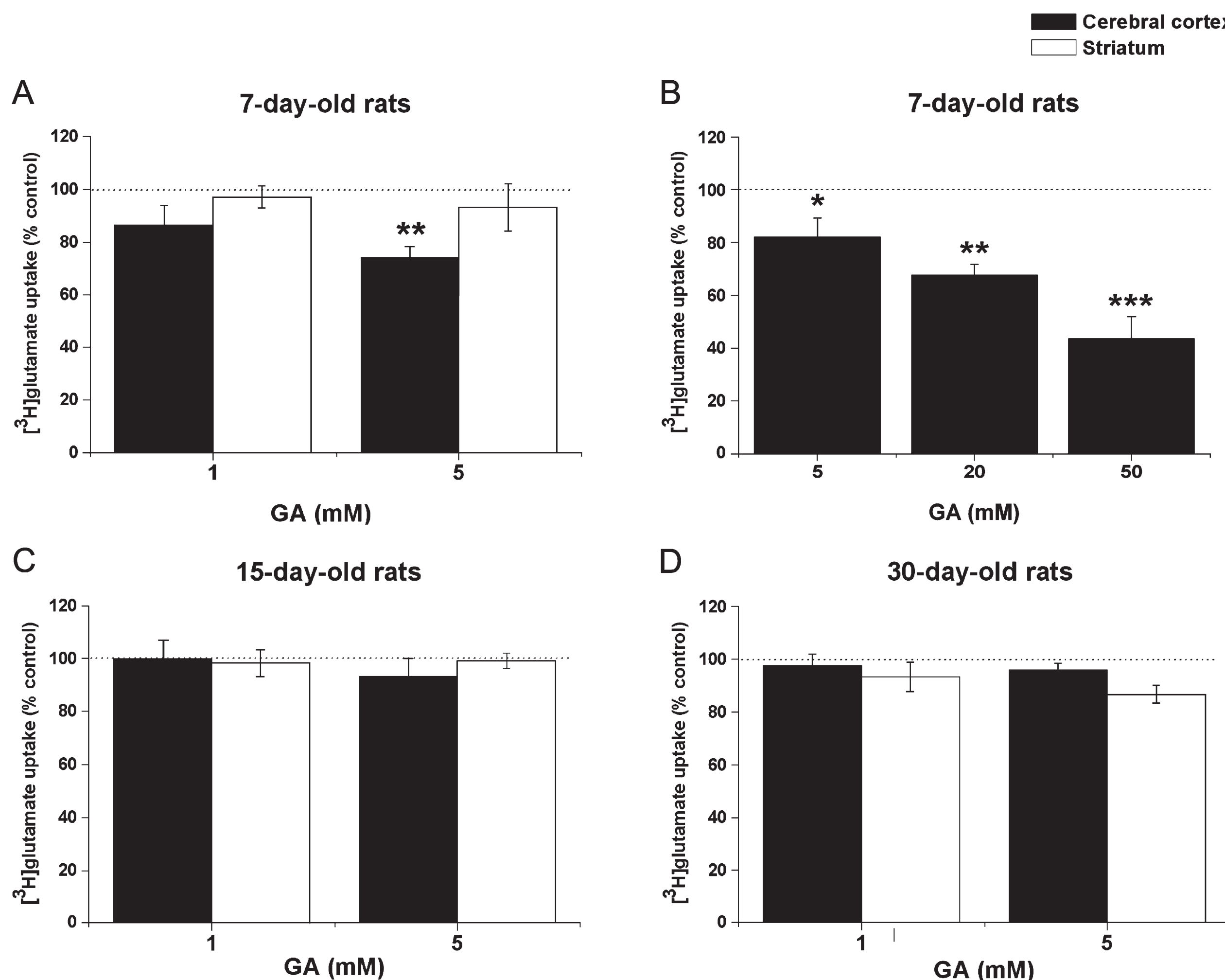

Fig. 1. Effect of glutaric acid (GA, 1–50 mM) on [^{3}H]glutamate uptake by tissue slices from cerebral cortex and striatum of rats aged 7 (A, B), 15 (C) and 30 (D) days. Results are presented as mean±SEM from three to six independent experiments performed in triplicate and are expressed as percentage of controls. Control (100%) values expressed as nmol [^{3}H]glutamate/min/mg protein correspond to: cerebral cortex 0.965±0.22 (7-day-old), 0.644±0.06 (15-day-old) and 0.282±0.06 (30-day-old); striatum 0.928±0.26 (7-day-old), 0.615±0.09 (15-day-old) and 0.321±0.06 (30-day-old). *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with controls (Duncan's multiple range test).

glutamate uptake was measured in a medium without sodium containing 0.32 M sucrose and 20 mM Triz/acetate, pH 7.4. Synaptosomal [^{3}H]glutamate uptake was calculated as the difference between the sodium-dependent and the sodium-independent uptake. All experiments were performed in triplicate in polycarbonated tubes. Results were calculated as pmol [^{3}H]glutamate/min/mg protein.

N-acetylcysteine administration

Four-day-old rats were divided into two groups and treated with one daily intraperitoneal injection of saline solution or *N*-acetylcysteine dissolved in saline (NAC, 150 mg/kg body weight) during three days (Farbiszewski et al., 2000; Ocal et al., 2004). The animals were sacrificed one day after

Table 1
Effect of 3-hydroxyglutaric acid (3-OHGA) on [^{3}H]glutamate uptake by cerebral cortex and striatum slices from rats aged 7, 15 and 30 days

3-OHGA (mM)	Animal age					
	7-day-old		15-day-old		30-day-old	
	Cerebral cortex	Striatum	Cerebral cortex	Striatum	Cerebral cortex	Striatum
0.1	98.8±3.99	103.5±3.26	97.2±5.60	96.3±1.95	94.0±5.01	93.2±6.11
1	88.8±6.12	91.9±8.00	94.1±4.86	95.8±5.34	82.3±7.94	90.5±6.33

Results are presented as mean±SEM of four to six independent experiments performed in triplicate and are expressed as percentage of controls. Control (100%) values (nmol [^{3}H]glutamate/min/mg protein) correspond to: cerebral cortex: 0.834±0.06 (7-day-old), 0.659±0.07 (15-day-old) and 0.671±0.11 (30-day-old); striatum: 0.816±0.12 (7-day-old), 0.373±0.05 (15-day-old) and 0.397±0.09 (30-day-old). No significant differences from controls were observed (ANOVA).

Table 2
Effect of glutaric acid (GA, 5 mM) on the viability of cortical slices from 7-day-old rats determined by MTT reduction and LDH release

Viability assays	Control	5 mM GA	Triton X-100
MTT reduction (A570–A630)	0.48±0.02	0.55±0.03	ND
LDH (% release)	27.0±7.4	29.5±5.38	99.7±3.44**

Results are reported as mean±SEM of five independent experiments in each group. No significant difference between control and GA was detected in MTT reduction (Student's *t*-test for paired samples). LDH release: $^{**}P<0.01$ compared to control and GA (Duncan's multiple range test). ND=not done.

treatment and glutamate uptake was measured as described before in the presence or absence of 5 mM GA.

Protein determination

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

Statistical analysis

Data were analyzed by one- or two-way analysis of variance (ANOVA) followed by the Duncan's multiple range test when appropriate. Linear regression analysis was performed to verify dose-dependent effects. Student's *t*-test for paired and independent samples was also used for comparison of two means. Only significant values are shown in the text. Analyses were performed using the SPSS (Statistical Package for the Social Sciences) software in a PC-compatible computer. A value of $P<0.05$ was considered to be significant.

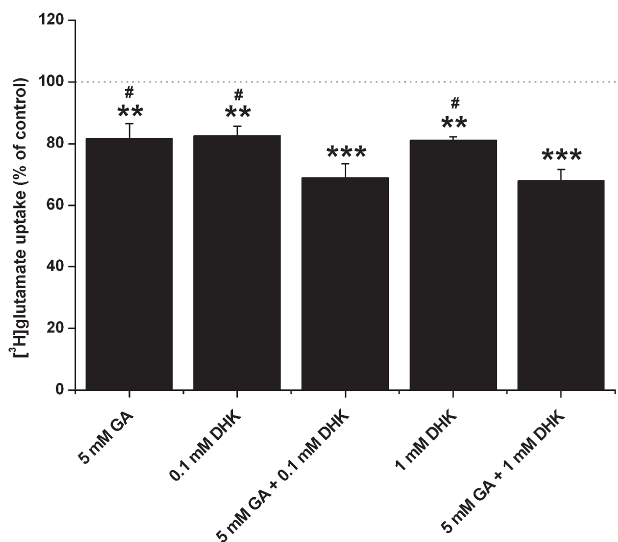

Fig. 2. Effect of glutaric acid (GA, 5 mM) and dihydrokainic acid (DHK, 0.1 mM and 1 mM) on [^{3}H]glutamate uptake by tissue slices from cerebral cortex of 7-day-old rats. Results are presented as mean±SEM from two to five independent experiments performed in triplicate and are expressed as percentage of controls. Control (100%) values correspond to: 0.768±0.11 nmol [^{3}H]glutamate/min/mg protein. $^{**}P<0.01$, $^{***}P<0.001$ vs control, $^{\#}P<0.05$ vs 5 mM GA+0.1 mM DHK and 5 mM GA+1 mM DHK (Duncan's multiple range test).

Results

GA inhibits glutamate uptake by cortical brain slices from 7-day-old rats

We first evaluated the effect of GA on [^{3}H]glutamate uptake by cerebral cortex and striatum slices from 7-, 15- and 30-day-old rats. Fig. 1 shows that GA, at 5 mM and higher concentrations, selectively decreased [^{3}H]glutamate uptake into cerebral cortex slices from 7-day-old rats (A) [$F(2,12)=6.97$; $P<0.05$] in a dose-dependent manner (B) [$F(3,15)=17.94$; $P<0.001$] ($\beta=-0.857$, $P<0.001$), but not from 15- (C) and 30-day-old animals (D). The figure also shows that GA had no effect on [^{3}H]glutamate uptake into striatum at all ages examined. In contrast, 3-OHGA did not change [^{3}H]glutamate uptake by cerebral cortex and striatum slices of rats aged 7, 15 and 30 days (Table 1).

The next experiments were performed in brain slices from 7-day-old rats since glutamate uptake was only altered by GA at this age. Thus, we evaluated the viability of brain slices measured by the MTT reduction and the LDH release assays in order to verify whether cellular death could be responsible for the GA-induced glutamate uptake reduction. We observed that 5 mM GA did not alter cellular viability in cortical slices from 7-day-old rats (Table 2).

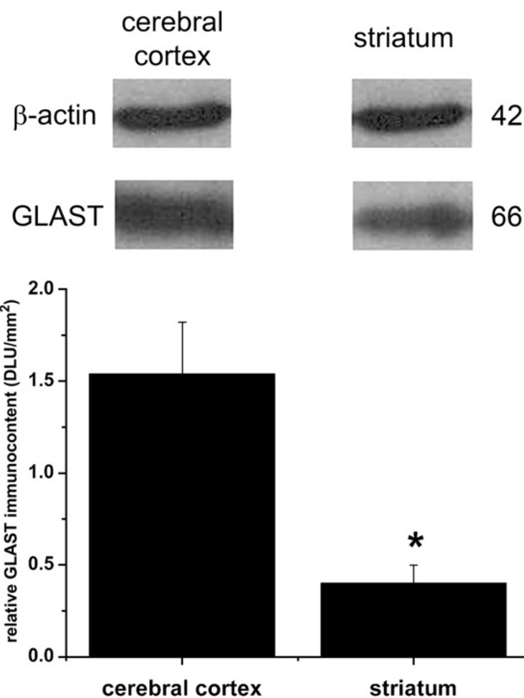

Fig. 3. Representative Western blots of GLAST transporters in tissue slices from cerebral cortex and striatum of 7-day-old rats. Equal amounts of protein samples were analyzed by polyacrylamide gel electrophoresis and immunoblotting with antibodies against GLAST and β-actin. The molecular weight of each protein (in kilodalton) is indicated on the right. Densitometric measurements were performed on individual immunoblot analysis obtained from two slices per structure. The densitometric values obtained were first normalized to their respective β-actin densitometric values and then expressed as total digital light units DLU/mm^2. Data represent relative optical density and are expressed as mean±SEM from three independent experiments. $^{*}P<0.05$ (Student's *t*-test for paired samples).

GA in vivo treatment does not affect glutamate uptake by cerebral cortex slices

We also investigated the effect of in vivo administration of GA on glutamate uptake by cerebral cortex slices from 7-day-old rats and verified that this uptake was not altered (data not shown).

Glial transporters are probably involved in GA-induced decrease of glutamate uptake by cortical slices

Next, we examined the effect of 5 mM GA on [^{3}H]glutamate uptake by cerebral cortex slices of 7-day-old rats in the presence of 0.1 mM to 1 mM dihydrokainate (DHK), a specific inhibitor of GLT1, in order to determine which glutamate transporter subtype was involved in GA-induced reduction of this uptake. It can be seen in Fig. 2 that 5 mM GA and DHK, at the distinct concentrations used, inhibited this uptake by approximately the same degree (20%), whereas the simultaneous addition of both compounds (5 mM GA plus 0.1 mM DHK or 5 mM GA plus 1 mM DHK) resulted in a significantly higher inhibition (32%) [$F(5,18)=11.47$; $P<0.001$]. In addition, immunoblot analysis revealed that GLAST immunocontent was approximately 3–4-fold higher in cerebral cortex of 7-day-old rats compared to the striatum [$t(2)=4.53$; $P<0.05$] (Fig. 3).

N-acetylcysteine (NAC) administration prevents the GA-induced inhibition of glutamate uptake into cortical slices

The involvement of oxidative damage in the reduced glutamate uptake caused by GA on cortical slices was investigated by pretreating 4-day-old rats with a daily administration of the glutathione precursor *N*-acetylcysteine (NAC, 150 mg/Kg body weight) for three days. Pretreatment with NAC completely prevented GA-induced inhibition of glutamate uptake [significant pretreatment (NAC or saline) X treatment (control or GA) interaction [$F(1,8)=8.62$; $P<0.05$] (Fig. 4).

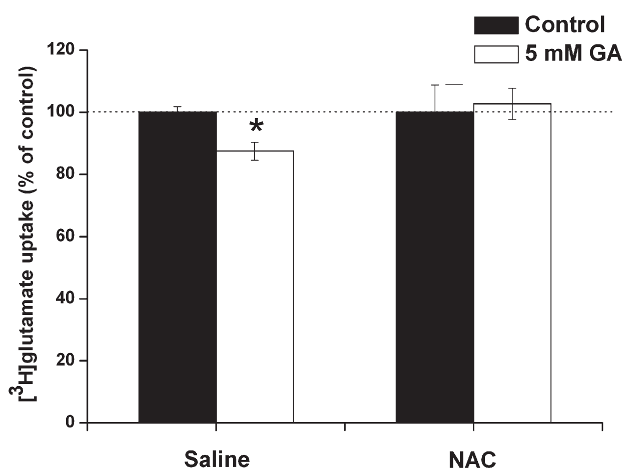

Fig. 4. Effect of *N*-acetylcysteine (NAC) pretreatment on in vitro glutaric acid (GA, 5 mM)-induced reduction of [^{3}H]glutamate uptake by cerebral cortex slices from 7-day-old rats. Results are presented as mean ± SEM from five independent experiments performed in triplicate and are expressed as percentage of controls. Control saline: 0.839 ± 0.02 nmol [^{3}H]glutamate/min/mg protein; Control NAC: 0.829 ± 0.07 nmol [^{3}H]glutamate/min/mg protein. *$P<0.05$ (two-way ANOVA).

GA and 3-OHGA do not alter synaptosomal glutamate uptake

We first observed that the viability of the synaptosomal preparations determined by LDH release was not altered by GA (5 mM) and 3-OHGA (1 mM). Furthermore, we evaluated the effect of GA (5 mM) and 3-OHGA (1 mM) on [^{3}H]glutamate uptake by synaptosomal preparations from cerebral cortex and striatum of 7-, 15- and 30-old rats. We observed that both GA and 3-OHGA did not affect [^{3}H]glutamate uptake by synaptosomal preparations from cerebral cortex and striatum at all studied ages (data not shown).

Discussion

Excitotoxicity has been proposed as an important neurotoxic mechanism in GDD pathophysiology (Flott-Rahmel et al., 1997; Kölker et al., 1999, 2004a; Wajner et al., 2004; Rosa et al., 2004), although this is disputed (Lund et al., 2004; Freudenberg et al., 2004). It is conceivable that these conflicting results 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 and 3-OHGA on glutamate transport in slices and synaptosomal preparations from cerebral cortex and striatum during rat brain development. The main objective was to verify whether age-dependent alterations of glutamate uptake could explain the window of vulnerability of striatum and cerebral cortex injury in GDD children.

We initially observed that glutamate uptake was selectively inhibited in cortical slices of 7-day-old rats by GA exposure in a dose-dependent manner, with no alteration of this uptake in cerebral cortex from 15- and 30-day-old rats and in striatum slices at all rat ages. Furthermore, this effect was not due to cellular death, as evidenced by the MTT and LDH viability tests. In contrast, 3-OHGA did not change glutamate uptake by slices at all rat ages and in the cerebral structures studied, indicating that GA effect was specific rather than an unspecific action due to an acidic compound. These results suggest that specific glutamate transporters localized in cerebral cortex at an early age of rat development could be responsible for this effect. Thus, considering that the glial GLAST and GLT1 glutamate transporters and the neuronal glutamate transporter EAAC1 are present in our slice preparations, one or more of these transporters could serve as a target for GA inhibitory effect. Our experiments with the simultaneous addition of the specific GLT1 inhibitor DHK (0.1 to 1 mM) and 5 mM GA resulted in a significantly higher inhibitory effect on glutamate uptake (32%) in cortical slices from 7-day-old animals compared to the effect (20%) elicited by DHK or GA alone. We also observed that 0.1 or 1 mM DHK provoked the same degree of glutamate uptake inhibition, implying that GLT1 transporters were totally inhibited by this compound at doses as low as 0.1 mM.

Therefore, it is feasible that GA, by provoking a higher inhibition when associated to DHK, probably acted on other carriers such as GLAST transporters.

Furthermore, synaptosomal glutamate uptake in cerebral cortex and striatum of rats aged 7–30 days was not altered by GA and 3-OHGA exposure. Previous studies have demonstrated that glutamate uptake into synaptosomal preparations is mediated by GLT1 transporters (Robinson et al., 1993; Bridges et al., 1999), which is in accordance with the fact that synaptosomes prepared from GLT1 deficient mutant mice have very low uptake activities (Tanaka et al., 1997; Danbolt, 2001). It has been also proposed that glutamate uptake into synaptosomal preparations is due to glial cell fragments contaminating these preparations (Nakamura et al., 1993). Taken together our observations in cortical slices and synaptosomal preparations, it may be presumed that the GLT1 carrier was not mainly involved in GA inhibitory effect.

On the other hand, Western blotting analysis revealed that cerebral cortex from 7-day-old rats presented 3–4-fold higher density of GLAST transporters as compared to the striatum, in accordance with the observations that GLAST predominates in the cerebral cortex compared to the striatum at early ages of rat development (Furuta et al., 1997). Therefore, it might be proposed that GLAST transporter were mainly involved in GA action. In this scenario, the reasons by which GA did not interfere with glutamate uptake in rat striatum may be related to the fact that the major carrier involved with glutamate transport in the striatum at early postnatal ages is EAAC1 (Furuta et al., 1997) and that at later ages the expression of GLT1 transporters in this cerebral structure is predominant (Ullensvang et al., 1997).

A further result of the present investigation was that in vivo pretreatment of *N*-acetylcysteine, a glutathione precursor (Karageorgos et al., 2006), fully prevented the GA-induced reduction of glutamate uptake by cortical slices, suggesting that oxidative damage of glutamate transporters may be also involved in this effect. Previous findings showing that glutamate transporter activity can be inhibited by oxidation (Volterra et al., 1994; Trotti et al., 1996, 1998) and that GA induces oxidative stress in brain of young rats (de Oliveira et al., 2003) reinforce this hypothesis.

On the other hand, in vivo administration of GA did not alter glutamate uptake into cortical slices from 7-day-old rats. These data suggest that higher brain GA concentrations than those achieved by subcutaneous GA administration (Ferreira et al., 2005) are necessary to impair glutamate uptake in vivo. It should be also considered that the final concentrations of GA in the brain slice preparations used to measure glutamate uptake after in vivo subcutaneous GA administration were lower than those achieved in our model (approximately 1 mM) because cerebral cortex slices were extensively washed with HBSS buffer.

With regard to the pathophysiological significance of our present data, we showed moderate alterations of glutamate transport caused by high doses of GA (5 mM and higher) in vitro, but not after in vivo GA administration, indicating that impairment of glutamate transport cannot solely explain the severe brain abnormalities of GDD patients. Probably distinct mechanisms, such as excitotoxicity (Flott-Rahmel et al., 1997; Kölker et al., 2004a; Wajner et al., 2004), energetic dysfunction (Ullrich et al., 1999; Kölker et al., 2004b; Latini et al., 2005a; Sauer et al., 2005), oxidative stress (Latini et al., 2002, 2005b; de Oliveira et al., 2003) and perhaps alterations of the kynurenine pathway (Heyes, 1987) act synergistically to cause neurodegeneration in this disorder.

In conclusion, to our knowledge this is the first report showing a regional and age-specific window of vulnerability for GA action reducing glutamate uptake by cerebral cortex from infant rats. The present data may explain at least partly why cortical neurons are more susceptible to damage at birth as evidenced by the frontotemporal cortical atrophy observed in newborns with GDD (Goodman, 2004). However, further investigation should be carried out to define the window of striatum susceptibility in this disorder.

Acknowledgements

This work was supported by CNPq and the FINEP research grant Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00.

References

Amara, S.G., Fontana, A.C., 2002. Excitatory amino acid transporters: keeping up with glutamate. Neurochemistry International 41 (5), 313–318.

Amir, N., el-Peleq, O., Shalev, R.S., Christensen, E., 1987. Glutaric aciduria type I: clinical heterogeneity and neuroradiologic features. Neurology 37 (10), 1654–1657.

Berger, U.V., Hediger, M.A., 1998. Comparative analysis of glutamate transporter expression in rat brain using differential double in situ hybridization. Anatomy and Embryology (Berl) 198 (1), 13–30.

Bjugstad, K.B., Goodman, S.I., Freed, C.R., 2000. Age at symptom onset predicts severity of motor impairment and clinical outcome of glutaric academia type 1. The Journal of Pediatrics 137 (5), 681–686.

Bridges, R.J., Kavanaugh, M.P., Chamberlin, A.R., 1999. A pharmacological review of competitive inhibitors and substrates of high-affinity, sodium-dependent glutamate transport in the central nervous system. Current Pharmaceutical Design 5 (5), 363–379.

Brismar, J., Ozand, P.T., 1995. CT and MR of the brain in glutaric academia type I: a review of 59 published cases and a report of 5 new patients. AJNR. American Journal of Neuroradiology 16 (4), 675–683.

Choi, D.W., 1992. Excitotoxic cell death. Journal of Neurobiology 23 (9), 1261–1276.

Chow, C.W., Haan, E.A., Goodman, S.I., Anderson, R.M., Evans, W.A., Kleinschimidt-DeMasters, B.K., Wise, G., McGill, J.J., Danks, D.M., 1988. Neuropathology in glutaric acidaemia type I. Acta Neuropathologica (Berl) 76 (6), 590–594.

Danbolt, N.C., 1994. The high affinity uptake system for excitatory amino acids in the brain. Progress in Neurobiology 44 (4), 377–396.

Danbolt, N.C., 2001. Glutamate uptake. Progress in Neurobiology 65 (1), 1–105.

de Oliveira Marques, F., Hagen, M.E., Pederzolli, C.D., Sgaravatti, A.M., Durigon, K., Testa, C.G., Wannmacher, C.M.D., de Souza Wyse, A.T., Wajner, M., Dutra-Filho, C.S., 2003. Glutaric acid induces oxidative stress in brain of young rats. Brain Research 964 (1), 153–158.

Farbiszewski, R., Witek, A., Skrzydlewska, E., 2000. *N*-acetylcysteine or trolox derivative mitigate the toxic effects of methanol on the antioxidant system of rat brain. Toxicology 156 (1), 47–55.

Ferreira, G., da, C., Viegas, C.M., Schuck, P.F., Tonin, A., Ribeiro, C.A., Coelho, D., de, M., Dalla-Costa, T., Latini, A., Wyse, A.T., Wannmacher, C.M., Vargas, C.R., Wajner, M., 2005. Glutaric acid administration impairs energy metabolism in midbrain and skeletal muscle of young rats. Neurochemical Research 30 (9), 1123–1131.

Flott-Rahmel, B., Falter, C., Schluff, P., Fingerhut, R., Christensen, E., Jakobs, C., Musshoff, U., Fautek, J.D., Ludolph, A., Ullrich, K., 1997. Nerve cell lesions caused by 3-hydroxyglutaric acid: a possible mechanism for neurodegeneration in glutaric acidaemia I. Journal of Inherited Metabolic Disease 20 (3), 387–390.

Fonnum, F., 1984. Glutamate: a neurotransmitter in mammalian brain. Journal of Neurochemistry 42 (1), 1–11.

Forstner, R., Hoffmann, G.F., Gassner, I., Heideman, P., De Klerk, J.B., Lawrenz-Wolf, B., Doringer, E., Weiss-Wichert, P., Troger, J., Colombo, J.P., Plochl, E., 1999. Glutaric aciduria type I: ultrasonographic demonstration of early signs. Pediatric Radiology 29 (2), 138–143.

Freudenberg, F., Lukacs, Z., Ullrich, K., 2004. 3-Hydroxyglutaric acid fails to affect the viability of primary neuronal rat cells. Neurobiology of Disease 16 (3), 581–584.

Frizzo, M.E.S., Lara, D.R., Prokopiuk, A.S., Vargas, C.R., Salbego, C.G., Wajner, M., Souza, D.O., 2002. Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. Cellular and Molecular Neurobiology 22 (3), 353–363.

Funchal, C., Rosa, A.M., Wajner, M., Wofchuk, S., Pureur, R.P., 2004. Reduction of glutamate uptake into cerebral cortex of developing rats by the branched-chain alpha-keto acids accumulating in maple syrup urine disease. Neurochemical Research 29 (4), 747–753.

Furuta, A., Rothstein, J.D., Martin, L.J., 1997. Glutamate transporter protein subtypes are expressed differentially during rat CNS development. The Journal of Neuroscience 17 (21), 8363–8375.

Gonçalves, M.L., Cunha, R.A., Ribeiro, J.A., 1997. Adenosine A_{2A} receptors facilitate $^{45}Ca^{2+}$ uptake through class A calcium channels in rat hippocampal CA3 but not CA1 synaptosomes. Neuroscience Letters 238 (1–2), 73–77.

Goodman, S.I., 2004. Development of pathogenic concepts in glutaryl-CoA dehydrogenase deficiency: the challenge. Journal of Inherited Metabolic Disease 27 (6), 801–803.

Goodman, S.I., Frerman, F.E., 2001. Organic acidemias due to defects in lysine oxidation: 2-ketoadipic acidemia and glutaric acidemia, In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds.), The Metabolic and Molecular Bases of Inherited Disease, 8th ed. McGraw-Hill, New York, pp. 2195–2204.

Goodman, S.I., Markey, S.D., Moer, P.G., Miles, B.S., Teng, C.C., 1975. Glutaric aciduria: a "new" disorder of amino acid metabolism. Biochemical Medicine 12 (1), 12–21.

Goodman, S.I., Norenberg, M.D., Shikes, R.H., Breslich, D.J., Moe, P.G., 1977. Glutaric aciduria: biochemical and morphological considerations. The Journal of Pediatrics 90 (5), 746–750.

Haberny, K.A., Paule, M.G., Scallet, A.C., Sistare, F.D., Lester, D.S., Hanig, J.P., Slikker Jr., W., 2002. Ontogeny of the *N*-methyl-D-aspartate (NMDA) receptor system and susceptibility to neurotoxicity. Toxicological Sciences 68 (1), 9–17.

Heyes, M.P., 1987. Hypothesis: a role for quinolinic acid in the neuropathology of glutaric aciduria type I. The Canadian Journal of Neurological Sciences 14 (3 suppl), 441–443.

Hoffmann, G.F., Zschocke, J., 1999. Glutaric aciduria type I: from clinical, biochemical and molecular diversity to successful therapy. Journal of Inherited Metabolic Disease 22 (4), 381–391.

Karageorgos, N., Patsoukis, N., Chroni, E., Konstantinou, D., Assimakopoulos, S.F., Georgiou, C., 2006. Effect of *N*-acetylcysteine, allopurinol and vitamin E on jaundice-induced brain oxidative stress in rats. Brain Research 1111 (1), 203–212.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 1999. 3-Hydroxyglutaric and glutaric acids are neurotoxic through NMDA receptors in vitro. Journal of Inherited Metabolic Disease 22 (3), 259–262.

Kölker, S., Koeller, D.M., Okun, J.G., Hoffmann, G.F., 2004a. Pathomechanisms of neurodegeneration in glutaryl-CoA dehydrogenase deficiency. Annals of Neurology 55 (1), 7–12.

Kölker, S., Koeller, D.M., Sauer, S., Horster, F., Schwab, M.A., Hoffmann, G.F., Ullrich, K., Okun, J.G., 2004b. Excitotoxicity and bioenergetics in glutaryl-CoA dehydrogenase deficiency. Journal of Inherited Metabolic Disease 27 (6), 805–812.

Kölker, S., Kohr, G., Ahlemeyer, B., Okun, J.G., Pawlak, V., Horster, F., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., 2002a. Ca(2+) and Na(+) dependence of 3-hydroxyglutarate-induced excitotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatric Research 52 (2), 199–206.

Kölker, S., Okun, J.G., Ahlemeyer, B., Wyse, A.T., Horster, F., Wajner, M., Kohlmuller, D., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., 2002b. Chronic treatment with glutaric acid induces partial tolerance to excitotoxicity in neuronal cultures from chick embryo telencephalons. Journal of Neurosciences Research 68 (4), 424–431.

Kugler, P., Schmitt, A., 1999. Glutamate transporter EAAC1 is expressed in neurons and glial cells in the rat nervous system. Glia 27 (2), 129–142.

Latini, A., Borba Rosa, R., Scussiato, K., Llesuy, S., Bello-Klein, A., Wajner, M., 2002. 3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats. Brain Research 956 (2), 367–373.

Latini, A., Rodriguez, M., Borba Rosa, R., Scussiato, K., Leipnitz, G., Reis de Assis, D., da Costa Ferreira, G., Funchal, C., Jacques-Silva, M.C., Buzin, L., Giugliani, R., Cassina, A., Radi, R., Wajner, M., 2005a. 3-hydroxyglutaric acid moderately impairs energy metabolism in brain of young rats. Neuroscience 135 (1), 111–120.

Latini, A., Scussiato, K., Leipnitz, G., Dutra-Filho, C.S., Wajner, M., 2005b. Pro \motion of oxidative stress by 3-hydroxyglutaric acid in rat striatum. Journal of Inherited Metabolic Disease 28 (1), 57–67.

Lipton, S.A., Rosenberg, P.A., 1994. Excitatory amino acids as a final common pathway for neurologic disorders. The New England Journal of Medicine 330 (9), 613–622.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. The Journal of Biological Chemistry 193 (1), 265–275.

Lund, T.M., Christensen, E., Kristensen, A.S., Schousboe, A., Lund, A.M., 2004. On the neurotoxicity of glutaric, 3-hydroxyglutaric, and trans-glutaconic acids in glutaric acidemia type 1. Journal of Neuroscience Research 77 (1), 143–147.

Maragakis, N.J., Rothstein, J.D., 2001. Glutamate transporters in neurologic disease. Archives of Neurology 58 (3), 365–370.

McDonald, J.W., Johnston, M.V., 1990. Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Research Brain Research Reviews 15 (1), 41–70.

Moussa, C.E., Rae, C., Bubb, W.A., Griffin, J.L., Deters, N.A., Balcar, V.J., 2007. Inhibitors of glutamate transport modulate distinct patterns in brain metabolism. Journal of Neuroscience Research 85 (2), 342–350.

Nakamura, Y., Iga, K., Shibata, T., Shudo, M., Kataoka, K., 1993. Glial plasmalemmal vesicles: a subcellular fraction from rat hippocampal homogenate distinct from synaptosomes. Glia 9 (1), 48–56.

Ocal, K., Avlan, D., Cinel, I., Unlu, A., Ozturk, C., Yaylak, F., Dirlik, M., Camdeviren, H., Aydin, S., 2004. The effect of *N*-acetylcysteine on oxidative stress in intestine and bacterial translocation after thermal injury. Burns 30 (8), 778–784.

Ozawa, S., Kamiya, H., Tsuzuki, K., 1998. Glutamate receptors in the mammalian central nervous system. Progress in Neurobiology 54 (5), 581–618.

Robinson, M.B., Sinor, J.D., Dowd, L.A., Kerwin, J.F., 1993. Subtypes of sodium-dependent high-affinity L-[^{3}H]glutamate transport activity — pharmacologic specificity and regulation by sodium and potassium. Journal of Neurochemistry 60 (1), 167–179.

Rosa, R.B., Schwarzbold, C., Dalcin, K.B., Ghisleni, G.C., Ribeiro, C.A., Moretto, M.B., Frizzo, M.E., Hoffmann, G.F., Souza, D.O., Wajner, M., 2004. Evidence that 3-hydroxyglutaric acid interacts with NMDA receptors in synaptic plasma membranes from cerebral cortex of young rats. Neurochemistry International 45 (7), 1087–1094.

Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., Kanai, Y., Hediger, M., Wang, Y., Schielke, J.P., Welty, D.F., 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16 (3), 675–686.

Sauer, S.W., Okun, J.G., Schwab, M.A., Crnic, L.R., Hoffmann, G.F., Goodman, S.I., Koeller, D.M., Kölker, S., 2005. Bioenergetics in glutaryl-coenzyme A dehydrogenase deficiency: a role for glutaryl-coenzyme A. The Journal of Biological Chemistry 280 (23), 21830–21836.

Sauer, S.W., Okun, J.G., Fricker, G., Mahringer, A., Müller, I., Crnic, L.R., Mühlhausen, C., Hoffmann, G.F., Hörster, F., Goodman, S.I., Harding, C.O., Koeller, D.M., Kölker, S., 2006. Intracerebral accumulation of glutaric and 3-hydroxyglutaric acids secondary to limited flux across the blood-brain barrier constitute a biochemical risk factor for neurodegeneration in glutaryl-CoA dehydrogenase deficiency. Journal of Neurochemistry 97 (3), 899–910.

Schlüter, K., Figiel, M., Rozyczka, J., Engele, J., 2002. CNS region-specific regulation of glial glutamate transporter expression. The European Journal of Neuroscience 16 (5), 836–842.

Strauss, K.A., Puffenberger, E.G., Robinson, D.L., Morton, H., 2003. Type I glutaric aciduria, part 1: natural history of 77 patients. American Journal of Medical Genetics Part C, Seminars in Medical Genetics 121 (1), 38–52.

Tanaka, K., Watase, K., Manabe, T., Yamada, K., Watanabe, M., Takahashi, K., Iwama, H., Nishikawa, T., Ichihara, N., Kikuchi, T., Okuyama, S., Kawashima, N., Hori, S., Takimoto, M., Wada, K., 1997. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 276 (5319), 1699–1702.

Trotti, D., Danbolt, N.C., Volterra, A., 1998. Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? Trends in Pharmacological Sciences 19 (8), 328–334.

Trotti, D., Rossi, D., Gjesdal, O., Levy, L.M., Racagni, G., Danbolt, N.C., Volterra, A., 1996. Peroxynitrite inhibits glutamate transporter subtypes. The Journal of Biological Chemistry 271 (11), 5976–5979.

Ullensvang, K., Lehre, K.P., Storm-Mathisen, J., Danbolt, N.C., 1997. Differential development expression of the two rat brain glutamate transporter proteins GLAST and GLT. The European Journal of Neuroscience 9 (8), 1646–1655.

Ullrich, K., Flott-Rahmel, B., Schluff, P., Musshoff, U., Das, A., Lucke, T., Steinfeld, R., Christensen, E., Jakobs, C., Ludolph, A., Neu, A., Roper, R., 1999. Glutaric aciduria type I: pathomechanisms of neurodegeneration. Journal of Inherited Metabolic Disease 22 (4), 392–403.

Volterra, A., Trotti, D., Tromba, C., Floridi, S., Racagni, G., 1994. Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes. The Journal of Neuroscience 14 (5 Pt 1), 2924–2932.

Wajner, M., Kölker, S., Souza, D.O., Hoffmann, G.F., Mello, C.F., 2004. Modulation of glutamatergic and GABAergic neurotransmission in glutaryl-CoA dehydrogenase deficiency. Journal of Inherited Metabolic Disease 27 (6), 825–828.

PARTE III

Discussão e Conclusões

III.1 DISCUSSÃO

Apesar das intensas investigações realizadas nos últimos anos, os mecanismos responsáveis pela atrofia frototemporal cortical presente ao nascimento e da degeneração estriatal aguda que ocorre durante os primeiros 36 meses de vida nos pacientes afetados pela deficiência da enzima glutaril-CoA desidrogenase (GCDH) permanecem ainda obscuros (Goodman, 2004). No entanto, efeitos dos ácidos glutárico (GA) e 3-hidroxiglutárico (3-OHGA), principais metabólitos acumulados nessa acidemia orgânica, sobre o metabolismo energético (Ullrich et al., 1999; Silva et al., 2000; Kölker et al., 2002a,b, 2004b; Das, Luche e Ullrich, 2003; Latini et al., 2005a; Ferreira et al., 2005; da C. Ferreira et al., 2005; Sauer et al., 2005; Ferreira et al., 2007; Zinnanti et al., 2007), parâmetros de estresse oxidativo (Kölker et al., 2001; de Oliveira Marques et al., 2003; Latini et al., 2002, 2005b, 2007) e particularmente sobre o sistema glutamatérgico (Flott-Rahmel et al., 1997; Lima et al., 1998; Ullrich et al., 1999; Kölker et al., 1999, 2000, 2002a,b, 2004a,b; Porciúncula et al., 2000, 2004; Frizzo et al., 2004) têm sido apontados como causas da disfunção neurológica encontrada nos pacientes com acidemia glutárica tipo I (GA I).

A hipótese de excitotoxicidade glutamatérgica como causa da neurodegeneração na GA I foi proposta inicialmente por Goodman e colaboradores (1977) baseados na semelhança dos achados neuropatológicos *postmortem* de pacientes afetados (vacuolizações cerebrais) e aqueles causados pelo glutamato. Vários trabalhos demonstraram que o GA e particularmente o 3-OHGA apresentam ações excitotóxicas em cérebro fresco e em neurônios

cultivados de embriões de ratos e de outras espécies, sendo então sugerido por esses investigadores que tais achados se deviam à similaridade estrutural existente entre essas substâncias e o glutamato (Flott-Rahmel et al., 1997; Lima et al., 1998; Hoffmann e Zschocke, 1999; Wajner et al., 2004; Goodman, 2004). Entretanto, outros estudos não relacionam a excitotoxicidade com esses ácidos orgânicos (Lund et al., 2004; Freudenberg, Lukacs e Ullrich, 2004) fazendo com que esta questão continue sob intenso debate.

Neste contexto, Kölker e colaboradores (1999, 2000) sugeriram que o GA e o 3-OHGA funcionariam como falsos neurotransmissores, agindo especificamente em receptores do tipo NMDA NR1/NR2B, os quais predominam durante o período inicial do desenvolvimento cerebral (McDonald, Silverstein e Johnston, 1988; Wenzel et al., 1997). Mais tarde, esses mesmos pesquisadores postularam que o 3-OHGA é capaz de ativar também receptores do tipo NMDA NR1/NR2A (Kölker et al., 2002a). Entretanto, Ullrich e colaboradores (1999) sugeriram que a ativação de receptores NMDA pelo 3-OHGA é secundária ao déficit energético causado por esse metabólito que provoca a saída do Mg^{2+} do sítio bloqueador dependente de voltagem do receptor NMDA, permitindo a permeabilidade do canal ao Ca^{2+} (Henneberry et al., 1989; Lipton e Rosenberg, 1994). Dessa forma, os mecanismos através dos quais o 3-OHGA exerce esse efeito não estão bem determinados ao nível molecular. Esse aspecto obscuro motivou a realização dos estudos apresentados no capítulo I do presente trabalho.

Nossos primeiros resultados evidenciaram a capacidade do 3-OHGA (10 e 100 μM), em concentrações similares às encontradas no cérebro de pacientes afetados pela GA I (Sauer et al., 2006), de inibir *in vitro* a ligação de glutamato a

seus receptores e transportadores de membrana plasmática sináptica de córtex cerebral de ratos de 30 dias de vida. No intuito de determinar o tipo de receptor envolvido nesse resultado, utilizamos o agonista de receptores glutamatérgicos ácido *N*-metil-D-aspártico (NMDA, 10 e 100 μM) que dá nome ao tipo de receptor. Com o uso desta abordagem, verificamos que o NMDA (10 e 100 μM) foi mais eficiente em deslocar o glutamato de seus receptores do que o 3-OHGA. Além disso, a co-incubação de 3-OHGA com NMDA não aumentou o grau de deslocamento de glutamato provocado pelo NMDA, sugerindo que os dois compostos interagem com receptores NMDA. O próximo passo foi investigar a capacidade do 3-OHGA de funcionar como um agonista desses receptores. Para isso, testamos o efeito desse ácido sobre a ligação de MK-801, a dizocilpina, um antagonista não-competitivo de receptores NMDA que para se fixar a este receptor necessita da ativação do mesmo (von Euler e Liu, 1993; Lipton e Rosenberg, 1994). Nossos resultados mostraram que o 3-OHGA funciona como um agonista dos receptores NMDA uma vez que aumentou a ligação de MK-801 a esses receptores. Além disso, observamos que a sua potência como agonista NMDA foi pequena, quando comparada a do glutamato. Em outros ensaios verificamos que o 3-OHGA (100 μM) aumentou o influxo de cálcio em fatias de córtex cerebral de ratos, reforçando os resultados anteriores de que esse ácido funciona como um agonista NMDA, permitindo o influxo de íons (principalmente Ca^{2+}) para o meio intracelular. É possível, portanto, presumir que o 3-OHGA pode funcionar como um agente excitotóxico, ativando enzimas de degradação de lipídios, proteínas e DNA que, quando estimuladas pelo aumento na concentração de Ca^{2+} intracelular,

podem levar ao dano celular (Lipton e Rosenberg, 1994; Sattler e Tymianski, 2000; Danbolt, 2001).

Acredita-se que a diversa expressão de receptores e transportadores glutamatérgicos ao longo do desenvolvimento nas diferentes estruturas cerebrais (McDonald e Johnston, 1990; Wenzel et al., 1997; Ullensvang et al., 1997; Danbolt, 2001; Schlüter et al., 2002) possa resultar em propriedades farmacológicas específicas, bem como insultos seletivos causados por distintas neurotoxinas (Lipton e Rosenberg, 1994; Ozawa, Kamiya e Tsuzuki, 1998; Danbolt, 2001; Haberny et al., 2002). Tomando como base tais achados, Goodman (2004) sugeriu que a diferente suscetibilidade das regiões cerebrais afetadas (córtex e estriado) na deficiência da GCDH poderia envolver a expressão variável dos tipos de receptores glutamatérgicos. Essa hipótese foi testada no capítulo II deste trabalho, onde investigamos a influência *in vitro* do GA e do 3-OHGA sobre a ligação de glutamato a seus receptores e transportadores de membrana plasmática de córtex cerebral e estriado de ratos ao longo do desenvolvimento.

Primeiramente, verificamos que o GA (0,01 a 1 mM) inibe a ligação de glutamato a seus receptores em córtex cerebral e estriado de ratos de 7 e 15 dias de vida, sem alterar esta ligação em animais de 60 dias. Além disso, esse ácido orgânico não interferiu com a ligação de glutamato a seus transportadores em todas as idades e estruturas cerebrais estudadas. Utilizando membranas sinápticas de córtex cerebral e estriado de ratos de 15 dias de vida e os antagonistas competitivos de receptores glutamatérgicos ionotrópicos 6-ciano-7-nitroquinoxalina-2,3-diona (CNQX, antagonista não-NMDA) e ácido DL-2-amino-

fosfonovalérico (AP5, antagonista NMDA), verificamos também que o efeito inibitório do GA sobre a ligação de glutamato parece estar dirigido aos receptores do tipo não-NMDA. Nossos resultados mostraram que a co-incubação de GA (1 mM) com CNQX (100 µM) não altera o efeito inibitório encontrado com o uso desse antagonista não-NMDA isoladamente. Por outro lado, a co-incubação de GA (1 mM) com AP5 (100 µM) provocou uma tendência de maior inibição da ligação do glutamato a seus receptores, quando comparada ao efeito isolado de AP5, sugerindo que o GA possa estar agindo em um sítio distinto dos receptores NMDA. Além disso, verificamos que o GA (1 mM) inibe marcadamente a ligação de cainato a seus receptores em membranas sinápticas de córtex cerebral de ratos de 15 dias de vida. Estes resultados reforçam o trabalho de Lima e colaboradores (1998), o qual demonstrou que as convulsões causadas por administração intraestriatal de GA podem ser prevenidas por pré-administração de DNQX, um antagonista de receptores não-NMDA. Outros estudos com o uso da eletrofisiologia não encontraram efeitos do GA sobre receptores não-NMDA do tipo AMPA (Ullrich et al., 1999; Kölker et al., 2002a). Tomados em seu conjunto, estes resultados indicam que o GA age preferencialmente sobre receptores não-NMDA do tipo cainato. Em contraste, Kölker e colaboradores (2000) demonstraram a ineficácia de CNQX na prevenção da morte celular observada em culturas neuronais na presença de GA. As diferenças encontradas nesses estudos, quando comparados com os nossos, podem estar relacionadas aos diferentes modelos estudados, pois Kölker e colaboradores (2000) utilizaram culturas de embriões de pintos e culturas mistas (neurônios e células gliais) de

hipocampo de ratos neonatos, enquanto nossos trabalhos foram realizados em córtex cerebral e estriado de ratos de diferentes idades de vida pós-natal.

Os efeitos do GA dependentes da idade podem estar relacionados com a expressão diferenciada de receptores glutamatérgicos durante o desenvolvimento, bem como, com as distintas propriedades desses receptores nas estruturas cerebrais estudadas (Ozawa, Kamiya e Tsuzuki, 1998; Anderson et al., 1999; Ritter, Vazquez e Meador-Woodruff, 2002). Neste contexto, os receptores não-NMDA (AMPA e cainato) são mais expressos em torno da segunda e terceira semana de vida pós-natal (7 a 15 dias) no rato, decaindo após (Insel, Miller e Gelhard, 1990; Miller et al., 1990; Kossut et al., 1993; Brennan et al., 1997), o que poderia explicar ao menos em parte nossos resultados. Além disso, é possível que a maior densidade de receptores glutamatérgicos durante os estágios iniciais de desenvolvimento pós-natal possa também ter contribuído para os resultados obtidos (Ritter, Vazquez e Meador-Woodruff, 2002).

No que se refere ao ácido 3-OHGA, este composto não interferiu com a ligação de glutamato a seus receptores em membranas de córtex cerebral e estriado de ratos de 7, 15 e 60 dias de vida. Entretanto, o 3-OHGA (100 µM) inibiu a ligação de glutamato a seus transportadores em estriado de ratos de 7 dias, sem alterar este parâmetros em animais mais velhos. Outros experimentos indicaram que este efeito foi dirigido aos transportadores de glutamato, visto que a co-incubação de 3-OHGA (100 µM) com L-*trans*-pirrolidina-2,4-dicarboxilato (PDC, 50 µM), um inibidor competitivo da captação de glutamato, não alterou o grau de inibição causado pelo PDC quando testado isoladamente. Além disso, nossos achados podem estar ligados ao fato de que transportadores de glutamato

apresentam afinidade por substâncias com grupamentos hidroxila no carbono 3 (Balcar, Johnston e Twitchin, 1977). Por outro lado, considerando que neste estágio de desenvolvimento cerebral (ratos de 7 dias) os principais transportadores de glutamato são os do tipo GLAST (Ullensvang et al., 1997) e o transportador EAAC1 é bem expresso no estriado de ratos jovens (Furuta, Rothstein e Martin, 1997), podemos inferir que essas proteínas estejam envolvidas no efeito inibitório causado pelo 3-OHGA.

O sistema de transporte de glutamato desempenha uma função crítica no metabolismo cerebral do glutamato (Danbolt, 2001; Schlüter et al., 2002). Alterações nos transportadores glutamatérgicos estão envolvidas em diversos processos neurodegenerativos que afetam o SNC adulto e imaturo (Furuta, Rothstein e Martin, 1997). Defeitos na captação de glutamato são sugeridos como fatores determinantes em diversas neuropatologias (trauma, esclerose lateral amiotrófica, epilepsia, doença de Alzheimer, isquemia, encefalopatia hepática e SIDA), nas quais a falência do transporte está relacionada ao estresse oxidativo, déficit energético e reduzida expressão desses transportadores (Danbolt, 2001).

Evidências da alteração do transporte de glutamato na presença de GA e 3-OHGA (Porciúncula et al., 2000; Frizzo et al., 2004) sugerem o envolvimento de transportadores glutamatégicos na fisiopatogenia da GA I. O efeito *in vitro* dos ácidos GA e 3-OHGA sobre a captação de glutamato por fatias e preparações sinaptossomais de cérebro de ratos ao longo do desenvolvimento foi estudado no capítulo III deste trabalho.

Observamos inicialmente que o GA (5 a 50 mM) inibiu seletivamente a captação de glutamato por fatias de córtex cerebral de ratos de 7 dias de vida de

maneira dose dependente, não alterando este parâmetro em córtex cerebral de ratos de 15 e 30 dias de vida, nem em estriado de ratos de todas as idades estudadas. Este efeito não foi resultante de morte celular na presença de GA, como evidenciado pelos testes de viabilidade de redução do azul de tiazolil (MTT) e da liberação da enzima lactato desidrogenase (LDH). Considerando que os transportadores astrocitários do tipo GLAST e GLT1 juntamente com o transportador neuronal EAAC1 estão presentes nas fatias obtidas, qualquer destas proteínas poderia estar envolvida neste efeito. Para determinar o tipo de transportador inibido pelo GA, realizamos o ensaio de captação de glutamato na presença de dihidrocainato (DHK, 0,1 e 1 mM), um inibidor específico de transportadores GLT1. Nossos resultados mostraram que ambas concentrações de DHK provocaram o mesmo grau de inibição na captação de glutamato por fatias de córtex cerebral de ratos de 7 dias de vida, sugerindo que o transporte via GLT1 foi totalmente inibido por concentrações de DHK tão baixas quanto 0,1 mM. Além disso, a co-incubação de GA (5 mM) com as duas concentrações de DHK utilizadas mostraram um aumento da inibição da captação de glutamato comparado ao uso das substâncias (GA e DHK) isoladas. Esse achado sugere que transportadores do tipo GLT1 não estão envolvidos no efeito inibitório sobre a captação de glutamato provocado pelo GA. Tais resultados podem ser reforçados pelo fato de que, segundo Ullensvang e colaboradores (1997), os transportadores GLT1 têm sua maior expressão por volta da segunda semana pós-natal em cérebro de ratos, tornando-se o principal transportador astrocitário de glutamato no cérebro adulto, com a exceção de algumas regiões como o cerebelo, onde o

transportador do tipo GLAST desempenha a função majoritária na captação de glutamato.

A captação de glutamato por preparações sinaptossomais não foi alterada na presença de GA. A idéia de que preparações de sinaptossomas são terminais nervosos fechados e metabolicamente ativos (Erecinska, Nelson e Silver, 1996; Robinson et al., 1998; Danbolt, 2001) praticamente excluiria a participação de transportadores astrocitários na captação de glutamato. Entretanto, diversos estudos têm demonstrado que a captação de glutamato por preparações sinaptossomais é mediada por transportadores GLT1 presentes em fragmentos gliais contaminantes dessas preparações (Nakamura et al., 1993; Robinson et al., 1993; Tanaka et al., 1997; Bridges, Kavanaugh e Chamberlin, 1999; Danbolt, 2001). A ausência de efeito do GA no transporte sinaptossomal de glutamato poderia ser mais um indício de que transportadores GLT1 não estão envolvidos na ação inibitória desse ácido em fatias de córtex cerebral de ratos de 7 dias de vida. Além disso, esse achado pode ser importante no que diz respeito ao envolvimento do transportador neuronal EAAC1 que, na ausência de uma relevante expressão de GLT1 (Ullensvang et al., 1997), seria o principal responsável pela captação de glutamato por preparações sinaptossomais nessa idade. Assim, as observações de que o GA não alterou a captação de glutamato por preparações sinaptossomais no cérebro de ratos de 7 dias de vida poderia sugerir a ausência de efeito desse ácido orgânico no transporte mediado por EAAC1. Podemos inferir então, que os transportadores glutamatérgicos do tipo GLAST são o principal alvo do efeito inibitório do GA sobre a captação de glutamato. De fato, nossos resultados demonstraram que a expressão de transportadores do tipo GLAST é

aproximadamente quatro vezes maior no córtex cerebral do que no estriado de ratos de 7 dias de vida. Neste particular, Furuta, Rothstein e Martin (1997) demonstraram que o transportador neuronal EAAC1 é muito expresso no estriado nos estágios iniciais de desenvolvimento, desempenhando um papel determinante na captação de glutamato nesta estrutura cerebral.

Nossos resultados demonstraram que o dano oxidativo parece ser um mecanismo importante pelo qual o GA inibe a captação de glutamato por fatias de córtex cerebral de ratos de 7 dias de vida, visto que a pré-administração *in vivo* de *N*-acetilcisteína, um precursor da síntese de glutationa (Karageorgos et al., 2006), preveniu totalmente o efeito inibitório do GA sobre o transporte de glutamato. Neste contexto, diversos trabalhos têm demonstrado que os transportadores de glutamato podem ser inibidos por oxidação (Volterra et al., 1994; Trotti et al., 1996; Trotti, Danbolt e Volterra, 1998) e que o GA é capaz de induzir estresse oxidativo *in vitro* e *in vivo* em cérebro de ratos (de Oliveira Marques et al., 2003; Latini et al., 2007).

Verificamos também que a administração *in vivo* de GA não alterou a captação *in vitro* de glutamato por fatias de córtex cerebral. Tais resultados podem dever-se às baixas concentrações de GA presentes no cérebro dos animais durante os ensaios de captação de glutamato.

Além disso, a captação de glutamato por fatias e preparações sinaptossomais de córtex cerebral e estriado de ratos de todas as idades estudadas não foi alterada pela presença do ácido 3-OHGA. É possível que estes resultados estejam relacionados ao fato de que a concentração de GA que provocou inibição na captação de glutamato (5 mM de GA) foi maior do que as

testadas para o 3-OHGA (0,1 e 1 mM). Alternativamente, considerando que o co-transportador de ácidos dicarboxílicos dependente de Na^+ 3 (NaDC3), um transportador de alta expressão no cérebro que está envolvido no transporte de GA e 3-OHGA, possui um K_m aproximadamente 25 vezes maior para o 3-OHGA quando comparado ao GA (Stellmer et al., 2007), podemos postular que, em nosso modelo, o 3-OHGA não atingiu concentrações intracelulares necessárias para afetar a captação de glutamato.

Como mostrado nos capítulos I e II deste trabalho, o 3-OHGA inibiu a ligação de glutamato a transportadores de membrana plasmática em estriado de ratos de 7 e em córtex cerebral de ratos de 30 dias de vida. Mesmo que experimentos de ligação de glutamato na presença de Na^+ indiquem a interação desse neurotransmissor com seus transportadores de membrana (Balcar e Li, 1992; Danbolt, 2001), eles devem ser distinguidos de estudos de captação (Danbolt, 2001). Os estudos de ligação são determinados no equilíbrio, enquanto ensaios de captação são determinados a partir de um estágio inicial, onde as concentrações extracelulares de $[^3H]$glutamato são bem superiores às do meio intracelular (Danbolt, 1994, 2001). Além disso, baixas concentrações de K^+ são capazes de inibir a ligação de glutamato a seus transportadores (Kramer e Baudry, 1984), enquanto esse cátion é extremamente necessário para o funcionamento da captação do neurotransmissor (Danbolt e Storm-Mathisen, 1986). Outro fator importante de distinção é o fato da captação ser dependente do estado de integridade das membranas e sofrer variações com a quantidade intracelular dos aminoácidos transportados (Danbolt, 1994). Portanto, efeitos encontrados nos diferentes ensaios podem não apresentar correlação.

Concluindo, ainda que nossos resultados não possam explicar a janela de vulnerabilidade estriatal característica dos indivíduos afetados pela GA I (Goodman, 2004), acreditamos que o diferenciado padrão regional (córtex cerebral e estriado) e temporal (diferentes estágios do desenvolvimento cerebral) apresentado nos efeitos causados pelos ácidos GA e 3-OHGA estão relacionados com o dano neurológico dos pacientes. Considerando diversos parâmetros do desenvolvimento cerebral, Anand e Scalzo (2000) relacionam a maturidade do sistema nervoso central de ratos de 6 - 9 dias de vida com o cérebro neonato humano. Portanto, nosso estudo demonstra uma maior suscetibilidade aos metabólitos que se acumulam na GA I em um estágio de desenvolvimento do SNC (7 - 30 dias de vida em ratos) que corresponde ao período crítico de vulnerabilidade ao dano neurológico dos pacientes afetados por esta doença, ou seja, os primeiros anos de vida (Hoffmann e Zschocke, 1999; Goodman, 2004). Demonstramos que o ácido 3-OHGA parece interagir diretamente com receptores NMDA em córtex cerebral de ratos de 30 dias de vida, como proposto por Kölker e colaboradores (1999, 2000, 2002a). Esses resultados não nos permitem inferir quais subunidades desses receptores estariam envolvidas nesse efeito (NR2A e/ou NR2B), porém a ausência de efeito em ratos adultos (60 dias) pode ser um indicativo de uma maior afinidade por receptores expressos no SNC imaturo, onde a subunidade NR2B é predominante (McDonald, Silverstein e Johnston, 1988; Wenzel et al., 1997). Por outro lado, a ausência de efeitos do 3-OHGA sobre receptores glutamatérgicos em idades precoces do desenvolvimento pós-natal (7 e 15 dias) pode estar relacionada ao fato de que nessas idades os receptores

NMDA NR2A e NR2B ainda não atingiram o seu pico de expressão que ocorre após os 21 dias de idade (Wenzel et al., 1997).

A proposta de que o ácido 3-OHGA é a principal neurotoxina acumulada no cérebro de pacientes com GA I (Ullrich et al., 1999; Kölker et al, 2002a) vem sendo intensamente discutida nos últimos anos. As maiores concentrações de GA no cérebro de pacientes (Sauer et al., 2006), a maior facilidade de transporte desse ácido quando comparado ao 3-OHGA (Stellmer et al., 2007) e diversos estudos mostrando efeitos deletérios do GA sobre diversos parâmetros do metabolismo energético (Silva et al., 2000; da C. Ferreira et al., 2005; Ferreira et al., 2005; Ferreira et al., 2007), de estresse oxidativo (de Oliveira Marques et al., 2003; Latini et al., 2007) e do sistema glutamatérgico (Lima et al., 1998; Porciúncula et al., 2000; Kölker et al., 2000; Porciúncula et al., 2004) juntamente com nossos resultados, indicam que esse ácido orgânico exerce efeitos neurotóxicos relevantes na fisiopatogenia da GA I. Os efeitos encontrados em nossos estudos corroboram os estudos de Lima e colaboradores (1998) e Porciúncula e colaboradores (2004) que sugerem que as alterações no sistema glutamatérgico provocadas pelo GA seriam exercidas principalmente via receptores não-NMDA. Como mostrado para o 3-OHGA, as ações do GA parecem ser mais efetivas no SNC imaturo, o que poderia ser explicado pela diminuição da densidade de receptores AMPA e cainato no cérebro de ratos adultos (Insel, Miller e Gelhard, 1990; Miller et al., 1990; Kossut et al., 1993; Brennan et al., 1997).

A inibição da captação de glutamato por GA parece ser dirigida especificamente aos transportadores do tipo GLAST, altamente expressos no cérebro imaturo (Ullensvang et al., 1997). Essa inibição pode se refletir em um

aumento das concentrações extracelulares de glutamato causando a estimulação exacerbada de receptores culminando com o dano neuronal (excitotoxicidade) (Lipton e Rosenberg, 1994, Danbolt, 2001). A ausência de efeito do 3-OHGA sobre a captação de glutamato indica a falta de correlação existente entre estudos de ligação a transportadores e o transporte de glutamato propriamente dito (Danbolt, 2001).

Apesar de extremamente relevantes no estudo da fisiopatogenia cerebral da GA I, os efeitos dos principais metabólitos acumulados nesta doença sobre parâmetros de bioenergética e estresse oxidativo falham em explicar a maior suscetibilidade das diferentes estruturas cerebrais afetadas (córtex cerebral e estriado) ao longo do desenvolvimento do SNC. Pela primeira vez, demonstramos efeitos do GA e do 3-OHGA sobre o sistema glutamatérgico de forma dependente da idade e da região cerebral. Dessa forma, é possível que o metabolismo energético e o estresse oxidativo, juntamente com outros mecanismos, ajam sinergicamente com a expressão diferenciada de receptores e transportadores de glutamato conferindo uma maior vulnerabilidade do cérebro imaturo às neurotoxinas acumuladas na GA I. Acreditamos que este trabalho possa auxiliar na elucidação dos mecanismos fisiopatogênicos que conduzem à neurodegeneração característica dos pacientes com GA I, servindo como base para o desenvolvimento de estratégias terapêuticas relevantes no tratamento e na melhora da qualidade de vida dos portadores desta doença.

III.2 CONCLUSÕES

- O ácido 3-hidroxiglutárico (3-OHGA), nas concentrações de 10 e 100 μM, inibiu a ligação de glutamato Na^+-independente (ligação a receptores) e Na^+-dependente (ligação a transportadores) a membranas plasmáticas sinápticas de córtex cerebral de ratos de 30 dias de vida.

- Estudos de ligação de glutamato na presença de *N*-metil-D-aspartato indicaram que o 3-OHGA parece interagir diretamente com receptores glutamatérgicos do tipo NMDA.

- O aumento da ligação de MK-801 a receptores NMDA de membranas plasmáticas sinápticas e o incremento do influxo de Ca^{2+} provocado pelo 3-OHGA em fatias de córtex cerebral de ratos de 30 dias de vida indicam que este ácido atua como um agonista de receptores glutamatérgicos do tipo NMDA.

- O ácido glutárico (GA, 0,01 a 1 mM) inibiu a ligação de glutamato Na^+-independente (ligação a receptores) a membranas plasmáticas sinápticas de córtex cerebral e estriado de ratos de 7 e 15 dias de vida, sem alterar essa ligação a membranas de ratos de 60 dias em ambas estruturas estudadas.

- A utilização dos antagonistas competitivos de receptores glutamatérgicos CNQX e AP5 nos estudos de ligação de glutamato a membranas

plasmáticas sinápticas na ausência de sódio e a inibição da ligação de cainato a essas membranas causada pelo GA indicam que o efeito desse ácido parece ser dirigido a receptores do tipo não-NMDA e mais especificamente a receptores do tipo cainato.

- O GA não interferiu na ligação de glutamato Na^+-dependente (ligação a transportadores) a membranas plasmáticas sinápticas de córtex cerebral e estriado de ratos de 7, 15 e 60 dias de vida.

- O 3-OHGA (100 μM) inibiu a ligação de glutamato Na^+-dependente (ligação a transportadores) a membranas plasmáticas sinápticas de estriado de ratos de 7 dias de vida, sem interferir com essa ligação em ratos de 15 e 60 dias.

- A utilização de PDC, um inibidor competitivo do transporte de glutamato, nos ensaios de ligação de glutamato Na^+-dependente (ligação a transportadores) a membranas plasmáticas de estriado de ratos de 7 dias de vida reforçam a idéia de que o 3-OHGA de fato desloca o glutamato de seus transportadores.

- O 3-OHGA não interferiu na ligação de glutamato Na^+-dependente (ligação a transportadores) a membranas plasmáticas sinápticas de córtex cerebral de ratos de 7, 15 e 60 dias de vida.

- O GA (5-50 mM) inibiu a captação de glutamato por fatias de córtex cerebral de ratos de 7 dias de vida, sem interferir nessa captação em ratos de 15 e 30 dias.

- Experimentos de captação de glutamato por fatias de córtex cerebral de ratos de 7 dias de vida na presença de DHK e a ausência de efeito do GA sobre a captação de glutamato por preparações sinaptossomais indicam que o efeito deste ácido orgânico parece estar dirigido a transportadores do tipo GLAST expressos nos estágios iniciais do desenvolvimento cerebral de ratos.

- A pré-administração *in vivo* de *N*-acetilcisteína, um precursor da glutationa, preveniu totalmente a captação de glutamato causada pelo GA, indicando um mecanismo oxidativo nessa inibição.

- A administração *in vivo* de GA não alterou a captação *in vitro* de glutamato por fatias de córtex cerebral de ratos de 7 dias de vida.

- O GA não interferiu com a captação de glutamato por fatias de estriado de ratos de todas as idades estudadas.

- O 3-OHGA não alterou a captação de glutamato por fatias e preparações sinaptossomais de córtex cerebral e estriado de ratos de todas as idades estudadas.

- Os efeitos do GA e do 3-OHGA parecem envolver seletivamente distintos tipos de receptores e transportadores de glutamato expressos nas diferentes estruturas cerebrais (córtex cerebral e estriado) ao longo do desenvolvimento de ratos.

- Esses resultados podem estar relacionados com a janela de vulnerabilidade cerebral apresentada pelos pacientes afetados pela acidemia glutárica tipo I, e explicar, ao menos em parte, o dano neurológico característico dessa doença.

REFERÊNCIAS BIBLIOGRÁFICAS

Amara, S.G., Fontana, A.C. Excitatory amino acid transporters: keeping up with glutamate. Neurochem Int 41: 313-318, 2002.

Amir, N., el-Peleq, O., Shalev, R.S., Christensen, E. Glutaric aciduria type I: clinical heterogeneity and neuroradiologic features. Neurology 37: 1654-1657, 1987.

Anand, K.J., Scalzo, F.M. Can adverse neonatal experiences alter brain development and subsequent behavior? Biol Neonate 77: 69-82 Review, 2000.

Anderson, K.J., Mason, K.L., McGraw, T.S., Theophilopoulos, D.T., Sapper, M.S., Burchfield, D.J. The ontogeny of glutamate receptors and D-aspartate binding sites in the CNS. Brain Res Dev Brain Res 118: 69-77, 1999.

Balcar, V.J., Johnston, G.A., Twitchin, B. Stereospecificity of the inhibition of L-glutamate and L-aspartate high affinity uptake in rat brain slices by threo-3-hydroxyaspartate. J Neurochem 28: 1145-1146, 1977.

Balcar, V.J., Li, Y. Heterogeneity of high affinity uptake of L-glutamate and L-aspartate in the mammalian central nervous system. Life Sci 51: 1467-1478 Review, 1992.

Baric, I., Furnic, K., Hoffmann, G.F. Inborn errors of metabolism at the turn of the millennium. Croat Med J 42: 379-383, 2001.

Baric, I., Zschocke, J. Christensen, E., Duran, M., Goodman, S.I., Leonard, J.V., Muller, E., Morton, D.H., Superti-Furga, A., Hoffmann, G.F. Diagnosis and management of glutaric aciduria type I. J Inherit Metab Dis 21: 326-340, 1998.

Berger, U.V., Hediger, M.A. Comparative analysis of glutamate transporter expression in rat brain using differential double in situ hybridization. Anat Embryol (Berl) 198: 13-30, 1998.

Bickel, H. Early diagnosis and treatment of inborn errors of metabolism. Enzyme 38: 14-26, 1987.

Biery, B.J., Stein, D.E., Morton, D.H., Goodman, S.I. Gene structure and mutations of glutaryl-coenzyme A dehydrogenase: impaired association of enzyme subunits that is due to an A421V substitution causes glutaric acidemia type I in the Amish. Am J Hum Genet 59: 1006-1011, 1996.

Bjugstad, K.B., Zawada, W.M., Goodman, S. Free, C.R. IGF-1 and bFGF reduce glutaric acid and 3-hydroxyglutaric acid toxicity in striatal cultures. J Inherit Metab Dis 24: 631-647, 2001.

Brennan, E.M., Martin, L.J., Johnston, M.V., Blue, M.E. Ontogeny of Non-NMDA glutamate receptors in rat barrel field cortex: II. α-AMPA and kainate receptors. J Comp Neurol 386: 29-45, 1997.

Bridges, R.J., Kavanaugh, M.P., Chamberlin, A.R. A pharmacological review of competitive inhibitors and substrates of high-affinity, sodium-dependent glutamate transport in the central nervous system. Curr Pharm Des 5: 363-379, 1999.

Brismar, J., Ozand, P.T. CT and MR of the brain in glutaric academia type I: a review of 59 published cases and a report of 5 new patients. AJNR Am J Neuroradiol 16: 675-683, 1995.

Burlina, A.P., Zara, G., Hoffmann, G.F., Zschocke, J., Burlina, A.B. Management of movement disorders in glutaryl-CoA dehydrogenase deficiency: anticholonergic drugs and botulinum toxin as additional therapeutic options. J Inherit Metab Dis 27: 911-915, 2004.

Busquets, C., Soriano, M., de Almeida, I.T., Garavaglia, B., Rimoldi, M., Rivera, I., Uziel, G., Cabral, A., Coll, M.J., Ribes, A. Mutation analysis of the GCDH gene in Italian and Portuguese patients with glutaric aciduria type I. Mol Genet Metab 71: 535-537, 2000.

Chalmers, R.A., Bain, M.D., Zschocke, J. Riboflavin-responsive glutaryl CoA dehydrogenase deficiency. Mol Genet Metab 88: 29-37, 2006.

Chalmers, R.A., Lawson, A.M. Organic acids in man. Analytical chemistry, biochemistry and diagnosis of the organic acidurias. London, Chapman & Hall. 221-229, 1982.

Chalmers, R.A., Purkiss, P., Watts, R.W., Lawson, A.M. Screening for organic acidurias and amino acidopathies in newborns and children. J Inherit Metab Dis 3: 27-43, 1980.

Chow, C.W., Haan, E.A., Goodman, S.I., Anderson, R.M., Evans, W.A., Kleinschimidt-DeMasters, B.K., Wise, G., McGill, J.J., Danks, D.M. Neuropathology in glutaric acidaemia type I. Acta Neuropathol (Berl) 76: 590-594, 1988.

Colwell, C.S., Cepeda, C., Crawford, C., Levine, M.S. Postnatal development of glutamate receptor-mediated responses in the neostriatum. Dev Neurosci 20: 154-163, 1998.

Conn, J.P., Pin, J.P. Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 37: 205-237, 1997.

da C. Ferreira, G., Viegas, C.M., Schuck, P.F., Latini, A., Dutra-Filho, C.S., Wyse, A.T.S., Wannmacher, C.M.D., Vargas, C.R., Wajner, M. Glutaric acid moderately compromises energy metabolism in rat brain. Int J Dev Neurosci 23: 687-693, 2005.

Daikhin, Y., Yudkoff, M. Compartmentation of brain glutamate metabolism in neurons and glia. J Nutr 130:1026S-1031S Review, 2000.

Danbolt, N.C. The high affinity uptake system for excitatory amino acids in the brain. Prog Neurobiol 44: 377-396, 1994.

Danbolt, N. C. Glutamate uptake. Prog Neurobiol 65: 1-105, 2001.

Danbolt, N.C., Storm-Mathisen, J. Inhibition by K^+ of Na^+-dependent D-aspartate uptake into brain membrane saccules. J Neurochem 47: 825-30, 1986.

Das, A.M., Luche, T., Ullrich, K. Glutaric aciduria I: creatine supplementation restores creatine phosphate levels in mixed cortex cells from rat incubated with 3-hydroxyglutarate. Mol Genet Metab 78: 108-111, 2003.

de Mello, C.F., Kölker, S., Ahlemeyer, B., de Souza, F.R., Fighera, M.R., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., Wajner, M. Intrastriatal administration of 3-hydroxyglutaric acid induces convulsions and striatal lesions in rats. Brain Res 916: 70-75, 2001.

de Oliveira Marques, F., Hagen, M.E., Pederzolli, C.D., Sgaravatti, A.M., Durigon, K., Testa, C.G., Wannmacher, C.M.D., de Souza Wyse, A.T., Wajner, M., Dutra-Filho, C.S. Glutaric acid induces oxidative stress in brain of young rats. Brain Res 964: 153-158, 2003.

Drigo, P., Piovan, S., Battistella, P.A., Della Puppa, A., Burlina, A.B. Macrocephaly, subarachnoid fluid collection, and glutaric aciduria type I. J Child Neurol 11: 414-417, 1996.

Erecinska, M., Nelson, D., Silver, I.A. Metabolic and energetic properties of isolated nerve ending particles (synaptosomes). Biochim Biophys Acta 1277: 13-34 Review, 1996.

Erecinska, M., Silver, I.A. Metabolism and role of glutamate in mammalian brain. Prog Neurobiol 35: 245-296 Review, 1990.

Ferreira, G. da C., Schuck, P.F., Viegas, C.M., Tonin, A., Latini, A., Dutra-Filho, C.S., Wyse, A.T., Wannmacher, C.M., Vargas, C.R., Wajner, M. Energy metabolism is compromised in skeletal muscle of rats chronically-treated with glutaric acid. Metab Brain Dis 22:111-123, 2007.

Ferreira, G.C., Viegas, C.M., Schuck, P.F., Tonin, A., Ribeiro, C.A.J., Coelho, D.M., Dalla-Costa, T., Latini, A., Wyse, A.T.S., Wannmacher, C.M.D., Vargas, C.R., Wajner, M. Glutaric acid administration impairs energy metabolism in

midbrain and skeletal muscle of young rats. Neurochem Res 30: 1123-1131, 2005.

Flott-Rahmel, B., Falter, C., Schluff, P., Fingerhut, R., Christensen, E., Jakobs, C., Musshoff, U., Deufel, T., Ludolph, A., Ullrich, K. Nerve cell lesions caused by 3-hydroxyglutaric acid: a possible mechanism for neurodegeneration in glutaric acidaemia I. J Inherit Metab Dis 20: 387-390, 1997.

Fonnum, F. Glutamate: a neurotransmitter in mammalian brain. J Neurochem 42: 1-11, 1984.

Forstner, R., Hoffmann, G.F., Gassner, I., Heideman, P., De Klerk, J.B., Lawrenz-Wolf, B., Doringer, E., Weiss-Wichert, P., Troger, J., Colombo, J.P., Plochl, E. Glutaric aciduria type I: ultrasonographic demonstration of early signs. Pediatr Radiol 29: 138-143, 1999.

Freudenberg, F., Lukacs, Z., Ullrich, K. 3-Hydroxyglutaric acid fails to affect the viability of primary neuronal rat cells. Neurobiol Dis 16: 581-584, 2004.

Frizzo, M. E., Schwarzbold, C., Porciuncula, L. O., Dalcin, K. B., Rosa, R. B., Ribeiro, C. A., Souza, D. O., Wajner, M. 3-hydroxyglutaric acid enhances glutamate uptake into astrocytes from cerebral cortex of young rats. Neurochem Int 44: 345-53, 2004.

Funk, C.B., Prasad, A.N., Frosk, P., Sauer, S., Kölker, S., Greenberg, C.R., Del Bigio, M.R. Neuropathological, biochemical and molecular findings in a glutaric acidemia type 1 cohort. Brain 128: 711-722, 2005.

Furuta, A., Rothstein, J.D., Martin, L.J. Glutamate transporter protein subtypes are expressed differentially during rat CNS development. J Neurosci 17: 8363-8375, 1997.

Goodman, S.I. Development of pathogenic concepts in glutaryl-CoA dehydrogenase deficiency: The challenge. J Inherit Metab Dis 27: 801-803, 2004.

Goodman, S.I., Frerman, F.E. Organic acidemias due to defects in lysine oxidation: 2-ketoadipic acidemia and glutaric acidemia. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds.). The Metabolic and Molecular Bases of Inherited Disease. 8th edition. New York, McGraw-Hill. 2195-2204, 2001.

Goodman, S.I., Markey, S.P., Moe, P.G., Miles, B.S., Teng, C.C. Glutaric aciduria: a 'new' disorder of amino acid metabolism. Biochem Med 12: 12-21, 1975.

Goodman, S.I., Norenberg, M.D., Shikes, R.H., Breslich, D.J., Moe, P.G. Glutaric aciduria: biochemical and morphologic considerations. J Pediatr 90: 746-750, 1977.

Goodman, S.I., Stein, D.E., Schlesinger, S., Christensen, E., Schwartz, M., Greenberg, C.R., Elpeleg, O.N. Glutaryl-CoA dehydrogenase mutations in glutaric acidemia (type I): review and report of thirty novel mutations. Hum Mutat 12: 141-144, 1998.

Greenberg, C.R., Reimer, D., Singal, R., Triggs-Raine, B., Chudley, A.E., Dilling, L.A., Philipps, S., Haworth, J.C., Seargeant, L.E., Goodman, S.I. A G-to-T transversion at the +5 position of intron 1 in the glutaryl-CoA dehydrogenase gene is associated with the Island Lake variant of glutaric acidemia type I. Hum Mol Genet 4: 493-495, 1995.

Haberny, K.A., Paule, M.G., Scallet, A.C., Sistare, F.D., Lester, D.S., Hanig, J.P., Slikker, W. Jr. Ontogeny of the N-methyl-D-aspartate (NMDA) receptor system and susceptibility to neurotoxicity. Toxicol Sci 68: 9-17, 2002.

Härtel, U., Eckel, E., Koch, J., Fuchs, G., Linder, D., Buckel, W. Purification of glutaryl-CoA dehydrogenase from Pseudomonas sp., an enzyme involved in the anareobic degradation of benzoate. Arch Microbiol 159: 174-181, 1993.

Henneberry, R.C., Novelli, A., Cox, J.A., Lysko, P.G. Neurotoxicity at the N-methyl-D-aspartate receptor in energy-compromised neurons. An hypothesis for cell death in aging and disease. Ann N Y Acad Sci 568: 225-233, 1989.

Heyes, M.P. Hypothesis: a role for quinolinic acid in the neuropathology of glutaric acidúria type I. Can J Neurol Sci 14: 441-443, 1987.

Hoffmann, G.F., Bohles, H.J., Burlina, A., Duran, M., Herwig, J., Lehnert, W., Leonard, J.V., Muntau, A., Plecko-Starting, F.K., Superti-Furga A, et al. Early signs and course of disease of glutaryl-CoA dehydrogenase deficiency. J Inherit Metab Dis 18:173-176, 1995.

Hoffmann, G.F. Selective screening for inborn errors of metabolism – past, present and future. Eur J Pediatr 153: S2-8, 1994.

Hoffmann, G.F., Zschocke, J. Glutaric aciduria type I: from clinical, biochemical and molecular diversity to successful therapy. J Inherit Metab Dis 22: 381-391, 1999.

Hoffmann, G.F., Athanassopoulos, S., Burlina, A.B., Duran, M., de Klerck, J.B.C., Lehnert, W., Leonard, J.V., Monavari, A.A., Müller, E., Muntau, A.C., Naughten, E.R., Plecko-Starting, B., Superti-Furga, A., Zschocke, J., Christensen, E. Clinical course, early diagnosis, treatment, and prevention of disease in glutaryl-CoA dehydrogenase deficiency. Neuropediatrics 27: 115-123, 1996.

Hoffmann, G.F., Seppel, C.K., Holmes, B., Mitchell, L., Christen, H.J., Hanefeld, F., Rating, D., Nyhan, W.L. Quantitative organic acid analysis in cerebrospinal fluid

and plasma: reference values in a pediatric population. J Chromatogr 617: 1-10, 1993.

Hoffmann, G.F., Trefz, F.K., Barth, P., Böhles, J.H., Biggemann, B., Bremer, H.J., Christensen, E., Frosch, M., Hanefeld, F., Hunneman, D.H., Jacobi, H., Kurlemann, G., Lawrenz-Wolf, B., Rating, D., Roe, C.R., Schutgens, R.B.H., Ullrich, K., Weisser, J., Wendel, U., Lehnert, W. Glutaril-CoA Dehydrogenase Deficiency: a distinct encephalopathy. Pediatrics 88: 1194-1203, 1991.

Hoffmann, G.F., Von Kries, R., Klose, D., Lindner, M., Schulze, A., Muntau, A.C., Röschinger, W., Liebl, B., Mayatepek, E., Roscher, A.A. Frequencies of inherited organic acidurias and disorders of mitochondrial fatty acid transport and oxidation in Germany. Eur J Pediatr 163: 76-80, 2004.

Hollmann, M., Heinemann, S. Cloned glutamate receptors. Annu Rev Neurosci 17: 31-108 Review, 1994.

Hurst, R.S., Villablanca, J.P., Levine, M.S. Late development of NMDA receptor-mediated synaptic current in the rat striatum. Neurosci Abstr 24:1644, 1998.

Insel, T.R., Miller, L.P., Gelhard, R.E. The ontogeny of excitatory amino acid receptors in the rat forebrain I: N-methyl-D-aspartate and quisqualate receptors. Neuroscience 35: 31-43, 1990.

Jensen, F.E. The role of glutamate receptor maturation in perinatal seizures and brain injury. Int J Dev Neurosci 20: 339-347, 2002.

Karageorgos, N., Patsoukis, N., Chroni, E., Konstantinou, D., Assimakopoulos, S.F., Georgiou, C. Effect of N-acetylcysteine, allopurinol and vitamin E on jaundice-induced brain oxidative stress in rats. Brain Res 1111: 203-212, 2006.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G. F. 3-Hydroxyglutaric and glutaric acids are neurotoxic through NMDA receptors in vitro. J Inherit Metab Dis 22: 259-262, 1999.

Kölker, S., Garbade, S.F., Greenberg, C.R., Leonard, J.V., Saudubray, J.M., Ribes, A., Kalkanoglu, H.S., Lund, A.M., Merinero, B., Wajner, M., Troncoso, M., Williams, M., Walter, J.H., Campistol, J., Marti-Herrero, M., Caswill, M., Burlina, A.B., Lagler, F., Maier, E.M., Schwahn, B., Tokatli, A., Dursun, A., Coskun, T., Chalmers, R.A., Koeller, D.M., Zschocke, J., Christensen, E., Burgard, P., Hoffmann, G.F. Natural history, outcome, and treatment efficacy in children and adults with glutaryl-CoA dehydrogenase deficiency. Pediatr Res 59: 840-847, 2006a.

Kölker, S., Koeller, D.M., Sauer, S., Horster, F., Schwab, M.A., Hoffmann, G.F., Ullrich, K., Okun, J.G. Excitotoxicity and bioenergetics in glutaryl-CoA dehydrogenase deficiency. J Inherit Metab Dis 27: 805-812, 2004b.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F. Contribution of reactive oxygen species to 3-hydroxyglutarate neurotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr Res 50: 76-82, 2001.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F. Maturation-dependent neurotoxicity of 3-hydroxyglutaric and glutaric acids in vitro: a new pathophysiologic approach to glutaryl-CoA dehydrogenase deficiency. Pediatr Res 47: 495-503, 2000.

Kölker, S., Koeller, D.M., Okun, J.G., Hoffmann, G.F. Pathomechanisms of neurodegeneration in glutaryl-CoA dehydrogenase deficiency. Ann. Neurol 55: 7-12, 2004a.

Kölker, S., Kohr, G., Ahlemeyer, B., Okun, J.G., Pawlak, V., Horster, F., Mayatepek, E., Krieglstein, J., Hoffmann, G.F. Ca2+ and Na+ dependence of 3-hydroxyglutarate-induced excitotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr Res 52: 199-206, 2002a.

Kölker, S., Okun, J.G., Ahlemeyer, B., Wyse, A.T., Horster, F., Wajner, M., Kohlmuller, D., Mayatepek, E., Krieglstein, J., Hoffmann, G.F. Chronic treatment with glutaric acid induces partial tolerance to excitotoxicity in neuronal cultures from chick embryo telencephalons. J Neurosci Res 68: 424-431, 2002b.

Kölker, S., Sauer, S.W., Okun, J.G., Hoffmann, G.F., Koeller, D.M. Lysine intake and neurotoxicity in glutaric aciduria type I: towards a rationale for therapy? Brain 129: e54, 2006b.

Koeller, D.M., Woontner, M., Crnic, L.S., Kleinschmidt-DeMasters, B., Stephens, J., Hunt, E.L., Goodman, S.I. Biochemical, pathologic and behavioral analysis of a mouse model of glutaric acidemia type I. Hum Mol Genet 11: 347-357, 2002.

Kossut, M., Glazewski, S., Siucinska, E., Skangiel, K.J. Functional plasticity and neurotransmitter receptor binding in the vibrissal barrel cortex. Acta Neurobiol Exp (Wars) 53: 161-173, 1993.

Kramer, K., Baudry, M. Low concentrations of potassium inhibit the Na-dependent [^{3}H]glutamate binding to rat hippocampal membranes. Eur J Pharmacol 102: 155-1588, 1984.

Kugler, P., Schmitt, A. Glutamate transporter EAAC1 is expressed in neurons and glial cells in the rat nervous system. Glia 27: 129-142, 1999.

Külkens, S., Harting, I., Sauer, S., Zschocke, J., Hoffmann, G.F., Gruber, S., Bodamer, O.A., Kölker, S. Late-onset neurologic disease in glutaryl-CoA dehydrogenase deficiency. Neurology 64: 2142-2144, 2005.

Latini, A., Ferreira, G.C., Scussiato, K., Schuck, P.F., Solano, A.F., Dutra-Filho, C.S., Vargas, C.R., Wajner, M. Induction of oxidative stress by chronic and acute glutaric acid administration to rats. Cell Mol Neurobiol 27: 423-438, 2007.

Latini, A., Rodriguez, M., Rosa, R.B., Scussiato, K., Leipnitz, G., de Assis, D.R., Ferreira, G.C., Funchal, C., Jacques-Silva, M.C., Buzin, L., Giugliani, R., Cassina, A., Radi, R., Wajner, M. 3-Hydroxygluraric acid moderately impairs energy metabolism in brain of young rats. Neuroscience 135: 111-120, 2005a.

Latini, A., Rosa, R.B., Scussiato, K., Llesuy, S., Bello-Klein, A., Wajner, M. 3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats. Brain Res 956: 367-373, 2002.

Latini, A., Scussiato, K., Leipnitz, G., Dutra-Filho, C.S., Wajner, M. Promotion of oxidative stress by 3-hydroxyglutaric acid in rat striatum. J Inherit Metab Dis 28: 57-67, 2005b.

Lehnert, W., Sass, J.O. Glutaconyl-CoA is the main toxic agent in glutaryl-CoA dehydrogenase deficiency (glutaric aciduria type I). Med Hypotheses 65: 330-333, 2005.

Leibel, R.L., Shih, V.E., Goodman, S., Bauman, M.L., McCabe, E.R., Zwerdling, R.G., Bergman, I., Costello, C. Glutaric acidemia: a metabolic disorder causing progressive choreoathetosis. Neurology 30: 1163-1168, 1980.

Lenich, A.C., Goodman, S.I. The purification and characterization of glutaryl-CoA dehydrogenase from porcine and human liver, J Biol Chem 261: 4090-4096, 1986.

Lima, T.T., Begnini, J., de Bastiani, J., Fialho, D.B., Jurach, A., Ribeiro, M.C., Wajner, M., de Mello, C.F. Pharmacological evidence for GABAergic and glutamatergic in involvement in the convulsant and behavioral effects of glutaric acid. Brain Res 802: 55-60, 1998.

Lipton, S.A., Rosenberg, P.A. Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 330: 613-622, 1994.

Lucas, D.R., Newhouse, J.P. The toxic effect of sodium L-glutamate on the inner layers of the retina. AMA Arch Ophthalmol 58: 193-201, 1957.

Lund, T.M., Christensen, E., Kristensen, A.S., Schousboe, A., Lund, A.M. On the neurotoxicity of glutaric, 3-hydroxyglutaric, and trans-glutaconic acids in glutaric acidemia type 1. J Neurosci Res 77: 143-147, 2004.

Maragakis, N.J., Rothstein, J.D. Glutamate transporters in neurologic disease. Arch Neurol 58: 365-370, 2001.

Mayatepek, E., Hoffmann, G.F., Baumgartner, R., Schulze, A., Jakobs, C., Trefz, F.K., Bremer, H.J. Atypical vitamin B12-uresponsive methylmalonic aciduria in

sibship with severe progressive encephalomyopathy: a new genetic disease? Eur J Pediatr 155: 398-403, 1996.

McDonald, J.W., Silverstein, F.S., Johnston, M.V. Neurotoxicity of N-methyl-D-aspartate is markedly enhanced in developing rat central nervous system. Brain Res 459: 200-203, 1988.

McDonald, J.W., Johnston, M.V. Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res Rev 15: 41-70, 1990.

Merinero, B., Perez-Cerda, C., Font, L.M., Garcia, M.J., Aparicio, M., Lorenzo, G., Martinez Pardo, M., Garzo, C., Martinez-Bermejo, A., Pascual Castroviejo, I. Variable clinical and biochemical presentation of seven Spanish cases with glutaryl-CoA dehydrogenase deficiency. Neuropediatrics 26: 238-242, 1995.

Miller, L.P., Johnson, A.E., Gelhard, R.E., Insel, T.R. The ontogeny of excitatory amino acid receptors in the rat forebrain II. Kainic acid receptors. Neuroscience 35: 45-51, 1990.

Muhlhausen, C., Ott, N., Chalajour, F., Tilki, D., Freudenberg, F., Shahhossini, M., Thiem, J., Ullrich, K., Braulke, T., Ergun, S. Endothelial effects of 3-hydroxyglutaric acid: implications for glutaric acidúria type I. Pediatr Res 59:196-202, 2006.

Nakamura, Y., Iga, K., Shibata, T., Shudo, M., Kataoka, K. Glial plasmalemmal vesicles: a subcellular fraction from rat hippocampal homogenate distinct from synaptosomes. Glia 9: 48-56, 1993.

Nakanishi, S. Molecular diversity of glutamate receptors and implications for brain function. Science 258: 597-603 Review, 1992.

Nansen, E.A., Jokel, E.S., Lobo, M.K., Micevych, P.E., Ariano, M.A., Levine, M.S. Striatal ionotropic glutamate receptor ontogeny in the rat. Dev Neurosci 22: 329-340, 2000.

Olney, J.W. Brain lesions, obesity, and others disturbances in mice treated with monosodium glutamate. Science 164: 719-721, 1969.

Ozawa, S., Kamiya, H., Tsuzuki, K. Glutamate receptors in the mammalian central nervous system. Prog Neurobiol 54: 581-618, 1998.

Porciúncula, L.O., Dal-Pizzol, Jr.A., Coitinho, A.S., Emanuelli, T., Souza, D.O., Wajner, M. Inhibition of synaptosomal [^{3}H]glutamate uptake and [^{3}H]glutamate binding to plasma membranes from brain of young rats by glutaric acid in vitro. J Neurol Sci 173: 93-96, 2000.

Porciúncula, L.O., Emanuelli, T., Tavares, R.G., Schwarzbold, C., Frizzo, M.E., Souza, D.O., Wajner, M. Glutaric acid stimulates glutamate binding and

astrocytic uptake and inhibits vesicular glutamate uptake in forebrain from young rats. Neurochem Int 45: 1075-1086, 2004.

Portera-Cailliau, C., Price, D.L., Martin, L.J. N-methyl-D-aspartate receptor proteins NR2A and NR2B are differentially distributed in the developing rat central nervous system as revealed by subunit-specific antibodies. J Neurochem 66: 692-700, 1996.

Ritter, L.M., Vazquez, D.M., Meador-Woodruff, J.H. Ontogeny of ionotropic glutamate receptor subunit expression in the rat hippocampus. Brain Res Dev Brain Res 139: 227-236, 2002.

Robinson, M.B., Sinor, J.D., Dowd, L.A., Kerwin, J.F. Subtypes of sodium-dependent high-affinity L-[^{3}H]glutamate transport activity – pharmacologic specificity and regulation by sodium and potassium. J Neurochem 60: 167-179, 1993.

Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., Kanai, Y., Hediger, M., Wang, Y., Schielke, J.P., Welty, D.F. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16: 675-686, 1996.

Sattler, R., Tymianski, M. Molecular mechanisms of calcium-dependent excitotoxicity. J Mol Med 78: 3-13, 2000.

Sauer, S.W., Okun, J.G., Schwab, M.A., Crnic, L.R., Hoffmann, G.F., Goodman, S.I., Koeller, D.M., Kölker, S. Bioenergetics in glutaryl-coenzyme A dehydrogenase deficiency: a role for glutaryl-coenzyme A. J Biol Chem 280: 21830-21836, 2005.

Sauer, S.W., Okun, J.G., Fricker, G., Mahringer, A., Müller, I., Crnic, L.R., Mühlhausen, C., Hoffmann, G.F., Hörster, F., Goodman, S.I., Harding, C.O., Koeller, D.M., Kölker, S. Intracerebral accumulation of glutaric and 3-hydroxyglutaric acids secondary to limited flux across the blood-brain barrier constitute a biochemical risk factor for neurodegeneration in glutaryl-CoA dehydrogenase deficiency. J Neurochem 97: 899-910, 2006.

Schlüter, K., Figiel, M., Rozyczka, J., Engele, J. CNS region-specific regulation of glial glutamate transporter expression. Eur J Neurosci 16: 836-842, 2002.

Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds). The Metabolic and Molecular Bases of Inherited Disease. 8th edition. New York, McGraw-Hill. 3-45, 2001.

Silva, C.G., Silva, A.R., Ruschel, C., Helegda, C., Wyse, A.T., Wannmacher, C.M., Dutra-Filho, C.S., Wajner, M. Inhibition of energy production in vitro by glutaric acid in cerebral córtex of young rats. Metab Brain Dis 15: 123-131, 2000.

Stellmer, F., Keyser, B., Burckhardt, B.C., Koepsell, H., Streichert, T., Glatzel, M., Jabs, S., Thiem, J., Herdering, W., Koeller, D.M., Goodman, S.I., Lukacs, Z., Ullrich, K., Burckhardt, G., Braulke, T., Mühlhausen, C. 3-Hydroxyglutaric acid is transported via the sodium-dependent dicarboxylate transporter NaDC3. J Mol Med 85: 763-70, 2007.

Stokke, O., Goodman, S.I., Moe, P.G. Inhibition of brain glutamate decarboxylase by glutarate, glutaconate, and beta-hydroxyglutarate: explanation of the symptoms in glutaric aciduria? Clin Chim Acta 66: 411-415, 1976.

Strauss, K.A., Morton, D.H. Type I glutaric aciduria, part 2: a model of acute striatal necrosis. Am J Med Genet C Semin Med Genet 121: 53-70, 2003.

Strauss, K.A., Puffenberger, E.G., Robinson, D.L., Morton, H. Type I glutaric aciduria, Part 1: Natural history of 77 patients. Am J Med Genet C Semin Med Genet 121: 38-52, 2003.

Tanaka, K., Watase, K., Manabe, T., Yamada, K., Watanabe, M., Takahashi, K., Iwama, H., Nishikawa, T., Ichihara, N., Kikuchi, T., Okuyama, S., Kawashima, N., Hori, S., Takimoto, M., Wada, K. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 276: 1699-1702, 1997.

Trotti, D., Danbolt, N.C., Volterra, A. Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? Trends Pharmacol Sci 19: 328-334, 1998.

Trotti, D., Rossi, D., Gjesdal, O., Levy, L.M., Racagni, G., Danbolt, N.C., Volterra, A. Peroxynitrite inhibits glutamate transporter subtypes. J Biol Chem 271: 5976-5979, 1996.

Ullensvang, K., Lehre, K.P., Storm-Mathisen, J., Danbolt, N.C. Differential development expression of the two rat brain glutamate transporter proteins GLAST and GLT. Eur J Neurosci 9: 1646-1655, 1997.

Ullrich, K., Flott-Rahmel, B., Schluff, P., Musshoff, U., Das, A., Lucke, T., Steinfeld, R., Christensen, E., Jakobs, C., Ludolph, A., Neu, A., Roper, R. Glutaric aciduria type I: pathomechanisms of neurodegeneration. J Inherit Metab Dis 22: 392-403, 1999.

Varadkar, S., Surtees, R. Glutaric aciduria type I and kynurenine pathway metabolites: a modified hypothesis. J Inherit Metab Dis 27: 835-842, 2004.

Volterra, A., Trotti, D., Tromba, C., Floridi, S., Racagni, G. Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes. J Neurosci 14: 2924-2932, 1994.

von Euler, G., Liu, Y. Glutamate and glycine decrease the affinity of [3H]MK-801 binding in the presence of Mg^{2+}. Eur J Pharmacol 245: 233-239, 1993.

Wajner, M., Kölker, S., Souza, D.O., Hoffmann, G.F., de Mello, C.F. Modulation of glutamatergic and GABAergic neurotransmission in glutary-CoA dehydrogenase deficiency. J Inherit Metab Dis 27: 825-828, 2004.

Wenzel, A., Fritschy, J.M., Mohler, H., Benke, D. NMDA receptor heterogeneity during postnatal development of the rat brain: differential expression of the NR2A, NR2B, and NR2C subunit proteins. J Neurochem 68: 469-478, 1997.

Yamaguchi, S., Orii, T., Yasuda, K., Yoshinori, K. A case of glutaric aciduria type 1 with unique abnormalities in the cerebral CT findings. Tohoku J Exp Med 151: 293-299, 1987.

Yudkoff, M., Daikhin, Y., Nissim, I., Horyn, O., Luhovyy, B., Lazarow, A., Nissim, I. Brain amino acid requirements and toxicity: the example of leucine. J Nutr 135: 1531S-1538S Review, 2005.

Ziadeh, R., Hoffman, E.P., Finegold, D.N., Hoop, R.C., Brackett, J.C., Strauss, A.W., Naylor, E.W. Medium chain acyl-CoA dehydrogenase deficiency in Pennsylvania: Neonatal screening shows high incidence and unexpected mutation frequencies. Pediatr Res 37: 675-678, 1995.

Zinnanti, W.J., Lazovic, J., Housman, C., Lanoue, K., O'callaghan, J.P., Simpson, I., Woontner, M., Goodman, S.I., Connor, J.R., Jacobs, R.E., Cheng, K.C. Mechanism of age-dependent susceptibility and novel treatment strategy in glutaric acidemia type I. J Clin Invest 117: 3258-3270, 2007.

Zinnanti, W.J., Lazovic, J., Wolpert, E.B., Antonetti, D.A., Smith, M.B., Connor, J.R., Woontner, M., Goodman, S.I., Cheng, K.C. A diet-induced mouse model for glutaric aciduria type I. Brain 129: 899-910, 2006a.

Zinnanti, W.J., Lazovic, J., Wolpert, E.B., Antonetti, D.A., Smith, M.B., Connor, J.R., Woontner, M., Goodman, S.I., Cheng, K.C. New insights for glutaric aciduria type I. Brain 129: e55, 2006b.

Zschocke, J., Quak, E., Guldberg, P., Hoffmann, G.F. Mutation analysis in glutaric aciduria type I. J Med Genet 37: 177-181, 2000.

Anexos

Trabalhos publicados relacionados à tese

ELSEVIER

Brain Research 956 (2002) 367–373

BRAIN RESEARCH

www.elsevier.com/locate/brainres

Research report

3-hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats

Alexandra Latini[a], Rafael Borba Rosa[a], Karina Scussiato[a], Susana Llesuy[b], Adriane Belló-Klein[b], Moacir Wajner[a,*]

[a]*Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Río Grande do Sul, Porto Alegre, RS, Brazil*
[b]*Departamento de Fisiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal de Río Grande do Sul, Rua Ramiro Barcelos, 2600 Anexo, CEP 90035-003, Porto Alegre, RS, Brazil*

Accepted 29 August 2002

Abstract

Glutaryl-CoA dehydrogenase deficiency (GDD) is an inherited neurometabolic disorder biochemically characterized by tissue accumulation of glutaric, 3-hydroxyglutaric (3-OHGA) and glutaconic acids and clinically by severe neurological symptoms and cerebral atrophy whose pathophysiology is poorly known. In the present study we investigated the effect of 3-OHGA, considered the main neurotoxin in GDD, on the lipoperoxidation parameters chemiluminescence and thiobarbituric acid-reactive species (TBA-RS), and on the amount of nitric oxide metabolites in cerebral cortex of young rats. Total radical-trapping antioxidant potential (TRAP), which reflects the tissue antioxidant defenses, was also examined. We observed that 3-OHGA significantly increased chemiluminescence, TBA-RS and nitric oxide metabolites, in contrast to TRAP, which was decreased by the metabolite. The data indicate a stimulation of lipid peroxidation and free radical production, and a reduction of the tissue antioxidant defenses caused by the metabolite. In case these findings also occur in the human condition, it may be presumed that oxidative stress is involved in the brain damage observed in GDD.
© 2002 Published by Elsevier Science B.V.

Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: Oxidative stress; Glutaric aciduria type I; Glutaryl-CoA dehydrogenase deficiency; 3-Hydroxyglutaric acid; Nitric oxide; Lipoperoxidation; Free radical

1. Introduction

Glutaryl-CoA dehydrogenase deficiency (GDD), also known as glutaric acidemia type I (GAI), is an autosomal recessive inherited neurometabolic disorder biochemically characterized by the accumulation of glutaric, 3-hydroxyglutaric and glutaconic acids in tissues and body fluids of affected patients due to a blockage in the L-lysine, hydroxy-L-lysine and tryptophan catabolic pathway [14]. GDD is included in a subgroup of organic acidurias called 'cerebral' since neurological manifestations without clear metabolic derangement are the predominant symptoms [14,21]. The mild neurological signs consisting of hypotonia with prominent head lag, irritability and macrocephaly occurring in the first few months of life become exacerbated by the occurrence of encephalopatic/encephalitis-like crises elicited by catabolic states following routine immunization, common infections or surgery. During these crises, which lead to irreversible destruction of vulnerable brain regions, i.e. striatum and cortex, the accumulating metabolites can reach millimolar concentrations [21,13]. Thereafter, the patients present a severe dystonic–dyskinetic disorder, sometimes associated to extreme hypotonia, rigidity and spasticity. Apart from acute neurodegenerative changes leading to striatal damage, neuroimaging of GDD patients usually present signs

*Corresponding author. Tel.: +55-51-3316-5571; fax: +55-51-3316-8010.
E-mail address: mwajner@ufrgs.br (M. Wajner).

0006-8993/02/$ – see front matter © 2002 Published by Elsevier Science B.V.
PII: S0006-8993(02)03573-4

of a delayed cerebral maturation (delayed myelination), spongy formation and attenuation of white matter signal (leukoencephalopathy) [7,6,11]. Besides, frontotemporal atrophy can be identified at birth, indicating that the impaired development of the central nervous system already begins in uterus and that the accumulating metabolites act very early during development [11].

Despite the marked alterations in the neuropathological findings, the mechanisms underlying the brain damage in GDD patients are poorly known. At first, an inhibition of neuronal glutamate decarboxylase activity decreasing GABA levels [35], and a toxic role of quinolinic acid [20] were hypothesized as pathogenetic mechanisms, but this was not subsequently confirmed [14].

On the other hand, excitotoxicity has been considered as a potential explanation for the neurological dysfunction of GDD since histological examination of the basal ganglia post-mortem in GDD has revealed postsynaptic vacuolation similar to that of glutamate-mediated damage [13]. Therefore, it has been proposed that the pathogenesis of GDD is related to an excitotoxic effect of the main pathological metabolites. In this context, substantial evidence relate the specific time course and localization of the neurological disease in GDD with a selective stimulation of the NR2B subtype of NMDA receptors by glutaric acid and 3-OHGA [24,28].

Recent in vitro and in vivo studies have pointed to 3-OHGA as the leading neurotoxin in GDD [9,27,26,36]. Mello and colleagues demonstrated that convulsions can be induced by 3-OHGA in a dose-dependent way [9]. Furthermore, it has recently been postulated that 3-OHGA may also induce oxidative stress [27,26]. Kölker and colleagues [27] have shown that 3-OHGA increased mitochondrial reactive oxygen species (ROS) generation in primary neuronal cultures from chick embryo telencephalons, which was prevented by the NMDA antagonist MK-801, suggesting an NMDA receptor-mediated mechanism. Moreover, when neuronal cultures were co-incubated with the free radical scavengers α-tocopherol and melatonine or when the cellular energy state was stabilized with creatine, free radical production was reduced or fully prevented [27]. In a subsequent study, Kölker and colleagues demonstrated that the antioxidant enzymes superoxide dismutase and catalase as well as the NMDA glutamate receptor antagonist MK-801 prevented 3-OHGA cytotoxicity in rat cortical and hippocampal astrocyte cultures, supporting a central role of NMDA receptor stimulation with subsequently increased superoxide anion production [26].

On the other hand, other studies performed in cortical cultures from neonatal rats have shown that 3-OHGA impairs the cellular energy by specifically inhibiting complexes II and V and therefore blocking the respiratory chain function, which was reflected by a decrease of creatine phosphate levels [36].

Taken together, these studies provide evidence that excitotocity, impairment of energy metabolism, and oxidative stress may be involved in the neuropathology of GDD. However, so far only scarce evidence of the participation of oxidative stress in the neurotoxicity of 3-OHGA has been observed. Therefore, in the present study we investigated the in vitro effect of 3-OHGA on some parameters of oxidative stress, namely chemiluminescence, thiobarbituric acid-reactive substances (TBA-RS), nitric oxide generation and finally on the total radical-trapping antioxidant potential (TRAP) which reflects the total antioxidant tissue defenses in the cerebral cortex of young rats. Our objective was therefore to evaluate the role of oxidative stress in 3-OHGA-induced neurotoxicity.

2. Material and methods

2.1. Animals and reagents

Male Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, were maintained on a 12:12 h light/dark cycle (lights on 07.00–19.00 h) in air conditioned constant temperature (22±1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow (Germani, Porto Alegre, RS, Brazil). The 'Principles of laboratory animal care' (NIH publication no. 85-23, revised 1985) were followed in all the experiments. All chemicals were purchased from Sigma (St. Louis, MO, USA) except thiobarbituric acid (TBA) which was purchased from Merck (Darmstadt, Germany). 3-OHGA was prepared on the day of the experiment in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The final concentrations of the acid in the incubation medium were 0.01, 0.1 or 1.0 mM. Chemiluminescence and TRAP were assayed using beta liquid scintillation spectrometers (Tri-Carb 2100TR and Wallac 1409, respectively), whereas the nitric oxide metabolites and the TBA-RS were measured with a double-beam spectrophotometer with temperature control (Hitachi U-2001).

2.2. Tissue preparation

On the day of the experiments the rats were sacrificed by decapitation without anesthesia and the brain was rapidly excised and placed on ice. The olfactory bulb, pons, medulla and cerebellum were discarded and the cerebral cortex was dissected, weighed and kept chilled until homogenization. The brain structure was homogenized in five volumes (1:5, w/v) of the incubation medium, consisting of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl, in a Teflon-glass homogenizer and centrifuged at 750 *g* for 10 min at 4 °C to discard nuclei and cell debris [12,30]. The pellet was discarded and the supernatant, a suspension of mixed and preserved

organelles such us mitochondria, was separated and immediately used for the measurements.

2.3. In vitro experiments

Cerebral cortex supernatants were incubated for 1 h at 37 °C in the presence of 3-OHGA at concentrations ranging from 0.01 to 1.0 mM. Controls did not contain 3-OHGA in the incubation medium. Immediately after incubation, aliquots were taken to measure chemiluminescence, TBA-RS, nitric oxide metabolites and TRAP.

2.4. Chemiluminescence

Samples were assayed for chemiluminescence in a dark room by the method of Gonzalez-Flecha and colleagues [12]. Incubation flasks contained 3.5 ml of medium consisting of 20 mM sodium phosphate buffer, pH 7.4 with 140 mM KCl. The background chemiluminescence was measured and 0.5 ml of supernatant was immediately added to the incubation medium. Chemiluminescence was measured for 30 min at room temperature. The background chemiluminescence was subtracted from the total value. Chemiluminescence was calculated as cpm/mg protein and expressed as percentage of controls. Cerebral cortex supernatants from five animals were used in these experiments.

2.5. Thiobarbituric acid-reactive species (TBA-RS)

TBA-RS were determined according to the method described by Esterbauer and Cheeseman [10]. Briefly, 300 µl of cold 10% (w/v) trichloroacetic acid were added to 150 µl of supernatant and centrifuged at 300 *g* for 10 min. Three hundred microlitres of the supernatant were transferred to a Pyrex tube and incubated with 300 µl of 0.67% (w/v) thiobarbituric acid in 7.1% (w/v) sodium sulphate, in a boiling water bath for 25 min. The mixture was allowed to cool on water for 5 min. The resulting pink stained TBA-RS were determined in a spectrophotometer at 535 nm. The acid did not produce color when tested without the addition of the supernatant, demonstrating the absence of a direct reaction to TBA. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as that of the supernatants. TBA-RS were calculated as nmol TBA-RS/mg protein and expressed as percentage of controls. For these experiments cerebral cortex supernatants from seven animals were used.

2.6. Nitric oxide metabolite measurement

The nitric oxide (NO) metabolites NO_3^- and NO_2^- were determined according to the method of Hevel and Marletta [19]. Five hundred µl of supernatant were deproteinized with 20 µl 25% (w/v) trichloroacetic acid and centrifuged at 18000 *g*. The supernatant was immediately neutralized with 35 µl 2 M potassium bicarbonate and used for the quantification of NO_3^- and NO_2^-. NO_3^- was reduced to NO_2^- by nitrate reductase. The total NO_2^- in the incubation medium was measured by a colorimetric assay at 540 nm based on the Griess reaction [19]. A standard curve was performed in the same way using sodium nitrate in order to calculate the concentration of the NO metabolites in the supernatants. Results from the evaluation of cerebral cortex supernatants from four animals were expressed as pmol (NO_3^- plus NO_2^-)/mg protein.

2.7. Total radical-trapping antioxidant potential (TRAP)

TRAP, representing the total antioxidant capacity of the tissue, was determined by measuring the luminol chemiluminescence intensity induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) according to the method of Lissi et al. [29]. The background chemiluminescence was measured by adding 4 ml 10 mM ABAP (in 0.1 M glycine buffer, pH 8.6) into a glass scintillation vial. Ten microlitres of luminol were added to each vial and the chemiluminescence was measured. This was considered to be the initial value. Ten microlitres of 300 µM Trolox or tissue supernatant were added and the chemiluminescence was measured until it reached the initial levels. The Trolox or supernatant addition to the incubation medium reduce the chemiluminescence. The time necessary for the chemiluminescence intensity returns to the initial value is called induction time (IT). IT is directly proportional to the antioxidant capacity of the tissue and the IT of each sample was compared with the IT of Trolox. TRAP values are expressed as nmol Trolox/mg protein. Supernatants of cerebral cortex from five animals were used for these experiments.

2.8. Protein determination

Protein concentrations were determined in the supernatant of cerebral cortex homogenates by the method of Lowry et al. [31], using bovine serum albumin as standard.

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the F value was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

3. Results

The in vitro effect of 3-OHGA on chemiluminescence, TBA-RS, NO metabolites and TRAP in cerebral cortex supernatants was studied. Lipoperoxidation was initially investigated by assessing chemiluminescence and TBA-RS

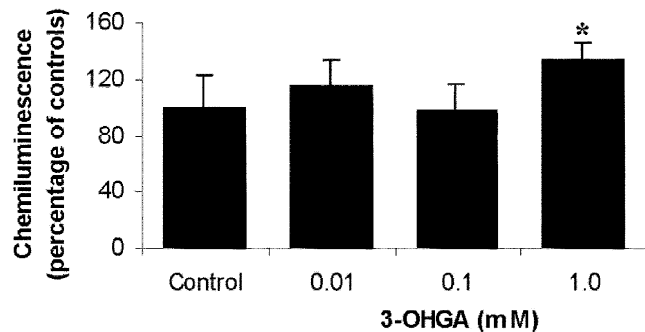

Fig. 1. Effect of 3-hydroxyglutaric acid (3-OHGA) on chemiluminescence in cerebral cortex from young rats. Data represent the mean±S.D. for five independent experiments (animals) performed in duplicate and are expressed as percentage of controls (1000±235 cpm/mg protein). Difference from control, *, $P<0.05$ (Duncan multiple range test).

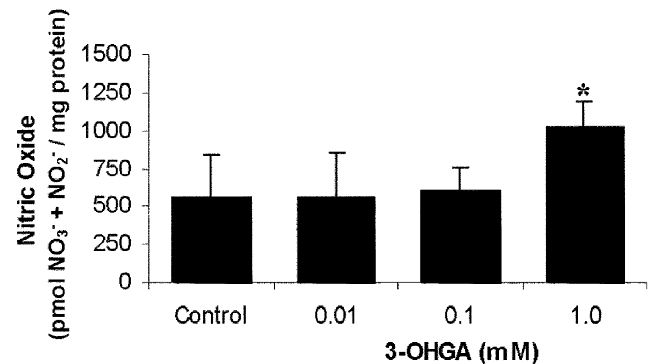

Fig. 3. Effect of 3-hydroxyglutaric acid (3-OHGA) on nitric oxide measurement in cerebral cortex from young rats. Data represent the mean±S.D. for four independent experiments (animals) performed in duplicate, expressed as pmol NO_3^- plus NO_2^-/mg protein. Difference from control, *, $P<0.05$ (Duncan multiple range test).

levels. Fig. 1 shows that 3-OHGA, at 0.01 to 1 mM concentrations, significantly increased (15–34%) chemiluminescence, as compared to controls [$F(3,16)=4.10$; $P<0.03$]. Similar results were obtained when measuring TBA-RS (Fig. 2). TBA-RS was significantly increased by 7–23%, as compared to controls [$F(3,24)=4.85$; $P<0.01$], when cortical supernatants were exposed to 0.01–1 mM concentrations of 3-OHGA, in a dose-dependent manner [$F=13.71$; $P<0.002$; $\beta=0.59$; $P<0.002$]. These results indicate that lipoperoxidation is stimulated by 3-OHGA.

Next, we determined the effect of 3-OHGA on NO production by measuring its derivatives NO_3^- plus NO_2^- in the cerebral cortex supernatants. Fig. 3 displays that NO_3^- plus NO_2^- concentrations significantly increased from 9–85%, as compared to control values when cortical supernatants were exposed to 0.01 to 1 mM 3-OHGA [$F(3,12)=3.77$; $P<0.05$], and this response was dose-dependent [$F=13.18$; $P<0.003$; $\beta=0.70$, $P<0.003$]. The results suggest that NO generation is induced by 3-OHGA.

Fig. 4 shows that the tissue antioxidant capacity (TRAP) was significantly diminished (29–34%) by 3-OHGA exposure at all tested concentrations (0.01 to 1 mM), as compared to controls [$F(3,16)=9.19$; $P<0.001$]. The data suggest that the tissue antioxidant defenses are reduced by 3-OHGA exposure.

4. Discussion

Glutaryl-CoA dehydrogenase deficiency (GDD) is a cerebral organic aciduria characterized by progressive neurodegeneration with neuroimaging findings of frontotemporal atrophy, delayed myelination with leukoencephalopathy, as well as caudate and putamen degeneration, which follows the characteristic encephalopathic crises when the levels of the accumulated metabolites glutaric and 3-OHGA dramatically increase to millimolar concentrations [21,13]. Although the neurological symptoms are predominant, its pathophysiology is far from understood. However, the available data in the literature points to 3-OHGA as the main neurotoxin in this disease. In this

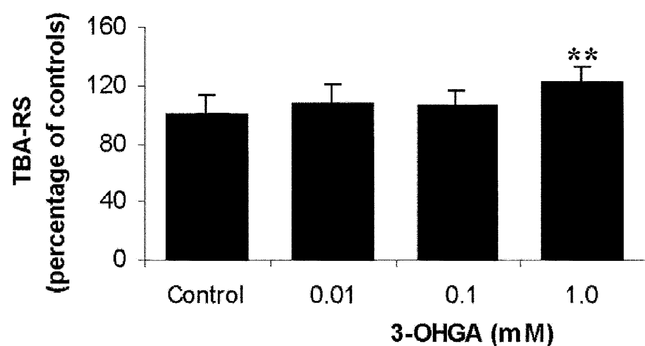

Fig. 2. Effect of 3-hydroxyglutaric acid (3-OHGA) on thiobarbituric acid-reactive species (TBA-RS) in cerebral cortex from young rats. Data represent the mean±S.D. for seven independent experiments (animals) performed in duplicate and are expressed as percentage of controls (1.3±0.2 nmol TBA-RS/mg protein). Difference from control, **, $P<0.01$ (Duncan multiple range test).

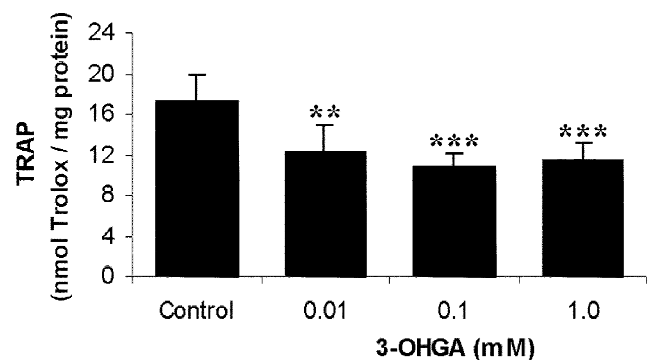

Fig. 4. Effect of 3-hydroxyglutaric acid (3-OHGA) on total radical-trapping antioxidant potential (TRAP) in cerebral cortex from young rats. Data represent the mean±S.D. for five independent experiments (animals) performed in duplicate and are expressed as nmol Trolox/mg protein. Difference from control, **, $P<0.01$; ***, $P<0.001$ (Duncan multiple range test).

context, recent in vitro studies have demonstrated that 3-OHGA induces neuronal damage involving excitotoxic mechanisms [24,22,28,9,25] and production of reactive oxygen species probably through stimulation of the NMDA receptor [27]. An inhibition of the respiratory chain by decreased activity of complex II and V and reduced levels of creatine phosphate provoked in vitro by 3-OHGA was also reported [36]. Despite these studies, the specific pathogenetic mechanisms of 3-OHGA on the central nervous system are still not fully established.

In the present study, we demonstrated that 3-OHGA significantly increased chemiluminescence and TBA-RS, as well as NO metabolites, and reduced TRAP levels in rat brain, indicating that this neurotoxin induced free radical generation and reduced the brain antioxidant defenses in vitro. Light in the chemiluminescence assay can arise from excited carbonyls, O_2^-, $ONOO^-$ and from peroxidizing lipids [18] as a result of increased oxygen and nitrogen free radical production. The increased value that we found in this parameter and in the TBA-RS measurement, which also reflects peroxidation of membrane fatty acids, is strongly suggestive that oxidative stress is induced in vitro by 3-OHGA. Furthermore, considering that the total antioxidant activity of the tissue (TRAP) gives a global picture of the non-enzymatic antioxidant status, the significant reduction of TRAP elicited by all 3-OHGA concentrations tested (0.01–1 mM) may therefore be due to a diminution of the brain tissue antioxidants of low-molecular mass (uric acid, selenium, protein SH groups, glutathione, α-tocopherol, ascorbic acid, coenzyme Q, lipoic acid and β-carotene, among others) which could be depleted by the increased levels of free radicals. However, we cannot rule out the possibility that other tissue antioxidant systems could be affected by the acid, such as the antioxidant enzymes catalase, glutathione peroxidase or superoxide dismutase and this should be a subject for future studies. Therefore, it is presumed that, because of the acute induction of free radical generation by 3-OHGA, some antioxidants defenses are consumed in the brain, giving rise to a reduction in the TRAP measurement.

Kölker and colleagues observed that 3-OHGA elicits ROS in primary neuronal cultures from chick embryo telencephalons and that 3-OHGA-induced neuronal death was significantly reduced or even prevented using α-tocopherol and melatonine, well known free radical scavengers [27]. These investigators also hypothesized that the increased mitochondrial ROS production was secondary to NMDA receptor activation by the acid because NMDA blockage by the antagonist MK-801, prevented ROS generation [27]. Our present findings provide evidence for the first time that acute exposure to 3-OHGA stimulates in vitro lipoperoxidation, a result of free radical generation, and decreases the antioxidant defenses in the cerebral cortex of young rats. Indirect production of ROS through stimulation of glutamate receptors is very unlikely in our case since we used in our experiments cerebral cortex supernatants which contained mitochondria and other subcellular fractions, which allow ROS production, but did not contain glutamate receptors. Kölker and collaborators used intact cells which, after appropriate glutamate receptor stimulation, result in Ca^{2+} entrance into the cell and activation of oxidative stress through distinct pathways including NO synthesis. We cannot therefore rule out ROS formation secondary to NMDA activation by 3-OHGA in systems using intact cells, such as those employed by Kölker and colleagues [27].

In addition, we also observed an increment of the NO metabolites NO_3^- plus NO_2^- in the brain tissue exposed to 3-OHGA, reflecting an increased NO production probably secondary to NOS stimulation. Taken together, the data presented in this investigation is strongly indicative of an in vitro stimulation of oxidative stress by 3-OHGA in the brain since this process is defined as an imbalance between free radical production and antioxidant defenses.

At this point, it should be emphasized that the brain has low cerebral antioxidant defenses compared to other tissues [15], a fact that makes this tissue more vulnerable to increased ROS. In fact, oxidative stress has been implicated in the pathophysiology of common neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, as well as epileptic seizures and demyelination [16,32,34,23].

We cannot determine at the present time whether energy deprivation, excitotoxicity or oxidative stress is the main pathogenetic mechanism leading to 3-OHGA brain injury. Regarding excitotoxicity, it is not fully established whether excessive activation of glutamate receptors is exclusively mediated via direct stimulation of NMDA receptors subtype NR2B by 3-OHGA [24,28,9,25] or through indirect mechanisms of NMDA receptor activation, such as secondary excitotoxicity due to cellular energy depletion caused by the metabolite [36], through upregulation of the NR2B subunit [8] or due to other alterations of cellular metabolism induced by 3-OHGA. With lack of energy the glutamate carriers may not function properly or may even reverse their operation and release glutamate into the extracellular space leading to activation of distinct glutamate receptor subtypes [37]. In this scenario, Ullrich and collaborators have demonstrated that 3-OHGA inhibits energy production by blocking the respiratory chain at various complexes. They also demonstrated that 3-OHGA activates NMDA receptors but does not directly interact with glutamate receptors and does not modify the binding of glutamate to receptors [36]. These findings indicate that the activation of NMDA receptors by 3-OHGA may be secondary to energy depletion (secondary excitotoxicity). In addition, it has been reported that inhibition of Ca^{2+}/calmodulin kinases upregulates the NR2B subunit of NMDA receptors in neurons [8], so that it would be of interest to test whether 3-OHGA exposure alter protein kinase and phosphatase activities in neural cells. On the other hand, overstimulation of glutamate receptors may

result in oxidative stress [2,3,33], and this was verified in previous experiments showing that induction of ROS and neuronal damage provoked by 3-OHGA is prevented by NMDA antagonists, but also by creatine, α-tocopherol and less efficiently by melatonine [27]. Therefore, it may be concluded that energy deprivation indirectly leads, at least in part, to NMDA activation and consequently to oxygen and nitrogen free radical generation.

Our present findings demonstrated that NO synthesis was significantly stimulated by 3-OHGA at the highest concentration used (1 mM), and this is in accordance with previous works demonstrating that 3-OHGA-induced NO generation is rather mild [27,26]. Therefore, considering that free radicals and particularly NO are potent inhibitors of the respiratory chain [5,4,1], our findings of an enhancement of NO generation would possibly result in a lower energy generation and further increase of free radical production since blockage of the electron transport chain leads to stimulation of superoxide and hydrogen peroxide formation [17].

It is therefore feasible that all three mechanisms (excitotoxicity, energy deprivation and oxidative stress) might act together with a complex intricate relationship and may contribute to the neuropathology of GDD. Even though the concentrations of 3-OHGA used in our experiments that significantly modified the oxidative stress parameters measured are similar to those found in CSF and plasma from individuals affected by GDD [14,21,13], it is difficult to extrapolate our findings to the human condition. However, if the effects detected in this study also occur in vivo, it is tempting to speculate that they may contribute, at least in part, to the neurological dysfunction found in GDD. Comparison between parameters of oxidative stress in CSF of GDD patients and of normal aged-matched controls would also be of interest to strengthen our findings pointing to stimulation of oxidative stress in GDD. Finally, it is proposed that the administration of antioxidants should be considered as an adjuvant therapy to specific diets or to other pharmacological agents for these patients.

Acknowledgements

This work was supported in part by grants from FAPERGS, PRONEX II and CNPq-Brazil.

References

[1] A. Almeida, S.J.R. Heales, J.P. Bolaños, J.M. Medina, Glutamate neurotoxicity is associated with nitric oxide-mediated mitochondrial dysfunction and glutathione depletion, Brain Res. Rev. 790 (1998) 209–216.

[2] M.F. Beal, Aging, energy, and oxidative stress in neurodegenerative diseases, Ann. Neurol. 38 (1995) 357–366.

[3] M.F. Beal, Mitochondria, free radicals, and neurodegeneration, Curr. Opin. Neurobiol. 6 (1996) 661–666.

[4] J.P. Bolaños, A. Almeida, J.M. Medina, Nitric oxide mediates brain mitochondrial damage during perinatal anoxia, Brain Res. 787 (1998) 117–122.

[5] J.P. Bolaños, A. Almeida, V. Stewart, S. Peuchen, J.M. Land, J.B. Clark, S.J.R. Heales, Nitric oxide-mediated mitochondrial damage in brain: mechanisms and implications for neurodegenerative diseases, J. Neurochem. 68 (1997) 2227–2240.

[6] J. Brismar, P.T. Ozand, CT, MR of the brain in glutaric acidemia type I: a review of 59 published cases and a report of 5 new patients, Am. J. Neuroradiol. 16 (1995) 675–683.

[7] C.W. Chow, E.A. Haan, S.I. Goodman, R.M. Anderson, W.A. Evans, B.K. Kleinschmidt-DeMasters, G. Wise, J.J. McGill, D.M. Danks, Neuropathology in glutaric acidaemia type 1, Acta Neuropathol. 76 (1988) 590–594.

[8] L. Corsi, J.H. Li, K.E. Krueger, Y.H. Wang, B.B. Wolfe, S. Vicini, Up-regulation of NR2B subunit of NMDA receptors in cerebellar granule neurons by Ca^{2+}/calmodulin kinase inhibitor KN93, J. Neurochem. 70 (1998) 1898–1906.

[9] C.F. de Mello, S. Kölker, B. Ahlemeyer, F.R. de Souza, M.R. Fighera, E. Mayatepek, J. Krieglstein, G.F. Hoffmann, M. Wajner, Intrastriatal administration of 3-hydroxyglutaric acid induces convulsions and striatal lesions in rats, Brain Res. 916 (2001) 70–75.

[10] H. Esterbauer, K.H. Cheeseman, Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal, Methods Enzymol. 186 (1990) 407–421.

[11] R. Forstner, G.F. Hoffmann, I. Gassner, P. Heideman, J.B. de Klerk, B. Lawrenz-Wolf, E. Doringer, P. Weiss-Wichert, J. Troger, J.P. Colombo, E. Plochl, Glutaric aciduria type I: ultrasonographic demonstration of early signs, Pediatr. Radiol. 29 (1999) 138–143.

[12] B. Gonzalez-Flecha, S. Llesuy, A. Boveris, Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of heart, liver and muscle, Free Rad. Biol. Med. 10 (1991) 93–100.

[13] S.I. Goodman, M.D. Norenberg, R.H. Shikes, D.J. Breslich, P.G. Moe, Glutaric aciduria type 1: biochemical and morphological considerations, J. Pediatr. 90 (1977) 746–750.

[14] S.I. Goodman, F.E. Frerman, Organic acidemias due to defects in lysine oxidation: 2-ketoadipic academia and glutaric acidemia, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Walle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, 8th Edition, McGraw-Hill, New York, 2001, pp. 2195–2204.

[15] B. Halliweel, Free radicals, proteins and DNA: oxidative damage versus redox regulation, Biochem. Soc. Trans. 24 (1996) 1023–1027.

[16] B. Halliwell, J.M.C. Gutteridge, Oxygen radicals and nervous system, Trends Neurosci. 8 (1996) 22–26.

[17] B. Halliwell, J.M.C. Gutteridge, Oxygen is a toxic gas—an introduction to oxygen toxicity and reactive oxygen species, in: B. Halliwell, J.M.C. Gutteridge (Eds.), Free Radicals in Biology and Medicine, 3rd Edition, Oxford University Press, Oxford, 2001, pp. 1–35.

[18] B. Halliwell, J.M.C. Gutteridge, Detection of free radicals and others reactive species: trapping and fingerprinting, in: B. Halliwell, J.M.C. Gutteridge (Eds.), Free Radicals in Biology and Medicine, 3rd Edition, Oxford University Press, Oxford, 2001, pp. 351–425.

[19] J.M. Hevel, M.A. Marletta, Nitric oxide synthase assays, Methods Enzymol. 233 (1994) 250–258.

[20] M.P. Heyes, Hypothesis: a role for quinolinic acid in the neuropathology of glutaric aciduria type I, Can. J. Neurol. Sci. 14 (1987) 441–443.

[21] G.F. Hoffmann, J. Zschocke, Glutaric aciduria type I: From clinical, biochemical and molecular diversity to successful therapy, J. Inherit. Metab. Dis. 22 (1999) 381–391.

[22] G.F. Hoffmann, S. Athanassopoulos, A.B. Burlina, M. Duran, J.B. de Klerk, W. Lehnert, J.V. Leonard, A.A. Monavari, E. Muller, A.C. Muntau, E.R. Naughten, B. Plecko-Starting, A. Superti-Furga, J. Zschocke, E. Christensen, Clinical course, early diagnosis, treatment, and prevention of disease in glutaryl-CoA dehydrogenase deficiency, Neuropediatrics 27 (1996) 115–123.

[23] E. Karelson, N. Bogdanovic, A. Garlind, B. Winblad, K. Zilmer, T. Kullisaar, T. Vihalemm, C. Kairane, M. Zilmer, The cerebrocortical areas in normal brain aging and in the Alzheimer's Disease: noticeable difference in the lipid peroxidation level and in antioxidant defense, Neurochem. Res. 26 (2001) 353–361.

[24] S. Kölker, B. Ahlemeyer, J. Kriegstein, G.F. Hoffmann, 3-Hydroxyglutaric and glutaric acids are neurotoxic trough NMDA receptors in vitro, J. Inherit. Metab. Dis. 22 (1999) 259–262.

[25] S. Kölker, J.G. Okun, B. Ahlemeyer, A.T. Wyse, F. Horster, M. Wajner, D. Kohlmuller, E. Mayatepek, J. Krieglstein, G.F. Hoffmann, Chronic treatment with glutaric acid induces partial tolerance to excitotoxicity in neuronal cultures from chick embryo telencephalons, J. Neurosci. Res. 685 (2002) 424–431.

[26] S. Kölker, B. Ahlemeyer, R. Huhne, E. Mayatepek, J. Kriegstein, G.F. Hoffmann, Potenciation of 3-hydroxyglutarate neurotoxicity following induction of astrocitic iNOS in neonatal rat hippocampal cultures, Eur. J. Neurosci. 13 (2001) 2115–2122.

[27] S. Kölker, B. Ahlemeyer, J. Kriegstein, G.F. Hoffmann, Contribution of reactive oxygen species to 3-hydroxyglutarate neurotoxicity in primary neuronal cultures from chick embryo telencephalons, Pediatr. Res. 50 (2001) 76–82.

[28] S. Kölker, B. Ahlemeyer, J. Kriegsteinm, G.F. Hoffmann, Maturation-dependent neurotoxicity of 3-hydroxyglutaric and glutaric acids in vitro: a new pathophysiologic approach to glutaryl-CoA dehydrogenase, Pediat. Res. 47 (2000) 495–503.

[29] E. Lissi, C. Pascual, M.D. Del Castillo, Luminol luminescence induced by 2,2′-azo-bis-(2-amidinopropane) thermolysis, Free Rad. Res. Commun. 17 (1992) 299–311.

[30] S.F. Llesuy, J. Milei, H. Molina, A. Boveris, S. Milei, Comparison of lipid peroxidation and myocardial damage induced by adriamycin and 4′-epiadriamycin in mice, Tumori 71 (1985) 241–249.

[31] O.H. Lowry, N.J. Rosebrough, A. Lewis-Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[32] E. Méndez-Álvarez, R. Soto-Otero, A. Hermida-Aeijeiras, A.M. López-Real, J.L. Labandeira-García, Effects of aluminium and zinc on the oxidative stress caused by 6-hydroxydopamine autoxidation: relevance for the pathogenesis of Parkinson's disease, Biochim. Biophys. Acta 1586 (2001) 155–168.

[33] D.G. Nicholls, S.L. Budd, Mitochondria and neuronal glutamate excitotoxicity, Biochim. Biophys. Acta 1366 (1998) 97–112.

[34] A.Z. Reznick, L. Packer, Free radicals and antioxidants in muscular neurological diseases and disorders, in: G. Poli, E. Albano, M.U. Dianzani (Eds.), Free Radicals: From Basic Science To Medicine, Birkhäuser Verlag, Basel, 1993, pp. 425–437.

[35] O. Stokke, S.I. Goodman, P.G. Moe, Inhibition of brain glutamate decarboxylase by glutarate, glutaconate and beta-hydroxyglutarate: explanations of the symptoms in glutaric aciduria?, Clin. Chim. Acta 66 (1976) 411–415.

[36] K. Ullrich, B. Flott-Rahmel, P. Schluff, U. Musshoff, A. Das, T. Lücke, R. Steinfeld, E. Christensen, C. Jacobs, A. Ludolph, A. Neu, R. Röper, Glutaric aciduria type I: pathomechanisms of neurodegeneration, J. Inherit. Metab. Dis. 22 (1999) 392–403.

[37] G.D. Zeevalk, W.J. Nicklas, Evidence that the loss of the voltage-dependent Mg^{2+} block at the *N*-methyl-D-aspartate receptor underlies receptor activation during inhibition of neuronal metabolism, J. Neurochem. 59 (1992) 1211–1220.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Neurochemistry International 44 (2004) 345–353
NEUROCHEMISTRY International
www.elsevier.com/locate/neuint

3-Hydroxyglutaric acid enhances glutamate uptake into astrocytes from cerebral cortex of young rats

M.E.S. Frizzo[a,b], C. Schwarzbold[a], L.O. Porciúncula[a], K.B. Dalcin[a], R.B. Rosa[a,b,c], C.A.J. Ribeiro[a], D.O. Souza[a], M. Wajner[a,c,*]

[a] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS, Porto Alegre, RS, CEP 900035-003, Brazil*
[b] *Departamento de Ciências Morfológicas, Instituto de Ciências Básicas da Saúde, UFRGS, Porto Alegre, RS, CEP 900035-003, Brazil*
[c] *Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil*

Received 24 October 2002; received in revised form 18 June 2003; accepted 1 July 2003

Abstract

A predominantly neurological presentation is common in patients with glutaric acidemia type I (GA-I). 3-Hydroxyglutaric acid (3-OHGA), which accumulates in affected patients, has recently been demonstrated to play a central role in the neuropathogenesis of this disease. In the present study, we investigated the in vitro effects of 3-OHGA at concentrations ranging from 10 to 1000 μM on various parameters of the glutamatergic system, such as the basal and potassium-induced release of [^{3}H]glutamate by synaptosomes, as well as on Na^+-dependent [^{3}H]glutamate uptake by synaptosomes and astrocytes and Na^+-independent [^{3}H]glutamate uptake by synaptic vesicles from cerebral cortex of 30-day-old Wistar rats. First, we observed that exposure of cultured astrocytes to 3-OHGA for 20 h did not reduce their viability. Furthermore, 3-OHGA significantly increased Na^+-dependent [^{3}H]glutamate uptake by astrocytes by up to 80% in a dose-dependent manner at doses as low as 30 μM. This effect was not dependent on the presence of the metabolite during the uptake assay, since it occurred even when 3-OHGA was withdrawn from the medium after cultured cells had been exposed to the acid for approximately 1 h. All other parameters investigated were not influenced by this organic acid, indicating a selective action of 3-OHGA on astrocyte transporters. Although the exact mechanisms involved in 3-OHGA-stimulatory effect on astrocyte glutamate uptake are unknown, the present findings contribute to the understanding of the pathophysiology of GA-I, suggesting that astrocytes may protect neurons against excitotoxic damage caused by 3-OHGA by increasing glutamate uptake and therefore reducing the concentration of this excitatory neurotransmitter in the synaptic cleft.
© 2003 Elsevier Ltd. All rights reserved.

Keywords: 3-Hydroxyglutaric acid; Glutaric acidemia type I; Glutaryl-CoA dehydrogenase; Excitotoxicity; Glutamate

1. Introduction

Glutaric aciduria type I (GA-I; synonym, glutaryl-CoA dehydrogenase (GCDH) deficiency; OMIM # 231670) is an autosomal recessive disorder in the catabolic pathway of lysine, hydroxylysine and tryptophan with an estimated prevalence of 1 in 40,000 newborns, caused by deficiency of the mitochondrial flavoenzyme glutaryl-CoA dehydrogenase (EC 1.3.99.7) (Hoffmann et al., 1996; Hoffmann and Zschocke, 1999; Goodman et al., 2001). Deficient GCDH activity results in an accumulation of glutaric acid (GA), 3-hydroxyglutaric acid (3-OHGA) and—to a lesser extent—glutaconic acid in body fluids and in brain (Goodman et al., 1977). The disorder is considered a *cerebral* organic acidemia because it predominantly presents with a neurologic symptomatology (Hoffmann et al., 1994). If untreated, most children with GA-I develop dystonic-dyskinetic movement disorders caused by striatal destruction during encephalopathic crises, which are precipitated by infectious diseases or vaccinations and usually occur between 3 and 18 months of age. Over subsequent years progressive involvement of the cortical white matter and the midbrain structures appears (Hoffmann and Zschocke, 1999).

At present, the exact mechanisms underlying the brain damage of the affected patients are only partly understood. A considerable body of evidence has emerged from recent studies indicating excitotoxic actions for GA and 3-OHGA (Flott-Rahmel et al., 1997; Lima et al., 1998; Kölker et al., 1999; Ullrich et al., 1999; Kölker et al., 2000a,b; Porciúncula et al., 2000; Mello et al., 2001; Kölker et al., 2002a,b). 3-OHGA has been shown in in vitro and in vivo

* Corresponding author. Tel.: +55-51-33165571; fax: +55-51-33168010.
E-mail address: mwajner@ufrgs.br (M. Wajner).

0197-0186/$ – see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0197-0186(03)00169-4

models to induce excitotoxic neuronal damage mediated by NMDA receptors (Kölker et al., 1999, 2000b, 2001a; Mello et al., 2001; Kölker et al., 2002a,b). *Post mortem* examinations of the basal ganglia and cerebral cortex of patients with GA-I has revealed postsynaptic vacuolization characteristic of glutamate-mediated brain injury. In addition, disturbances of brain energy metabolism caused by these metabolites have also been reported (Al Essa et al., 1998; Ullrich et al., 1999; Silva et al., 2000; Das and Ullrich, 2003). This is in line with reduced brain glucose utilization and increased urinary excretion of lactate and dicarboxylic acids encountered in patients with GA-I (Gregersen and Brandt, 1979). Furthermore, 3-OHGA was demonstrated to decrease ATP synthase activity and creatine phosphate concentration in cultured mixed cortical cells from neonatal rats and suggested to secondarily amplify NMDA receptor activation via removal of the voltage-dependent Mg^{2+} block of NMDA receptors leading secondarily to neuronal damage (Ullrich et al., 1999; Das and Ullrich, 2003).

It has been also hypothesized that the maturation-dependent and regional vulnerability in this disease is determined by a variety of factors, e.g. expression of NMDA receptors (in particular of the NR1/NR2B subtype), high excitotoxic susceptibility of striatal medium spiny neurons, the high energy demand of the basal ganglia, and the glutamatergic input via the corticostriatal pathway. Although 3-OHGA is a neurotoxin, its neurotoxic properties are quite weak compared to other compounds inducing primary or secondary excitotoxic cell damage, such as glutamate, NMDA, malonate, or 3-nitropropionate. Therefore, it has been suggested that 3-OHGA can only exert relevant neurotoxicity under pathophysiological conditions if additional trigger factors are present. It has been shown that hyperthermia as well as cytokine-induced stimulation of astrocytic iNOS expression strongly enhanced 3-OHGA neuronal cell damage (Kölker et al., 2001b, 2000c).

Glutamate receptors mediate most of the excitatory neurotransmission in mammalian central nervous system, participating in cerebral plasticity, memory and learning, and in the formation of neural networks during development (Ozawa et al., 1998). The appropriate activation of these receptors is essential for normal brain development and function, whereas their overstimulation potentially leads to excitotoxicity, as found in neurodegenerative disorders and brain injuries (Olney, 1980; Meldrum and Garthwaite, 1990). Therefore, maintaining the extracellular glutamate concentrations below neurotoxic levels is critical to brain function (Anderson and Swanson, 2000; Danbolt, 2001). The synaptic actions of glutamate are terminated by its uptake mainly by astrocytes, where it is converted to glutamine and subsequently transported to presynaptic terminals, reconverted to glutamate and finally stored into synaptic vesicles. The glutamate-glutamine cycle involves two transport systems: (a) high affinity Na^+-dependent carriers located in the cell membranes of neural cells, and (b) a low affinity Na^+-independent carriers located in the membrane of synaptic vesicles (Robinson and Dowd, 1997). Glutamate inside the synaptic vesicles is available for release during depolarization.

We have recently observed that GA inhibits [^{3}H]glutamate uptake in synaptosomes and [^{3}H]glutamate binding to plasma membranes from rat brain (Porciúncula et al., 2000). In the present study, we investigated the effects of 3-OHGA, considered the main neurotoxin in GA-I, on glutamate release by synaptosomes, as well as on glutamate uptake by synaptosomal preparations, synaptic vesicles and cultured astrocytes from rat brain.

2. Material and methods

Thirty-day-old male Wistar rats were used in the experiments. They were maintained at approximately 25 °C, on a 12:12 h light/dark cycle, with free access to food and water. Animals were killed by decapitation without anesthesia, the brain was rapidly removed and the cerebral cortices were dissected.

[^{3}H]Glutamate (48 Ci/mmol) was purchased from Amersham International, UK. Fetal bovine serum (FBS) certified (USA) and Eagle's minimal essential media (MEM Earle's salts) were from Gibco. All other chemicals were of analytical reagent grade and were obtained from Sigma (St Louis, MO, USA).

2.1. Synaptosomal preparation

Synaptosomal preparations were obtained by isotonic Percoll/sucrose discontinuous gradients at 4 °C, as previously described with modifications (Dunkley et al., 1986). Homogenates (10% (w/v)) from cerebral cortices were made in 1.28 M sucrose, 4 mM EDTA and 25 mM DDT (pH 7.4), and centrifuged at $800 \times g$ for 10 min The supernatant containing synaptosomes were subjected to 23, 15, 7 and 3% Percoll solution density gradient centrifugation at $24{,}000 \times g$ for 10 min. The synaptosomal fractions were isolated from the 23, 15 and 7% Percoll bands. The pooled fraction was suspended, homogenized in HBSS buffered low K^+ with Ca^{2+} (pH 7.4), containing in mM: 133 NaCl, 2.4 KCl, 1.2 KH_2PO_4, 1.09 $MgSO_4$, 27.7 HEPES, 0.012 glucose and 0.001 $CaCl_2$ and centrifuged at $21{,}000 \times g$ for 15 min The supernatant was removed and the pellet gently resuspended in HBSS buffer (0.3 ml/cerebral cortex). The synaptosomal fraction used contained approximately 2.2 mg of protein/ml of incubation mixture. 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; Nagi 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 synaptosomes with 3-OHGA for 15 min The LDH activity in the incubation medium and the total LDH content, which was determined by synaptosomal disruption using 1.5% Triton X-100, were assayed spectrophotometrically at a wavelength of 340 nm (Labtest reagents, Brazil).

2.1.1. Synaptosomal [^{3}H]glutamate release

Determination of [^{3}H]glutamate release was accomplished as described by Migues et al. (1999). Prior to release assay, synaptosomal preparations were loaded with labeled [^{3}H]glutamate for 15 min at 37 °C. Incubation was performed in a non-depolarizing medium, consisting of HBSS (HEPES buffered salt solution, composition in mM: 27 HEPES, 133 NaCl, 2.4 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 12 glucose, 1.0 $CaCl_2$) in the presence of 0.1 μCi [^{3}H]glutamate (Amersham, specific activity 53 Ci/mmol, final concentration 5×10^{-7} mM). Aliquots of labeled synaptosomal preparations (1.4 mg protein) were centrifuged at 16,000 × *g* for 1 min Supernatants were discarded, and the pellets were washed four times in HBSS by centrifugation at 16,000 × *g* for 1 min (at 4 °C). To assess the basal release of [^{3}H]glutamate, the final pellet was resuspended in HBSS and incubated for 1 min in the absence (control) or presence of 3-OHGA (10–1000 μM). K^+-stimulated [^{3}H]glutamate release was assessed as described for basal release, except that the incubation medium contained 40 mM KCl to induce synaptosomal depolarization. Incubation was terminated by immediate centrifugation (16,000 × *g* for 1 min). Radioactivity present in supernatants and pellets was separately determined in a Wallac scintillation counter. [^{3}H]Glutamate release was calculated as the percentage of total amount of radiolabel glutamate present at the start of the incubation period in preloaded synaptosomes. The total amount of synaptosomal [^{3}H]glutamate under these conditions was 9.9 nmoles/(mg protein).

2.1.2. [^{3}H]Glutamate uptake into synaptosomal preparation

[^{3}H]Glutamate uptake was measured in a medium containing 110 mM NaCl, 25 mM glucose, 5.3 mM KCl, 1 mM $MgSO_4 \cdot 6H_2O$, 1.8 mM $CaCl_2$, 6 mM sucrose in 40 mM Tris/acetate, pH 7.4, 1 μM [^{3}H]glutamate, and 3-OHGA (10–1000 μM). Controls did not contain 3-OHGA. The reaction was started by adding the synaptosomal preparation (100–200 μg of protein). After 1 min of incubation at 37 °C, the uptake was stopped by filtration through GF/B filters. Filters were rinsed three times with 4 ml of ice-cold 15 mM Tris/acetate, pH 7.4, containing 155 mM ammonium acetate and the bound radioactivity was determined with a Wallac scintillation spectrometer. Sodium independent uptake (10–20% of the total uptake) was determined by incubating the samples in 20 mM Tris/acetate, pH 7.4, containing 0.32 M sucrose. Specific uptake was considered as the difference between total uptake and nonspecific uptake. All experiments were performed in triplicate.

2.2. Synaptic vesicle preparation

Synaptic vesicles were prepared from cerebral cortex as described by Fykse and Fonnum (1996), with some modifications. Briefly, homogenates (10% (w/v)) from cerebral cortex of rats were made in buffer containing 0.32 M sucrose, 10 mM MOPS/Tris buffer, pH 7.4, and 1 mM EGTA and centrifuged twice for 10 min at 1000 × *g*. Both supernatants were pooled and centrifuged for 30 min at 20,000 × *g* to obtain the crude synaptosomal fraction (P2). This fraction was osmotically shocked by resuspension in 10 mM MOPS/Tris, pH 7.4, containing 0.1 mM EGTA (approximately 0.8 ml/g of fresh tissue) and centrifuged at 17,000 × *g* for 30 min The supernatant containing synaptic vesicles was subjected to 0.4 M and 0.6 M sucrose density gradient centrifugation at 65,000 × *g* for 2 h. The synaptic vesicle fraction was isolated from the 0.4 M sucrose band and stored at −70 °C for up to 4 weeks, with no loss of activity. We performed controls with addition of high sodium concentration in order to evaluate the presence of possible contaminating vesicles formed from plasma membranes. In all synaptic vesicle preparations assayed, the uptake was not stimulated by the addition of sodium (data not shown).

2.2.1. Synaptic vesicular [^{3}H]glutamate uptake

Synaptic vesicular uptake experiments were performed in a standard medium (final volume of 200 μl) composed of 10 mM MOPS/Tris, pH 7.4, 4 mM KCl, 140 mM potassium gluconate, 0.12 M sucrose, 2 mM $MgCl_2$, and 2 mM ATP, as described before (Wolosker et al., 1996). Glutamate uptake was performed in standard medium, with 50 μM [^{3}H]glutamate (3 μCi/ml). In experiments performed to study the effects of 3-OHGA the metabolite (10–1000 μM) was added to the uptake medium. The uptake was started by addition of synaptic vesicles (30–40 μg of protein/tube). Incubation was carried out for 10 min at 35 °C, and the reaction was stopped by rapid filtration of the assay medium through 0.45 μm Millipore filters. The filters were quickly flushed three times with 4 ml of 10 mM MOPS/Tris buffer, pH 7.4, at room temperature. Specific uptake was calculated as [^{3}H]glutamate uptake measured in the absence of ATP. Radioactivity was measured with a Wallac scintillation counter.

2.3. Astrocyte cultures

Primary astrocyte cultures were prepared from the cortices of 1-day-old Wistar rats as described previously (Sanetto and De Vellis, 1985). The plating medium was MEM with 10% FBS plus 10 ng/ml EGF. In each preparation, cortices from six pups were dissociated with trypsin followed by DNAse I (all obtained from Gibco) and the cells were maintained in an incubator at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. When confluence was achieved (6–7 days in vitro (DIV)), the medium was replaced by MEM and 5% FBS. Furthermore, 10 μM cytosine arabinoside (Ara-C) was added for 48 h to eliminate mitotic cells. The subsequent

medium change was performed in Ara-C-free media. EGF, which has been shown to induce GLT-1 protein expression in astrocytes (Zelenaia et al., 2000), was added to cell plating. Cultures were used at 9–12 days in vitro with astrocytes showing a confluent monolayer aspect, being >95% of glial fibrillary acidic protein (GFAP)-positive cells. The culture media were replaced with fresh media on the evening before glutamate uptake assays. Experiments were repeated at least three times and each experiment was performed in quadruplicate.

2.3.1. Measurement of cell viability

Cell viability of cultured astrocytes was determined by determination of the lactic dehydrogenase (LDH, EC 1.11.27) release from the cells in the medium and by the MTT test (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolic bromide), measuring the formation of formazan, which is dependent on the activity of mitochondrial dehydrogenases. Astrocytes were incubated for 20 h with 1 mM 3-OHGA. At the end of the incubation period, LDH release and MTT test were performed. LDH release was assayed spectrophotometrically (Labtest reagents, Brazil), as it was the formazan formation (570 and 630 nm).

2.3.2. Astrocyte [^{3}H]glutamate uptake

[^{3}H]Glutamate uptake assays were initiated by rinsing and pre-incubating the cultures for 53 min in Hank's balanced salt solution (HBSS), pH 7.2 in an incubator at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Various concentrations of 3-OHGA (3–1000 μM) were used in the pre-incubation period (53 min) and maintained in the medium during the uptake assay (7 min). The uptake was assessed by adding 0.33 μCi/ml L-[2,3-^{3}H]glutamate (American Radiochemical; 45 Ci/mmol) with 100 μM unlabeled glutamate to the culture wells. Incubation was stopped after 7 min by two ice-cold washes with 1 ml HBSS followed by immediate lyses during 15 min (0.5N NaOH). Glutamate uptake experiments were performed in the linear range of time curve (data not shown). Aliquots of lysates were taken for scintillation counting. Non-specific uptake was determined by using choline chloride instead of sodium chloride, being subtracted from the total uptake to obtain the specific uptake. In some experiments the astrocytes were washed after being exposed to 3-OHGA for 53 min and the uptake assay carried out in the absence of this organic acid.

2.4. Protein measurement

The protein content of synaptosomal, synaptic vesicle and astrocyte preparations was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.5. Statistical analysis

All experiments were performed at least in triplicate. Statistical significance of three or more groups was assessed by one-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test. Regression analysis was used to detect dose-dependent effects. The Student's *t*-test for paired samples was also used to compare two groups. A value of $P < 0.05$ was considered to be significant. Data were expressed as mean ± S.E.M.

3. Results

Synaptosomes incubated for 15 min with 1 mM 3-OHGA showed no significant leakage of the cytosolic marker LDH as compared to controls [$t(8) = 0.18$, $P > 0.05$). Percentages of the total (100%) LDH content achieved by synaptosomal disruption with Triton X-100 were: 26.4 ± 3.8 (control); 25.3 ± 3.3 (1 mM 3-OHGA, $n = 6$ experiments).

3-OHGA (10–1000 μM) did not alter the basal [^{3}H]glutamate release [$F(3, 28) = 0.5634$, $P > 0.05$] nor the K^+-stimulated [^{3}H]glutamate release [$F(3, 28) = 1.1103$, $P > 0.05$] from synaptosomal preparations (Fig. 1). Furthermore, at the same concentration range 3-OHGA did not modify [^{3}H]glutamate uptake by synaptosomal preparations [$F(5, 47) = 0.5163$, $P > 0.05$] (Table 1).

Next, we investigated the effect of 3-OHGA on [^{3}H]glutamate uptake by synaptic vesicles. In a previous study, we observed that the kinetic parameters of glutamate uptake by rat brain synaptic vesicles revealed a K_m of 0.80 ± 0.15 mM and a V_{max} of 4.60 ± 0.51 nmol/(mg min protein). 3-OHGA did not affect the vesicular glutamate uptake (10–1000 μM) [$F(3, 24) = 0.689$, $P > 0.05$] (Fig. 2).

We also observed that 3-OHGA (50 and 1000 μM) exposure for 20 h to cultured astrocytes did not affect cell viability, as determined by LDH release and the MTT test. Values of LDH release (expressed as percent of control) were: 100 ± 14 (controls) and 104 ± 19 (1 mM 3-OHGA) ($n = 5$) ($t(4) = 0.256$, $P > 0.05$). Values of formazan formation (MTT test; expressed as the difference between the absorbances at 570 and 630 nm) were 0.15 ± 0.01 (control), 0.15 ± 0.01 (50 μM 3-OHGA), 0.12 ± 0.01 (1000 μM 3-OHGA) ($n = 5$) [$F(2, 11) = 1.323$, $P > 0.05$].

Then we investigated whether 3-OHGA influenced astrocyte Na^+-dependent [^{3}H]glutamate uptake. Firstly, we found that 3-OHGA (50–1000 μM) significantly increased (35%)

Table 1
Effect of 3-hydroxyglutaric acid (3-OHGA) exposure on [^{3}H]glutamate uptake into rat cerebral cortex synaptosomal preparations

Treatment	[^{3}H]Glutamate uptake (pmol/(min mg protein))
Control	3.28 ± 0.63
10 μM 3-OHGA	4.03 ± 0.47
50 μM 3-OHGA	4.01 ± 0.47
100 μM 3-OHGA	3.78 ± 0.54
500 μM 3-OHGA	3.88 ± 0.37
1000 μM 3-OHGA	3.37 ± 0.37

Results are presented as means ± S.E.M. of eight experiments (animals) performed each in triplicate and are expressed as pmol/(min mg protein). No significance between groups was detected (one-way ANOVA).

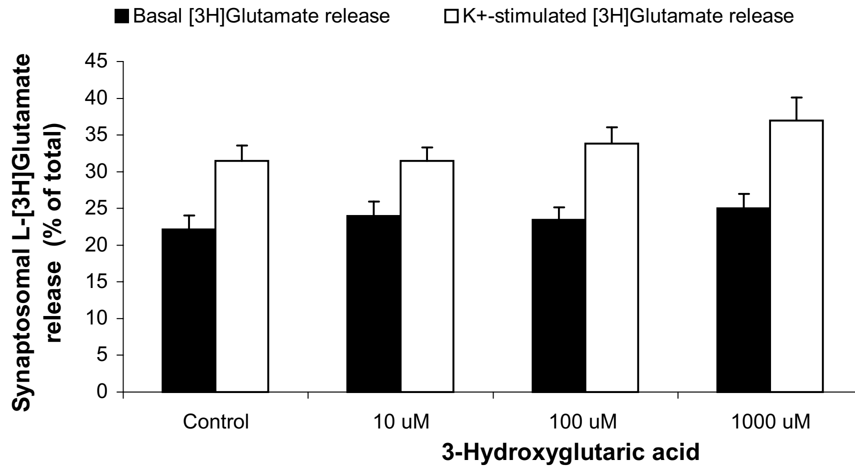

Fig. 1. Effect of 3-hydroxyglutaric acid (3-OHGA) exposure (10–1000 μM) on basal and K^+-stimulated [^{3}H]glutamate release from rat cerebral cortex synaptosomes. Results are expressed as percentage of total release in 1 min. Values are means ± S.E.M. of synaptosomal preparations from eight animals, each experiment performed in triplicate. No significance among groups was detected (one-way ANOVA).

[^{3}H]glutamate uptake by cultured astrocytes [$F(2, 15) = 9.654$, $P < 0.01$] (Fig. 3A). Then, we performed a new set of experiments using lower doses of 3-OHGA (3–100 μM) and observed that the acid significantly stimulated glutamate uptake into astrocytes in a dose-dependent manner ($F(1, 13) = 11.19$; $P < 0.05$; $\beta = 0.6803$; $P < 0.01$) at concentrations as low as 30 μM (Fig. 3B). The non-specific uptake (medium in the absence of sodium) was not influenced by 3-OHGA (results not shown), indicating that the effect of 3-OHGA in a medium supplemented by sodium was a true effect of this organic acid on astrocyte transporters which are sodium-dependent.

Finally, in order to determine whether the presence of 3-OHGA during the uptake assay was necessary for its stimulatory action, we preincubated astrocytes with 100–200 μM of the acid or 100 μM unlabeled glutamate for 53 min, after which the cells were washed and the uptake assays were performed as previously described. Glutamate was used because previous studies have shown that this neurotransmitter up-regulates its own transport by astrocytes (Duan et al., 1999). Fig. 4 shows that preincubation of astrocytes with 3-OHGA significantly increased glutamate uptake into these cells by up to 80%, whereas glutamate preincubation also increased (20%) its own uptake [$F(4, 14) = 20.245$, $P < 0.001$]. It can be also seen in the figure that the effects of 3-OHGA and glutamate were addictive (100% stimulation).

4. Discussion

GA-I is a *cerebral* organic acidemia characterized by progressive neurodegeneration, in particular following acute

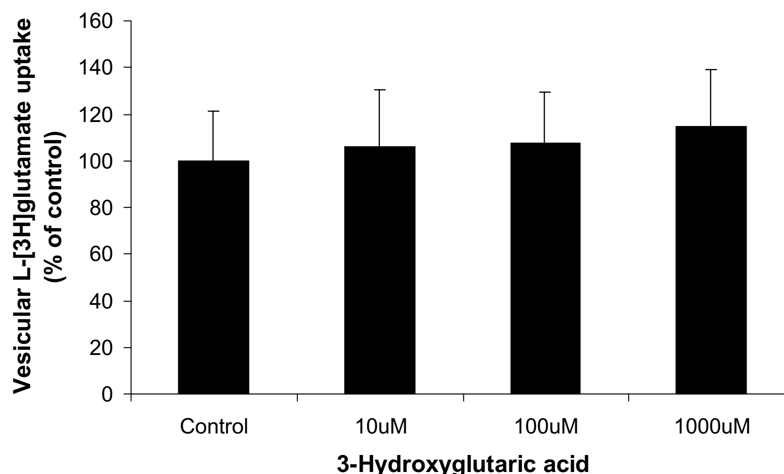

Fig. 2. Effect of 3-hydroxyglutaric acid (3-OHGA) exposure (10–1000 μM) on [^{3}H]glutamate uptake into rat brain synaptic vesicles. Results are presented as means ± S.E.M. of seven experiments from different synaptic vesicle preparations (animals) performed in triplicate and are expressed as percentage of control (control: 0.82 pmol/(min mg protein)). No significance among groups was detected (one-way ANOVA).

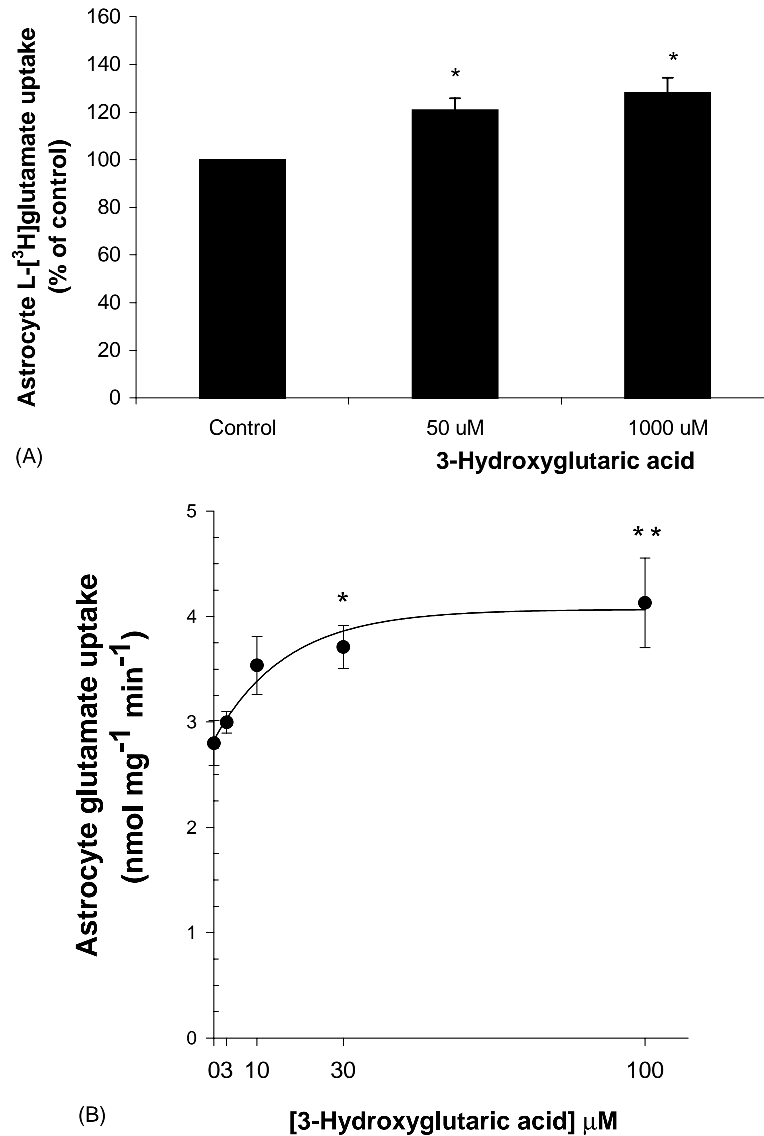

Fig. 3. Effect of 3-hydroxyglutaric acid (3-OHGA) exposure on [^{3}H]glutamate uptake into rat cerebral cortex astrocytes. Results are presented as means ± S.E.M. of 4–6 experiments (animals) performed each in triplicate and are expressed as percentage of control (A) or as nmol/(min mg protein) (B) $^*P < 0.05$, $^{**}P < 0.01$ vs. control, one-way analysis of variance (ANOVA) with post hoc Duncan's multiple range test.

encephalopathic crises (Goodman et al., 1977; Hoffmann and Zschocke, 1999). Previous in vitro studies have considered 3-OHGA as the main neurotoxin in this disease, inducing excitotoxic neuronal damage and death mediated by NMDA receptors, as well as generation of reactive species in the brain (Kölker et al., 1999; Kölker et al., 2000a,b, 2001a, 2002a,b; Latini et al., 2002). It has been also reported that in vivo intrastriatal administration of 3-OHGA to rats provoked convulsions and brain damage, which were prevented by pre-administration of the NMDA glutamate antagonist MK-801 (Mello et al., 2001). However, a recent report has shown that exposure of mixed cultured neural cells from rat cerebral cortex to 3-OHGA at concentrations as high as 8 mM for up to 96 h did not induce cell death, whereas exposure of these cultures to 1.0 mM glutamate caused a 20–45% death (Freudenberg et al., 2002). These investigators concluded that other alternative underlying mechanisms than excitotoxicity should be investigated to explain neurodegeneration in GA-I. Furthermore, inhibition of ATP synthase activity and intracellular depletion of creatine phosphate by 3-OHGA was reported (Ullrich et al., 1999; Das and Ullrich, 2003). Das and Ullrich (2003) showed that cell viability, as measured by increased LDH release from neurons, and creatine phosphate were reduced by 4.0 mM 3-OHGA, and that MK-801 prevented these effects. These investigators suggested that slow onset (secondary) excitotoxicity may possibly play a role in 3-OHGA action.

Therefore, because of these previous findings concerning the excitotoxic effect of 3-OHGA and since these studies did not evaluate a modulation of the glutamatergic

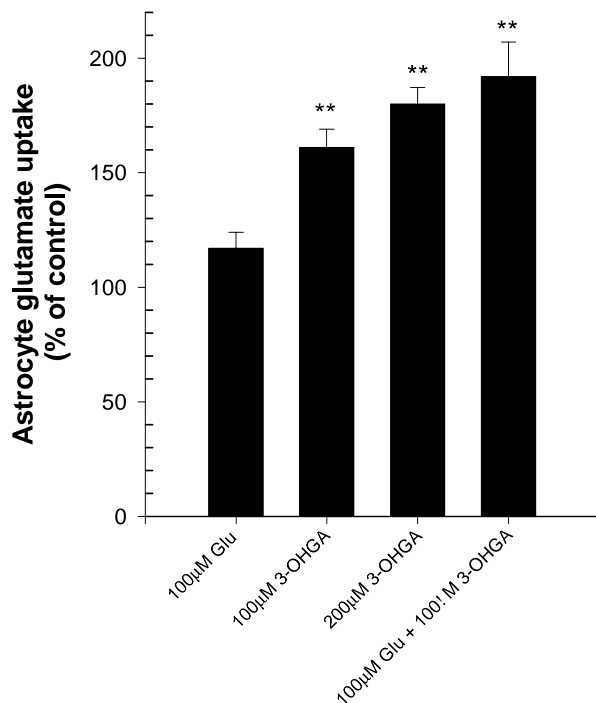

Fig. 4. Effect of preincubation of 3-hydroxyglutaric acid (3-OHGA) on [^{3}H]glutamate uptake into rat cerebral cortex astrocytes. Results are presented as means ± S.E.M. of three experiments (animals) performed each in triplicate and are expressed as percentage of control (2.80 ± 0.37 nmol/(min mg protein)). 3-OHGA or glutamate were preincubated with astrocytes for 53 min and withdrawn from the incubation medium before the uptake assays. $^{**}P < 0.01$ vs. control, one-way analysis of variance (ANOVA) with post hoc Duncan's multiple range test.

neurotransmission by 3-OHGA, the present investigation was conducted to test whether this metabolite could interfere with glutamate release and uptake. We have previously demonstrated that glutaric acid, which also accumulates in GA-I, inhibits both Na^+-dependent [^{3}H]glutamate binding to synaptic plasma membranes and [^{3}H]glutamate uptake into synaptosomal preparations (Porciúncula et al., 2000).

Here, we demonstrated that 3-OHGA (10–1000 µM) neither affected basal and K^+-stimulated [^{3}H]glutamate release from synaptosomal preparations, nor [^{3}H]glutamate uptake by synaptosomal preparations and synaptic vesicles. Thus, activation of NMDA receptors by 3-OHGA cannot be explained by stimulation of presynaptic glutamate release.

Furthermore, we showed that 3-OHGA significantly increased glutamate uptake by astrocytes in a dose-dependent manner and at very low concentrations (from 30 µM). When sodium was not present in the incubation medium (non-specific uptake), 3-OHGA did not stimulate glutamate uptake, indicating that its effect was true and directed towards astrocyte membrane transporters, which are dependent on sodium for its activity (Duan et al., 1999; Kondo et al., 1995). Since the acid did not affect glutamate uptake by synaptosomes or synaptic vesicles, it is possible to suppose that its action was selectively directed to astrocyte membrane transporters. We also observed that the presence of 3-OHGA during the uptake assay was not necessary to increase glutamate incorporation into astrocytes. Indeed, when 3-OHGA was not present during the uptake assays, its stimulatory effect was more pronounced, a fact that may suggest two distinct actions, one stimulating glutamate uptake as demonstrated, and the other somehow competing with glutamate for its uptake into astrocytes. Studies to test the action of 3-OHGA on glutamate binding in the presence and absence of sodium should be performed to clarify these mechanisms and are presently being undertaken in our laboratory.

We cannot establish at the present which underlying mechanisms are involved in the effect elicited by 3-OHGA facilitating glutamate uptake by astrocytes. In this context, it has been shown that glutamate uptake is regulated by the expression of transporter proteins, which is influenced by a variety of factors (Gegelashvili et al., 1996; Swanson et al., 1997; Gegelashvili and Schousboe, 1998; Schlag et al., 1998), and by the activity of the expressed transporters (Casado et al., 1993; Zerangue et al., 1995; Trotti et al., 1997). Furthermore, it has recently been demonstrated that glutamate triggers its own uptake into astrocytes and neurons (Gegelashvili et al., 1996; Duan et al., 1999; Anderson and Swanson, 2000; Munir et al., 2000) and stimulates astrocytic glutamate capacity by increasing GLAST expression in murine astrocytes (Duan et al., 1999). Therefore, whether an induction of GLAST expression or alternative mechanisms—such as glutamate receptor stimulation (Anderson and Swanson, 2000; Munir et al., 2000), the redox state of essential sulfhydryl groups (Volterra et al., 1994), protein phosphorylation (Danbolt, 2001), alteration of protein kinase C activity (Conradt and Stoffel, 1997), arachidonic acid (Volterra et al., 1992; Khan et al., 1995; Breukel et al., 1997) and guanine derivatives (Frizzo et al., 2001, 2002, 2003) are involved in 3-OHGA effect, remains to be elucidated.

On the other hand, since glutamate triggers its own uptake into astrocytes via a feedback mechanism, thereby modulating the astrocytic glutamate transport capacity (Duan et al., 1999) and considering the structural similarity between glutamate and 3-OHGA, it could be presumed that 3-OHGA could similarly increase astrocytic glutamate uptake. Our experiments revealed that, when pre-exposed to astrocytes, 3-OHGA more than glutamate, increased glutamate uptake. It may therefore be presumed that 3-OHGA-induced increase of astrocyte glutamate uptake may become an important mechanism to withdraw glutamate from the synaptic cleft and protect neurons from excitotoxicity induced by the metabolite. In this context, our present results may explain why viability of cultured neurons is decreased when these cells are exposed to 3-OHGA (Kölker et al., 1999; Kölker et al., 2000a,b, 2001; Kölker et al., 2002a,b), whereas mixed neural cultures containing neurons and glial cells (Freudenberg et al., 2002) or cultured astrocytes alone (as demonstrated in the present study) are not susceptible to 3-OHGA cytotoxic action. Therefore, it may be hypothesized that astrocytes protect neuronal cells against

excitotoxic damage provoked by 3-OHGA by enhancing the uptake of glutamate. In other words, the increased astrocytic glutamate uptake could reflect a response to the toxicity of 3-OHGA, as previously shown for glutamate (Gegelashvili et al., 1996; Duan et al., 1999; Anderson and Swanson, 2000; Munir et al., 2000).

In conclusion, we demonstrated that 3-OHGA at the concentrations usually found in GA-I alters glutamatergic neurotransmission by stimulation of astrocytic glutamate uptake, whereas glutamate release from synaptic vesicles was unaffected. These results virtually exclude that 3-OHGA-induced neurodegeneration involves enhanced presynaptic glutamate release or glutamate uptake inhibition. Rather, they indicate that 3-OHGA induced a neuroprotective astrocytic response to protect neurons from excitotoxicity by increasing glutamate uptake. Finally, although more research is necessary to clarify whether excitotoxicity, oxidative stress or energy deficit is the main pathomechanism involved in the neurodegeneration of GA-I patients, it is feasible that all these factors may act together to produce neurological damage in GA-I.

Acknowledgements

This study was supported by grants from the Brazilian National Research Council (CNPq), Financing Agency for Research of Rio Grande do Sul (FAPERGS) and The Excellence Nuclei Program (PRONEX). We are also grateful to Stefan Kölker and George F. Hoffmann for valuable discussion and evaluation of the manuscript.

References

Al Essa, M., Bakheet, S., Patay, Z., Al-Watban, J., Powe, J., Joshi, S., Ozand, P.T., 1998. Fluoro-2-deoxyglucose (18FDG) PET scan of the brain in glutaric aciduria type 1: clinical and MRI correlations. Brain Dev. 20, 295–301.

Anderson, C.M., Swanson, R.A., 2000. Astrocyte glutamate transport: review of properties, regulation and physiological functions. Glia 32, 1–14.

Breukel, A.I.M., Besselsen, E., Dasilva, F.H.L., Ghijsen, W.E.J.M., 1997. Arachidonic acid inhibits uptake of amino acids and potentiates PKC effects on glutamate but not GABA, exocytosis in isolated hippocampal nerve terminals. Brain Res. 773, 90–97.

Casado, M., Bendahan, A., Zafra, F., Danbolt, N.C., Aragon, C., Jiménez, C., Kanner, B.I., 1993. Phosphorylation and modulation of brain glutamate transporters by protein kinase C. J. Biol. Chem. 268, 27313–27317.

Conradt, M., Stoffel, W., 1997. Inhibition of the high-affinity brain glutamate transporter GLAST-1 via direct phosphorylation. J. Neurochem. 68, 1244–1251.

Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. 65, 1–65.

Das, A.M., Ullrich, K., 2003. Glutaric aciduria I: creatine supplementation restores creatinephosphate levels in mixed cortex cells from rat incubated with 3-hydroxyglutarate. Mol. Gen. Metabol. 78, 108–111.

Duan, S., Anderson, C.M., Stein, B.A., Swanson, R.A., 1999. Glutamate induces rapid upregulation of astrocyte glutamate transport and cell-surface expression of GLAST. J. Neurosci. 19, 10193–10200.

Dunkley, P.R., Heath, J., Harrison, S.M., Jarvie, P.E., Glenfield, P.J., Rostas, J.A.P., 1986. A rapid Percoll gradient procedure for isolation of synaptosomes directly from na S_1 fraction: homogeneity and morphology of subcellular fractions. Brain Res. 441, 59–71.

Flott-Rahmel, B., Falter, C., Schluff, P., Fingerhut, R., Christensen, E., Jakobs, C., Musshoff, U., Fautek, J.D., Ludolph, A., Ullrich, K., 1997. Nerve cell lesions caused by 3-hydroxyglutaric acid: a possible mechanism for neurodegeneration in glutaric academia I. J. Inherit. Metab. Dis. 20, 387–390.

Freudenberg, F., Lukacs, Z., Ullrich, K., Braulke, T., 2002. Primary cell culture of mixed rat cortex cells as an *in vitro* model for glutaric aciduria type I. J. Inherit. Metab. Dis. 25 (1), 150.

Frizzo, M.E.S., Lara, D.R., Dahm, K.S.C., Prokopiuk, A.S., Swanson, R.A., Souza, D.O., 2001. Activation of glutamate uptake by guanosine in primary astrocyte cultures. NeuroReport 426, 879–881.

Frizzo, M.E.S., Lara, D.R., Prokopiuk, A.S., Vargas, C.R., Salbego, C.G., Wajner, M., Souza, D.O., 2002. Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. Cell. Mol. Neurobiol. 22 (3), 353–363.

Frizzo, M.E.S., Soares, F.A., Dall'Onder, L.P., Lara, D.R., Swanson, R.A., Souza, D.O., 2003. Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake. Brain Res. 972 (1–2), 84–89.

Fykse, E.M., Fonnum, F.J., 1996. Amino acid neurotransmission: dynamics of vesicular uptake. Neurochem. Res. 21, 1053–1060.

Gegelashvili, G., Schousboe, A., 1998. Cellular distribution and kinetic properties of high-affinity glutamate transporters. Brain Res. Bull. 45, 233–238.

Gegelashvili, G., Civenni, G., Racagni, G., Danbolt, N.C., Schousboe, I., Schousboe, A., 1996. Glutamate receptor agonists up-regulate glutamate transporter GLAST in astrocytes. NeuroReport 8, 261–265.

Goodman, S.I., Norenberg, M.D., Shikes, R.H., Breslich, D.J., Moe, P.G., 1977. Glutaric aciduria type 1: biochemical and morphological considerations. J. Pediatr. 90, 746–750.

Goodman, S.I., Frerman, F.E., 2001. Organic acidemias due to defects in lysine oxidation: 2-ketoadipic academia and glutaric acidemia. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Walle, D. (Eds.), The Metabolic and Molecular Bases of Inherited Disease, eighth ed. McGraw-Hill, New York, 2001, pp. 2195–2204.

Gregersen, N., Brandt, N.J., 1979. Ketotic episodes in glutaryl-CoA dehydrogenase deficiency (glutaric aciduria). Pediatr. Res. 13, 977–981.

Hoffmann, G.F., Zschocke, J., 1999. Glutaric aciduria type I: from clinical biochemical and molecular diversity to successful therapy. J. Inherit. Metab. Dis. 22, 381–391.

Hoffmann, G.F., Gibson, K.M., Trekz, F.K., Nyhan, W.L., Bremer, H.J., Rating, D., 1994. Neurological manifestations of organic acid disorders. Eur. J. Pediatr. 153, S94–S100.

Hoffmann, G.F., Athanassopoulos, S., Burlina, A.B., Duran, M., de Klerk, J.B., Lehnert, W., Leonard, J.V., Monavari, A.A., Müller, E., Muntau, A.C., Naughten, E.R., Plecko-Starting, B., Superti-Furga, A., Zschocke, J., Christensen, E., 1996. Clinical course, early diagnosis, treatment, and prevention of disease in glutaryl-CoA dehydrogenase deficiency. Neuropediatrics 27, 115–123.

Khan, W.A., Blobe, G.C., Hannun, Y.A., 1995. Arachidonic acid and free fatty acids as second messengers and the role of protein kinase C. Cell. Signal. 7, 171–184.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 1999. 3-Hydroxyglutaric and glutaric acids are neurotoxic through NMDA receptors *in vitro*. J. Inherit. Metab. Dis. 22, 259–262.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2000a. Cerebral organic acid disorders induce neuronal damage via excitotoxic organic acids *in vitro*. Amino Acids 18, 31–40.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2000b. Maturation-dependent neurotoxicity of 3-hydroxyglutaric and glutaric acids *in vitro*: a new pathophysiologic approach to glutaryl-CoA dehydrogenase deficiency. Pediatr. Res. 47, 495–503.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2000c. Evaluation of trigger factors of acute encephalopathy in glutaric aciduria type I: fever and tumour necrosis factor-alpha. J. Inherit. Metab. Dis. 23 (4), 359–362.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2001a. Contribution of reactive oxygen species to 3-hydroxyglutarate neurotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr. Res. 50, 76–82.

Kölker, S., Ahlemeyer, B., Hühne, R., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., 2001b. Potentiation of 3-hydroxyglutarate neurotoxicity following induction of astrocytic iNOS in neonatal rat hippocampal cultures. Eur. J. Neurosci. 13, 2115–2122.

Kölker, S., Köhr, G., Ahlemeyer, B., Okun, J.G., Pawlak, V., Horster, F., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., 2002a. Ca^{2+} and Na^{+} dependence of 3-hydroxyglutarate-induced excitotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr. Res. 52 (2), 199–206.

Kölker, S., Okun, J.G., Ahlemeyer, B., Wyse, A.T., Horster, F., Wajner, M., Kohlmuller, D., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., 2002b. Chronic treatment with glutaric acid induces partial tolerance to excitotoxicity in neuronal cultures from chick embryo telencephalons. J. Neurosci. Res. 685, 424–431.

Kondo, K., Hashimoto, H., Kitanara, J., Sawada, M., Sukumura, A., Marunovchi, T., Baba, A., 1995. Expression of glutamate transporters in cultured glial cells. Neurosci. Lett. 188, 140–142.

Latini, A., Rosa, R.B., Scussiato, K., Llesuy, S., Belló-Klein, A., Wajner, M., 2002. 3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats. Brain Res. 956, 367–373.

Lima, T.T.F., Begnini, J., Bastiani, J., Fíalo, D.B., Jurach, A., Ribeiro, M.C., Wajner, M., de Mello, C.F., 1998. Pharmacological evidence for GABAergic and glutaminergic involvement in the convulsant and behavioral effects of glutaric acid. Brain Res. 802, 55–60.

Lowry, O.H., Roseborough, N.R., Farr, A.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Meldrum, B., Garthwaite, J., 1990. Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends Pharmacol. Sci. 11, 379–387.

Mello de, C.F., Kölker, S., Ahlemeyer, B., de Souza, F.R., Fighera, M.R., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., Wajner, M., 2001. Intrastriatal administration of 3-hydroxyglutaric acid induces convulsions and striatal lesions in rats. Brain Res. 916, 70–75.

Migues, P.V., Leal, R.B., Mantovani, M., Nicolau, M., Gabilan, N.H., 1999. Synaptosomal glutamate release induced by the fraction BC2 from the venom of the sea anemone Bunadosoma caissarum. NeuroReport 10, 67–70.

Munir, M., Correale, D.M., Robinson, M.B., 2000. Substrate-induced up-regulation of Na^{+}-dependent glutamate transport activity. Neurochem. Int. 37, 147–162.

Nagi, A.K., Shuster, T.A., Delgado-Escueta, A.V., 1986. Ecto-ATPase of mammalian synaptosomes: identification and enzymic characterization. J. Neurochem. 47, 976–986.

Olney, J.W., 1980. Excitotoxic mechanisms of neurotoxicity. In: Spencer, P.S., Schaumburg, H.H. (Eds.), Clinical and Experimental Neurotoxicology. Williams & Wilkins, Baltimore, MD, pp. 272–294.

Ozawa, S., Kamyiar, K., Tsuzuki, K., 1998. Glutamate receptors in the mammalian cerebral nervous system. Prog. Neurobiol. 54, 581–618.

Porciúncula, L.O., Dal-Pizzol Jr, A., Tavares, R.G., Coitinho, A.S., Emanuelli, T., Souza, D.O., Wajner, M., 2000. Inhibition of synaptosomal $[^3H]$glutamate and $[^3H]$glutamate binding to plasma membranes from brain of young rats by glutaric acid in vitro. J. Neurol. Sci. 173, 93–96.

Robinson, M.B., Dowd, L.A., 1997. Heterogeneity and functional properties of subtypes of sodium-dependent glutamate transporters in the mammalian cerebral nervous system. Adv. Pharmacol. 37, 69–115.

Sanetto, R.P., De Vellis, 1985. Characterization of cultured rat oligodendrocytes proliferating in a serum-free, chemically defined medium. Proc. Natl. Acad. Sci. U.S.A. 82, 3509–3913.

Schlag, B.D., Vondrasek, J.R., Munir, M., Kalandadze, A., Zelenaia, O.A., Rothstein, J.D., Robinson, M.B., 1998. Regulation of the glial Na^{+}-dependent glutamate transporters by cyclic AMP analogs and neurons. Mol. Pharmacol. 53, 355–369.

Silva, C.G., da Silva, A.R., Ruschel, C., Helegda, C., Wyse, A.T.S., Wannmacher, C.M.D., Dutra Filho, C.S., Wajner, M., 2000. Inhibition of energy production in vitro by glutaric acid in cerebral cortex of young rats. Metab. Brain Dis. 15, 123–131.

Swanson, R.A., Liu, J., Miller, J.W., Rothstein, J.D., Farrell, K., Stein, B.A., Longuemare, M.C., 1997. Neuronal regulation of glutamate transporter subtype expression in astrocytes. J. Neurosci. 17, 932–940.

Trotti, D., Rizzini, B.L., Rossi, D., Haugeto, O., Racagni, G., Danbolt, N.C., Volterra, A., 1997. Neuronal and glial glutamate transporters possess an SH-based redox regulatory mechanism. Eur. J. Neurosci. 9, 1236–1243.

Ullrich, K., Flott-Rahmel, B., Schluff, P., Musshoff, U., Das, A., Lücke, T., Steinfeld, R., Christensen, E., Jacobs, C., Ludolph, A., Neu, A., Röper, R., 1999. Glutaric aciduria type I: pathomechanisms of neurodegeneration. J. Inherit. Metab. Dis. 22, 392–403.

Volterra, A., Trotti, D., Cassutti, P., Tromba, C., Salvaggio, A., Melcangi, R.C., Racagni, G., 1992. High sensitivity of glutamate uptake to extracellular free arachidonic acid levels in rat cortical synaptossomes and astrocytes. J. Neurochem. 59, 600–606.

Volterra, A., Trotti, D., Tromba, C., Floridi, S., Racagni, G., 1994. Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes. J. Neurosci. 14, 2924–2932.

Wolosker, H., Souza, D.O., De Méis, L.J., 1996. Regulation of glutamate transport into synaptic vesicles by chloride and proton gradient. J. Biol. Chem. 271, 11726–11731.

Zelenaia, O., Schlag, B.D., Gochenauer, G.E., Ganel, R., Song, W., Beesley, J.S., Grinspan, J.B., Rothstein, J.D., Robinson, M.B., 2000. Epidermal growth factor receptor agonists increase expression of glutamate transporter GLT-1 in astrocytes through pathways dependent on phosphatidylinositol 3-kinase and transcription factor NF-kappaB. Mol. Pharmacol. 57, 667–678.

Zerangue, N., Arrizza, J.L., Amara, S.G., Kavanaugh, M.P., 1995. Differential modulation of human glutamate transporter subtypes by aracdonic acid. J. Bol. Chem. 270, 6433–6435.

Neuroscience 135 (2005) 111–120

3-HYDROXYGLUTARIC ACID MODERATELY IMPAIRS ENERGY METABOLISM IN BRAIN OF YOUNG RATS

A. LATINI,[a,b] M. RODRIGUEZ,[c] R. BORBA ROSA,[a] K. SCUSSIATO,[a] G. LEIPNITZ,[a] D. REIS DE ASSIS,[a] G. DA COSTA FERREIRA,[a] C. FUNCHAL,[a] M. C. JACQUES-SILVA,[a] L. BUZIN,[a] R. GIUGLIANI,[b] A. CASSINA,[c] R. RADI[c] AND M. WAJNER[a,b,d]*

[a]*Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul, Rua Ramiro Barcelos N° 2600, Anexo, CEP 90035-003, Porto Alegre, RS, Brazil*

[b]*Hospital de Clínicas, Serviço de Genética Médica, Porto Alegre, RS, Brazil*

[c]*Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay*

[d]*Universidade Luterana do Brasil, Canoas, Brazil*

Abstract—3-Hydroxyglutaric acid (3HGA) accumulates in the inherited neurometabolic disorder known as glutaryl-CoA dehydrogenase deficiency. The disease is clinically characterized by severe neurological symptoms, frontotemporal atrophy and striatum degeneration. Because of the pathophysiology of the brain damage in glutaryl-CoA dehydrogenase deficiency is not completed clear, we investigated the *in vitro* effect of 3HGA (0.01–5.0 mM) on critical enzyme activities of energy metabolism, including the respiratory chain complexes I–V, creatine kinase isoforms and Na^+,K^+-ATPase in cerebral cortex and striatum from 30-day-old rats. Complex II activity was also studied in rat C6-glioma cells exposed to 3HGA. The effect of 3HGA was further investigated on the rate of oxygen consumption in mitochondria from rat cerebrum. We observed that 1.0 mM 3HGA significantly inhibited complex II in cerebral cortex and C6 cells but not the other activities of the respiratory chain complexes. Creatine kinase isoforms and Na^+,K^+-ATPase were also not affected by the acid. Furthermore, no inhibition of complex II activity occurred when mitochondrial preparations from cerebral cortex or striatum homogenates were used. In addition, 3HGA significantly lowered the respiratory control ratio in the presence of glutamate/malate and succinate under stressful conditions or when mitochondria were permeabilized with digitonin. Since 3HGA stimulated oxygen consumption in state IV and compromised ATP formation, it can be presumed that this organic acid might act as an endogenous uncoupler of mitochondria respiration. Finally, we observed that 3HGA changed C6 cell morphology from a round flat to a spindle-differentiated shape, but did not alter cell viability neither induced apoptosis. The data provide evidence that 3HGA provokes a moderate impairment of brain energy metabolism and do not support the view that 3HGA-induced energy failure would solely explain the characteristic brain degeneration observed in glutaryl-CoA dehydrogenase deficiency patients. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: 3-hydroxyglutaric acid, mitochondrial respiratory chain, creatine kinase, Na^+;K^+-ATPase, mitochondrial oxygen consumption.

Glutaryl-CoA dehydrogenase deficiency (GDD) is an autosomal recessive neurometabolic disorder due to a blockage in the L-lysine, hydroxy-L-lysine and tryptophan catabolic pathway, leading to tissue accumulation of glutaric (GA), 3-hydroxyglutaric (3HGA) and occasionally glutaconic acids (Goodman and Frerman, 2001). GDD clinical presentation is predominantly neurological. During the first months of life the signs of the disease are non-specific and include hypotonia with prominent head lag, irritability and macrocephaly. If untreated, the disease course is usually complicated by the occurrence of encephalopathic/encephalitis-like crises which cause irreversible destruction of vulnerable brain regions, predominantly the striatum (Brismar and Ozand, 1995). Thereafter, the clinical picture is characterized by a severe dystonic-dyskinetic disorder, sometimes associated to extreme hypotonia, rigidity and spasticity (Hoffmann and Zschocke, 1999; Strauss et al., 2003; Kölker et al., 2004; Hoffman et al., 1996). Neuroimaging of GDD patients presents signs of frontotemporal atrophy, striatum degeneration, delayed cerebral maturation (cortical atrophy), spongy formation and attenuation of the white matter signal (leukoencephalopathy) (Amir et al., 1987; Chow et al., 1988; Brismar and Ozand, 1995; Hoffmann and Zschocke, 1999).

Despite the marked neurological alterations, the mechanisms underlying the brain damage in GDD patients are poorly defined. In this context, a neurotoxic role for quinolinic acid, a metabolite of the tryptophan kynurenine pathway and inhibition of the neuronal glutamate decarboxylase activity were first postulated to provoke the neurological sequelae in GDD (Stokke et al., 1976; Heyes, 1987; Varadkar and Surtees, 2004). On the other hand, evidence from recent *in vitro* and *in vivo* animal model studies suggested that other mechanisms are involved in the brain abnormalities of GDD patients. These studies indicated that 3HGA is the leading neurotoxin, acting probably through three main mechanisms, excitotoxicity, oxidative stress and energy impairment (Flott-Rahmel et al., 1997;

*Correspondence to: M. Wajner, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul, Rua Ramiro Barcelos N° 2600, Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Tel: +55-51-3316-5571; fax: +55-51-3316-5535.
E-mail address: mwajner@ufrgs.br (M. Wajner).

Abbreviations: CK, creatine kinase; Cy-CK, cytosolic creatine kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GA, glutaric acid; GDD, glutaryl-CoA dehydrogenase deficiency; Mi-CK, mitochondrial creatine kinase; NO, nitric oxide; PI, propidium iodide; RCR, respiratory control ratio; 3HGA, 3-hydroxyglutaric acid; 3NPA, 3-nitropropionic acid.

0306-4522/05$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.05.013

Ullrich et al., 1999; Silva et al., 2000; Kölker et al., 1999, 2000, 2001a,b, 2002; de Mello et al., 2001; Bjugstad et al., 2001; Latini et al., 2002, 2005; de Oliveira Marques et al., 2003; Frizzo et al., 2004; Rosa et al., 2004). As regards to energy metabolism, studies performed in rat neuronal cultures have shown that 3HGA impairs cellular energy generation by inhibiting complexes II and V of the respiratory chain and decreasing creatine phosphate levels (Ullrich et al., 1999; Das et al., 2003). On the other hand, Kölker and collaborators (2002) found a mild inhibition of complex V only at high concentrations of 3HGA (10 mM) with no alteration of the other activities of the electron transfer chain and more recently the same investigators did not observe any interference of 3HGA on all isolated activities of the respiratory chain in submitochondrial particles from bovine heart (Sauer et al., 2005). Apart from these experimental data, some GDD patients excrete increased concentrations of lactate, 3-hydroxybutyrate, acetoacetate and dicarboxylic acids, reflecting a mitochondrial dysfunction (Gregersen and Brandt, 1979; Floret et al., 1979). Because of this, Strauss and Morton (2003) postulated that the nature of the striatal damage in GDD is similar to the brain injury that occurs in infants after hypoxia–ischemia or systemic intoxication with 3-nitropropionic acid (3NPA), in which complex II activity is irreversible blocked. These investigators proposed an experimental model of GDD based on 3NPA administration.

The objective of the present work was therefore to investigate the *in vitro* effect of 3HGA on several enzyme activities of brain energy metabolism, namely the respiratory chain complexes I–V, total, mitochondrial and cytosolic creatine kinase (CK) and Na^{+},K^{+}-ATPase in homogenates and/or in mitochondrial preparations from cerebral cortex and striatum of young rats, as well as in C6-glioma cell homogenates. The effect of 3HGA on the rate of oxygen consumption in rat cerebrum mitochondria was also evaluated. Finally, cell morphology and viability, as well as apoptosis were investigated in C6-glioma cells exposed to 3HGA.

EXPERIMENTAL PROCEDURES

Animals and reagents

Male Wistar rats of 30 days of life obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, were used. The animals were maintained on a 12-h light/dark cycle (lights on 07:00–19:00 h) in a constant temperature (22 °C±1 °C) colony room, with free access to water and a 20% (w/w) protein commercial chow (Germani, Porto Alegre, RS, Brazil). The “Principles of laboratory animal care” (NIH publication no 80-23, revised 1996) were followed in all experiments. All efforts were made to minimize the number of animals used and their suffering. All chemicals were purchased from Sigma (St. Louis, MO, USA) except Dulbecco’s modified Eagle’s medium (DMEM) purchased from Gibco BRL (Carlsbad, CA, USA) and fetal bovine serum (FBS) from Cultilab (Campinas, SP, Brazil). 3HGA was prepared on the day of the experiments in the incubation medium used for each technique. The final concentrations of 3HGA used in the various assays were from 0.01–5.0 mM. The activities of the respiratory chain complexes, CK and Na^{+},K^{+}-ATPase were measured with a U-2001 Hitachi spectrophotometer with temperature control. For brain tissue preparations Sorval DC-2B and OTD-65B Sorval centrifuges were used.

Maintenance of cell line

C6-glioma cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were grown and maintained in DMEM (pH 7.4), containing 2.5 mg/ml Fungizone® and 100 U/l gentamicin, and supplemented with 5% FBS. Cells were kept at a temperature of 37 °C, with 95% humidity in an atmosphere of 5% CO_2 in air. Immunocytochemistry for glial fibrillary acidic protein (GFAP) was positive in more than 90% in rat C6-glioma cells, reflecting the high percentage of astrocytic cell type (data not shown).

Tissue preparation

Animals were killed by decapitation without anesthesia and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulb, pons, medulla and cerebellum were discarded, and the cerebral cortex and striatum were dissected, weighed and kept chilled until homogenization which was performed using a ground glass type Potter-Elvejhem homogenizer.

Brain preparation for measurement of respiratory chain complex activities and respiratory parameters

Rat cerebral cortex and striatum were homogenized in 20 volumes of SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Tris, 50 UI/ml heparin). The homogenates were centrifuged at 800×*g* for 10 min at 4 °C, the pellet was discarded and the supernatants were kept at −70 °C until enzyme activity determination.

Mitochondria from cerebral cortex were also purified for measurement of complexes I, II and V activities and for oxygen consumption experiments. Briefly, the cerebral cortex was homogenized in 10 volumes of phosphate buffer pH 7.4 containing 0.3 M sucrose, 5 mM MOPS, 1 mM EGTA and 0.1% bovine serum albumin. The homogenates were centrifuged at 1500×*g* for 10 min at 4 °C and the pellet was discarded. The supernatant was centrifuged at 15,000×*g* in order to isolate mitochondria present in the pellet, which was finally dissolved in the same buffer. Mitochondria from rat cerebrum were also isolated according to Cassina and Radi (1996) to measure the mitochondrial respiratory parameters state IV, state III and the respiratory control ratio (RCR).

C6-glioma cells were also homogenized in SETH buffer to a final protein concentration of 1–2 mg/ml for the enzymatic determinations.

The maximum period between these preparations and enzyme analysis was always less than a week, except for oxygen consumption experiments which were performed within the same day.

Brain preparations for CK activities measurement

Total homogenates from rat cerebral cortex were prepared in 10 volumes of saline solution, pH 7.5. The mitochondrial and cytosolic fractions were obtained by first centrifuging the homogenates at 800×*g* for 10 min at 4 °C and discarding the pellet. The supernatant was then centrifuged at 27,000×*g* for 30 min at 4 °C. The pellet was washed three times with saline solution and used as the mitochondrial fraction for the mitochondrial creatine kinase (Mi-CK) enzymatic assay. The supernatant was further centrifuged at 125,000×*g* for 60 min at 4 °C, the microsomal pellet was discarded, and the cytosol (supernantant) was used for the cytosolic creatine kinase (Cy-CK) enzymatic assay.

Synaptic plasma membrane preparation for Na^+, K^+-ATPase and Mg^{2+}-ATPase measurement

Cerebral cortex was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4. Membranes were prepared according to the method of Jones and Matus (1974) using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000×*g* for 2 h, the fraction at the 0.8–1.0 mM sucrose interface was taken as the membrane enzyme preparation.

Incubation of the various cell preparations with 3HGA

Cerebral cortex and striatum homogenate preparations, mitochondrial fractions and homogenates from rat glioma cells were incubated in the appropriate buffer for each assay for the measurement of the various enzyme activities involved in energy metabolism in the presence of 3HGA (0.01–5.0 mM). In some experiments 1.0 mM malonic acid was used. After reaching confluence (1.0×10^6 cells/Petri dish), C6 glial cells were incubated at 37 °C in an atmosphere of 5% CO_2 in air with increasing concentrations of 3HGA (0.01–1.0 mM) in DMEM, pH 7.4 free of FBS. Controls did not contain 3HGA or malonic acid in the incubation medium.

Morphological studies

Morphological studies were performed using phase contrast microscopy. C6-glioma cells were exposed to 3HGA (0.01–1.0 mM) for 6–24 h and morphology was analyzed.

Viability assays

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) stain, as described previously (Funchal et al., 2004). Cells were treated during 6–24 h with 1.0 mM 3HGA at 37 °C in an atmosphere of 5% CO_2/95% air in DMEM free of FBS, after which they were supplemented with 7.5 μM PI and photographed after 1 h with a Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory. Optical density was determined with the Optiquant version 02.00 software (Packard Instrument Company, USA). Density values obtained were expressed as density light unit. Cell death was also assessed by the presence of bright cell aggregates on microscopy.

Nuclear morphology assay

C6 cells were cultured in circular glass coverslips and treated with 1.0 mM 3HGA for 6 h. The confluent cells were fixed for 20 min with 4% paraformaldehyde in saline phosphate buffer stained with Hoechst (0.1 μg) for 90 min and visualized in a fluorescent microscope (Nikon inverted microscope using a TE-FM Epi-Fluorescent accessory). Apoptotic cells were morphologically defined by nuclear shrinkage and chromatin condensation and/or fragmentation.

Measurement of the respiratory chain enzyme activities

The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate:cytochrome *c* oxidoreductase (complex II-CoQ-complex III) were determined in homogenates from cerebral cortex and striatum, in purified mitochondria from cerebral cortex and in C6 cells homogenates according to the method of Fischer et al. (1985). The activity of NADH:cytochrome *c* oxidoreductase (complex I-CoQ-complex III) was assayed in cerebral cortex homogenates according to the method described by Schapira et al. (1990) and that of cytochrome *c* oxidase (complex IV) according to Rustin et al. (1994). The methods described to measure these activities were slightly modified, as described in details in a previous report (da Silva et al., 2002). NADH dehydrogenase (complex I) and ATPase (complex V) were measured in purified mitochondrial from cerebral cortex. Complex I activity was measured by the rate of NADH-dependent ferricyanide reduction at 420 nm (ε=1 mM^{-1} cm^{-1}) according to Cassina and Radi (1996). Complex V activity was assayed by coupling the reaction of pyruvate kinase with lactate dehydrogenase and following NADH oxidation at 340 nM (ε=6.22 mM^{-1} cm^{-1}) (Rustin et al., 1994). The activities of the respiratory chain complexes were calculated as nmol/min/mg protein or mmol/min/mg protein.

Determination of CK activities

CK activity was measured in total homogenates, as well as in cytosolic and mitochondrial preparations from cerebral cortex homogenates, based on the creatine formation determined according to the colorimetric method of Hughes (1962), with slight modifications as described previously (da Silva et al., 2004).

Na^+, K^+-ATPase and Mg^{2+}-ATPase activity assay

The reaction mixture for the Na^+,K^+-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, in a final volume of 200 μl. The reaction was started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. Mg^{2+}-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays (Tsakiris and Deliconstantinos, 1984). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Enzyme-specific activities were expressed as nmol Pi released/min/mg protein.

Respiratory parameters

The rate of oxygen consumption was measured polarographically using a Clark-type electrode in a thermostatically controlled (37 °C) and magnetically stirred incubation chamber of 1.6 ml capacity (Cassina and Radi, 1996). The assay was performed with 1.0 mg/ml of mitochondrial protein incubated in the same buffer used for mitochondrial isolation in the presence of NAD-linked (glutamate/malate: 2.5 mM each) and FAD-linked (5.0 mM succinate) substrates. The rate of oxygen consumption in these conditions corresponded to state IV mitochondrial respiration. State III was initiated by adding 250 nmol ADP. The RCR (state III/state IV) was then calculated. Some experiments were performed by using digitonin at a final concentration of 1.8 mg/10 mg mitochondrial protein.

Protein determination

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

Statistical analysis

Results are presented as means±standard deviation. Assays were performed in duplicate and the mean was used for statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc Duncan multiple range test when *F* was significant. Only significant *F* values are given in the text. For analysis of dose-dependent effects, linear regression was used. The Student's *t*-test was also used for comparison of two means. Differences between the groups were rated significant at $P<0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

RESULTS

Inhibition of complex II activity by 3HGA

Fig. 1A shows that 1.0 mM 3HGA provoked a moderate but significant inhibition of complex II [$F_{(3,16)}=3.42$; $P<0.05$] activity in rat cortical homogenates. This inhibitory action was investigated in more detail by varying the concentration of succinate (1.0–16 mM). Fig. 1B shows that 3HGA significantly inhibited complex II activity only at low concentrations of succinate (1.0 mM) [$F_{(4,15)}=15.87$; $P<0.001$]. It can also be observed in the figure that the inhibition provoked by 3HGA on this activity was around 25%, while the inhibition caused by malonic acid, the classical competitive inhibitor of complex II, was always higher than 50% at all succinate concentrations. We also observed that complex II activity was inhibited by approximately 20% after 6-h-incubation of C6 cell homogenates with 3HGA [$F_{(3,8)}=4.12$; $P<0.05$] (Fig. 1C). On the other hand, when complex II was determined in mitochondrial preparations from cerebral cortex in the presence of 3HGA, no inhibition was detected, whereas 1.0 mM malonic acid inhibited this complex by approximately 95% (data not shown). Furthermore, complex II activity was not modified in 3HGA-treated striatum homogenates (data not shown).

Next, we observed that 3HGA did not alter t-CK, Mi-CK and Cy-CK activities (Table 1), as well as Mg^{2+}-ATPase and Na^{+},K^{+}-ATPase activities in cerebral cortex homogenates and synaptic plasma membranes, respectively (Table 2).

Effect of 3-HGA on oxygen consumption parameters

The respiratory state IV (NAD and FAD-linked substrates), state III (ADP stimulated respiration) and RCR parameters were then measured in rat cerebrum mitochondria. Fig. 2 shows that the RCR values calculated from glutamate/malate- and succinate-respiring mitochondria were significantly reduced by 1.0 mM 3HGA up to 35% and 50%, respectively, as compared with controls [$RCR_{(glutamate/malate)}$: $t_{(3)}=4.98$; $P<0.05$; $RCR_{(succinate)}$: $t_{(4)}=3.52$; $P<0.05$] when $RCR_{(glutamate/malate)}$ values from controls were lower than 3 (Fig. 2A). Similar results were obtained when mitochondria were treated with digitonin (1.8 mg/10 mg mitochondrial protein) [$RCR_{(glutamate/malate)}$: $t_{(3)}=3.30$; $P<0.05$; $RCR_{(succinate)}$: $t_{(4)}=6.79$; $P<0.01$] (Fig. 2C). In contrast, no alterations of the respiratory parameters were observed in respiring mitochondria with RCR values of 3 or higher (Fig. 2B). Fig. 3A also shows that state IV was significantly increased (up to 30%) [$t_{(4)}=3.79$; $P<0.05$] and state III respiration was significantly lowered (>30%) [$t_{(4)}=3.32$; $P<0.05$] with the FAD-linked substrate succinate. Similar but not significant findings occurred when glutamate/malate was used in the incubation medium in low RCR mitochondria exposed to 3HGA. Digitonin-treated mitochondria also showed significantly increased state IV respiration (up to 40%) with the NAD-linked substrate glutamate/malate [$t_{(4)}=3.32$; $P>0.05$] and moderately increased (up to 30%) when succinate was used as the respiratory substrate. Under these conditions, state III was

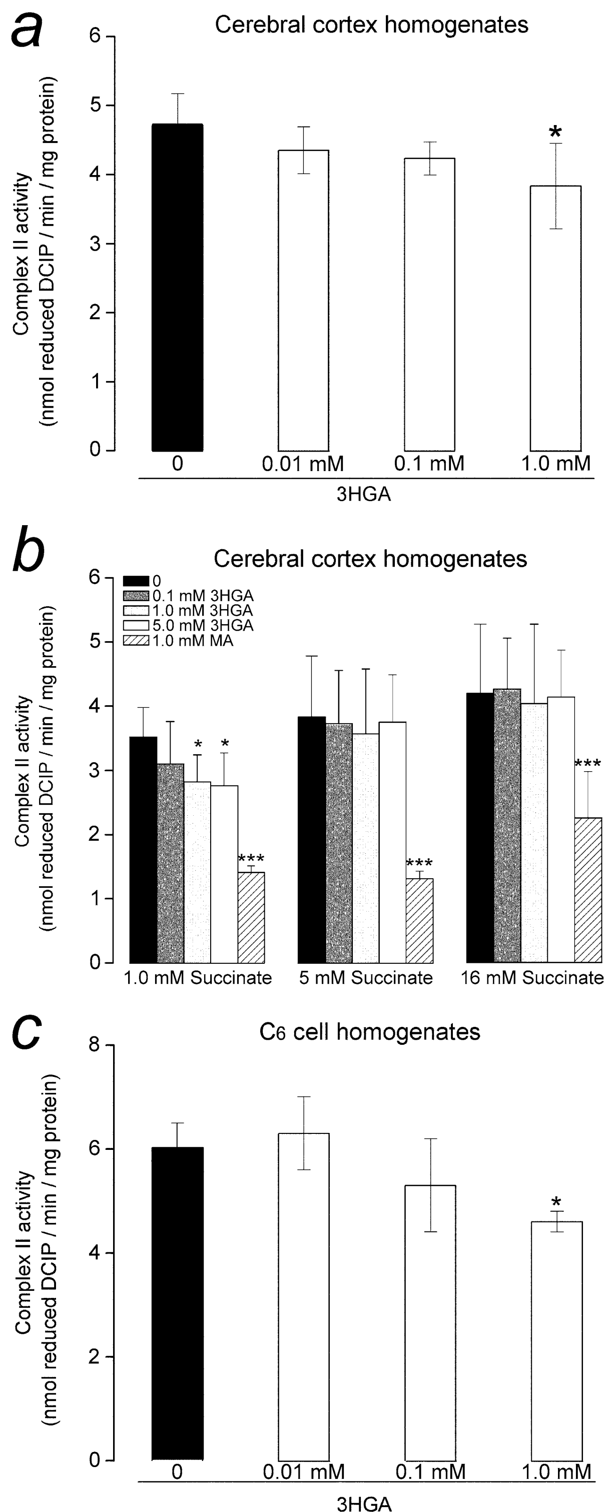

Fig. 1. Effect of 3HGA on the activity of complex II in cerebral cortex homogenates from 30-day-old rats using 1.0 mM succinate (a), a dose-response substrate curve with 1–16 mM succinate (b) and in C6 rat glioma cell homogenates with 1.0 mM succinate (c). The effect of 1.0 mM malonic acid (MA) on complex II in cortical homogenates was also studied (b). Values are mean±standard deviation for three to five independent experiments. * $P<0.05$, *** $P<0.001$, compared with controls (Duncan multiple range test).

Table 1. Creatine kinase activities in rat cerebral cortex supernatants exposed to 3HGA

Creatine kinase activities (μmol creatine/min/mg protein)	3HGA			
	0	0.01 mM	0.1 mM	1.0 mM
t-CK ($n = 6$)	3.1±0.3	2.9±0.3	2.9±0.4	2.9±0.3
Mi-CK ($n = 6$)	2.8±0.6	2.6±0.5	2.7±0.7	2.5±0.5
Cy-CK ($n = 5$)	23.4±3.7	21.3±2.5	20.6±3.7	21.6±3.3

t-CK, total creatine kinase. Values are mean±standard deviation for five to six independent experiments (animals) per group of creatine kinase activities measured in the presence or absence of 3HGA. No significant differences were detected between control and the 3HGA-treated groups (one-way ANOVA).

moderately diminished up to 20% leading to significant reduction of RCR values (Fig. 2C).

Effect of 3HGA exposition on C6 cell morphology and viability

We evaluated whether 3HGA could alter cell morphology of rat C6-glioma cells. It was observed that after 6-h-exposition cells changed from a round flat shape (Figs. 3A and B) to a spindle-differentiated shape in the presence of 0.1 and 1.0 mM 3HGA (Fig. 3C and D). However, no significant cell death (necrosis) occurred, as determined by the PI stain (Fig. 4A) and by the lack of bright cell aggregates. We also observed that C6-glioma cells exposed to 3HGA for up to 24 h behaved similarly to those in the presence of the organic acid for 6 h regarding cell morphology and viability (data not shown). Nuclear morphology was further studied in this cell line in order to assess apoptosis. We observed neither nuclear shrinkage nor chromatin condensation or fragmentation, characteristic morphological findings of apoptosis, in cells exposed for up to 6 h to 1.0 mM 3HGA (Fig. 4B and C).

DISCUSSION

Recently, it has been postulated that the nature of the striatal damage in GDD is similar to the brain injury that occurs in infants after hypoxia–ischemia or systemic intoxication with 3NPA, in which complex II activity is irreversibly blocked (Strauss and Morton, 2003). Therefore, in the present study we investigated whether 3HGA, considered the main neurotoxin in GDD, is able to disrupt brain energy metabolism, by evaluating the effect of this organic acid on critical enzyme activities responsible for ATP production and transfer within the cell, namely the respiratory chain complexes, Na^+,K^+-ATPase and CK in rat brain and in C6-glioma cells. We also examined the effect of 3HGA on mitochondrial oxygen consumption, as well as on the morphology and viability of rat C6-glioma cells.

We initially observed that complex II activity was mildly but significantly reduced by 1.0 mM 3HGA in brain homogenates from cerebral cortex, whereas the other activities of the respiratory chain were not changed by the acid at all tested doses (0.1–1.0 mM). Moreover, this inhibition only occurred at 1.0 mM of succinate, which corresponds to the actual *in vivo* concentration of succinate in the brain (1/μmol/g≅1.0 mM) (Goldberg et al., 1966). Furthermore, when complex II activity was measured in mitochondrial preparations from cerebral cortex, no inhibitory effect of 3HGA was detected, in contrast to 1.0 mM malonic acid, which severely affected this activity. These data suggest that 3HGA-induced inhibition on complex II activity is mild and probably due to a cytosolic-dependent mechanism. Considering that 3HGA is able to induce reactive species formation (Kölker et al., 2001a,b; Latini et al., 2002, 2005) and that complex II activity is vulnerable to free radical attack (Rustin and Rötig, 2002), it might be presumed that the inhibitory property of 3HGA on this activity in brain homogenates may have occurred via free radical formation. In this context, it has been described that deficiencies in the activity of complex II are associated to reactive oxygen species (Rustin and Rötig, 2002). The inactivation of complex II might have occurred via nitric oxide (NO) or NO derivatives such as peroxynitrite which is formed from the controlled reaction between NO and the mitochondrial-derived superoxide radical (Radi et al., 2002). In this scenario, it has previously been shown that 3HGA elicits NO generation in supernatants from cerebral cortex and striatum from young rats as well as in neuronal cultures (Kölker et al., 2001a,b; Latini et al., 2002, 2005) and that NO specifically inhibit complexes I and II of the respiratory chain by binding and potentially disrupting iron–sulfur clusters of these complexes (Moncada and Higgs, 1993).

We also showed here that 3HGA did not affect complex II activity in striatum and moderately inhibited this activity in C6 cell homogenates. Therefore, according to our data it seems unlikely that the striatum degeneration of

Table 2. Mg^{2+} and Na^+, K^+-ATPases activities in rat synaptic plasma membranes from cerebral cortex exposed to 3HGA

ATPases activities (μmol Pi/h/mg protein)	3HGA			
	0	0.01 mM	0.1 mM	1.0 mM
Mg^{2+}-ATPase ($n = 4$)	767±56	904±239	769±113	900±192
Na^+, K^+-ATPase ($n = 4$)	1083±112	897±168	1045±102	1092±139

Values are mean ± standard deviation for four independent experiments (animals) per group measured in the presence or absence of 3HGA. No significant differences were detected between control and the 3HGA-treated groups (one-way ANOVA).

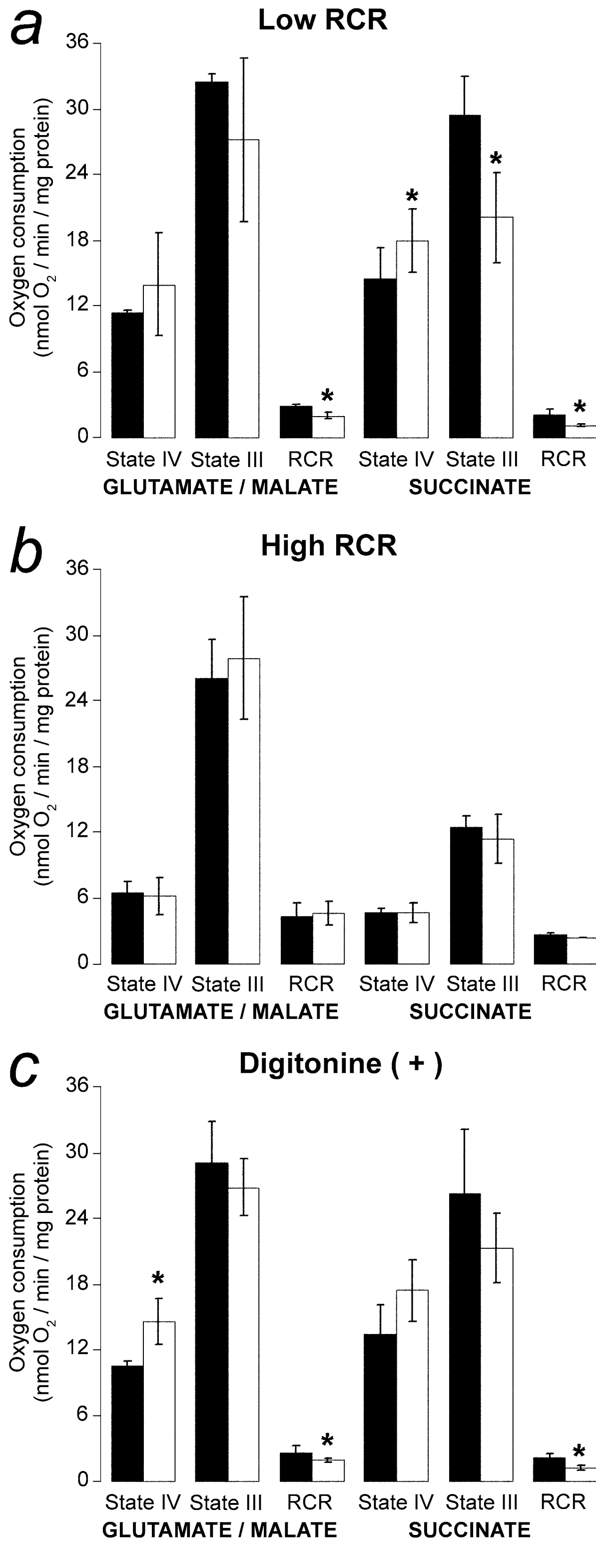

Fig. 2. Effect of 3HGA on respiratory parameters in rat cerebrum mitochondrial preparations. Respiratory parameters in mitochondria with: low (<3) RCR (a); high RCR (>3) (b) and; digitonin-treated mitochondria (c). Values are mean±standard deviation for four independent experiments per group in the presence or absence of the metabolite. * $P<0.05$, compared with controls (Duncan multiple range test).

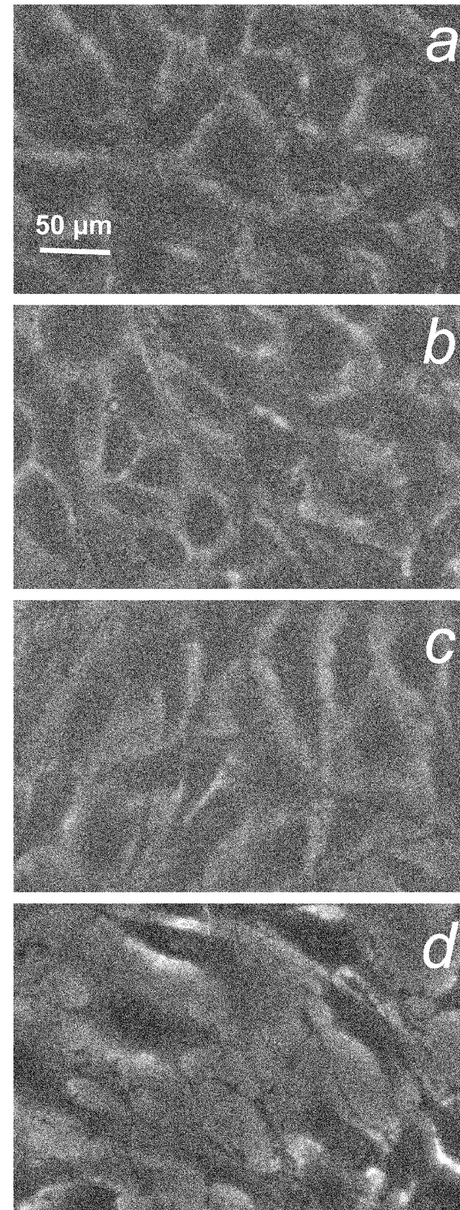

Fig. 3. Effect of 3HGA on C6-glioma cell morphology. Cells were cultured to confluence in DMEN+5% FBS. The medium was then changed to DMEN free of FBS and cells were incubated for further 6–24 h with different concentrations of 3HGA (a: basal; b: 0.01; c: 0.1 and d: 1.0 mM). Images are representative of three separated experiments with 6-h 3HGA incubation.

GDD occurs via inhibition of complex II of the respiratory chain by 3HGA. Taken together, it appears that 3HGA provokes a moderate inhibition of complex II activity, which is tissue specific and dependent on the experimental conditions utilized.

Inhibitory properties for 3HGA on the activities of complexes II and V and decreased creatine phosphate levels have also been reported in mixed cortical cultures from neonatal rats by 2.0 and 4.0 mM of this organic acid (Ullrich et al., 1999; Das et al., 2003). However, other investigators found only a mild complex V inhibition at high

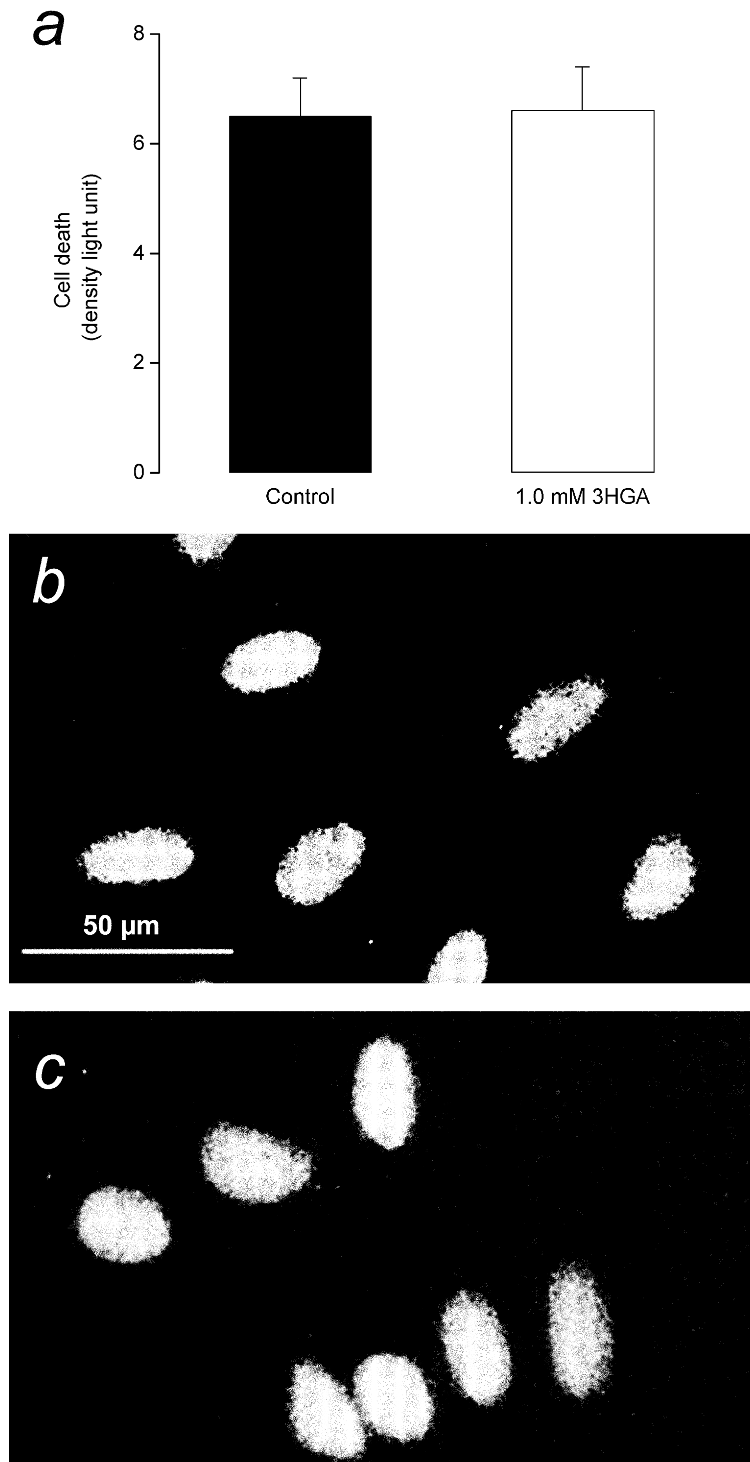

Fig. 4. Effect of 3HGA on viability and nuclear morphology of C6-glioma cells. Cell damage (a) was assessed by fluorescent image analysis of PI stain after cell exposition to 1.0 mM 3HGA for 6–24 h. Values are mean±standard deviation for optical density values obtained from images of three independent experiments at 6-h 3HGA incubation. Apoptosis was assessed at 6-h incubation with 3HGA by using the Hoechst stain (0.1 μg) during 90 min and visualized by fluorescence (b: basal; c: 1.0 mM 3HGA).

levels of 3HGA (10 mM) (Kölker et al., 2002) or no direct inhibition of the isolated respiratory chain complex activities in submitochondrial particles from bovine heart (Sauer et al., 2005). Since creatine phosphate levels are reduced by exposure of primary cultures of neuronal cells to 3HGA and since this effect can be prevented by pre-incubation of these cultures with creatine (Ullrich et al., 1999; Das et al., 2003), we tested whether 3HGA, at concentrations ranging

from 0.01–1.0 mM, could inhibit CK activities and verified that this organic acid did not change these activities. Therefore, it is unlikely that the reduction of neuronal creatine phosphate levels could be related to an inhibition of CK isoforms by 3HGA. Similarly, 3HGA did not alter Na^{+},K^{+}-ATPase activity from synaptic plasma membrane from cerebral cortex, a critical enzyme for neurotransmission and normal brain function.

In order to assess the overall consequences of 3HGA inhibitory effect on the electron transfer chain on mitochondrial respiration, we tested the effect of 1.0 mM 3HGA on the rate of mitochondrial oxygen consumption in the presence of glutamate/malate and succinate as substrates. To assure the entrance of the acid into the mitochondria, some mitochondrial preparations were also treated with digitonin, which makes the outer mitochondrial membrane permeable but preserves the inner membrane and mitochondria function (Elias et al., 1978). Treatment with 3HGA significantly lowered the RCR values in mitochondria showing low RCR and also in digitonin-treated mitochondria, by moderately reducing state III respiration and moderately increasing state IV respiration in the presence of NAD and FAD-linked substrates. These findings indicate that ATP synthesis (reduction of state III respiration) is compromised in mitochondria exposed to 3HGA and that this metabolite uncouples oxidative phosphorylation (reduction of RCR values). In contrast, when high RCR values were obtained, which indicate more tightly coupled mitochondria and therefore intact outer and inner mitochondrial membranes, 3HGA did not cause any effect on oxygen consumption. These observations might possibly indicate that 3HGA was not able to enter mitochondria and therefore not able to modify the respiratory parameters studied. Coupled respiration is known to be regulated by the outer mitochondrial membrane permeability which fully controls state IV respiration (Groen et al., 1982; Vander Heiden et al., 2000). Taken together, we may assume that 3HGA might act as an endogenous uncoupler of mitochondrial respiration under certain conditions that disrupt membrane mitochondria, such as, for example, lipid peroxidation, which has been previously demonstrated to be elicited by 3HGA (Latini et al., 2002, 2005).

On the other hand, although a mild inhibition of complex V activity caused by 3HGA has been previously described (Kölker et al., 2002), the effect of 3HGA on the respiratory parameters as shown here is unlikely to be mediated by complex V inhibition, since this would lead to a lower oxygen consumption reflected by a diminution of state IV respiration in 3HGA-treated mitochondria. We conclude that under our experimental conditions ATPase activity inhibition is not physiologically relevant.

We also found that 3HGA altered cell morphology of cultivated C6-glioma cells. Cells changed from a round flat to a spindle-differentiated shape, indicating injury due to cytoskeletal reorganization which may be a consequence of energy deficit or oxidative stress (Dalle-Donne et al., 2001). Furthermore, 3HGA incubation for up to 24 h did not decrease cell viability, as measured by the PI and by the absence of bright cell aggregates by phase contrast microscopy, or cause apoptosis, as evaluated by the Hoechst stain. It may be concluded that viability of C6-glioma cells was not decreased by up to 24-h incubation with 1.0 mM 3HGA. However, we cannot exclude that longer exposition with the acid would lead to C6-cell death, as verified previously with cultured neurons (Kölker et.al, 1999, 2001a, 2002).

The concentrations of 3HGA found to exert inhibitory actions on energy metabolism in our assays (1.0 mM) may be considered supraphysiological since the literature has demonstrated that 3HGA levels in postmortem brain are about 0.15 mM (Funk et al., 2005) or even lower (Kölker et al., 2003), a fact that may be explained by the differential adherence to the dietary treatment restricted in lysine and tryptophan of GDD patients. However, considering that 3HGA can be produced inside the brain and neurons have a limit capacity to extrude them (Kölker et al., 2003; Funk et al., 2005), it is conceivable that these levels may be much greater especially during crises of metabolic decompensation, in which very high production of the accumulating metabolites occurs (Goodman et al., 1977; Goodman, 2004). In addition, it should be noted that postmortem determination of chemical substances may result in lower levels than the actual *in vivo* concentrations because of their degradation and/or transformation. Therefore, it is presumed that greater *in vivo* concentrations of 3HGA than those previously reported may be reached in the brain. In this context, we cannot rule out that our results may be relevant to the understanding of the neuropathology of GDD, particularly the brain damage occurring during the acute episodes of encephalopathy.

Finally, we do not underestimate the role of GA and other related metabolites that accumulate in GDD inhibiting energy metabolism or inducing free radical attack (Okuda et al., 1998; Ullrich et al., 1999; Silva et al., 2000; de Oliveira Marques et al., 2003; Baran et al., 2003; Varadkar and Surtees, 2004; Sauer et al., 2005). We cannot also establish at this stage whether energy depletion, oxidative stress or excitotoxicity is mainly responsible for the brain damage in GDD patients. Most likely, primary or secondary excitotoxicity, oxidative stress and energy depletion cooperate in a synergistic way to result in the dramatic neuropathological findings and more particularly in the acute striatal neurodegeneration found in GDD patients occurring during acute encephalopathic crises which usually follow catabolic states due mainly to infections or vaccinations and in which the levels of the accumulating metabolites and pro-inflammatory toxins dramatically rise. In this context, exogenous conditions such as inflammation, has been demonstrated to act synergistically with excitotoxicity to induce neuronal death probably via metabolites of the kynurenine pathway (Kölker et al., 2001b; Varadkar and Surtees, 2004).

Acknowledgments—This work was supported in part by grants from FAPERGS, PRONEX II and CNPq-Brazil.

REFERENCES

Amir N, el-Peleg O, Shalev RS, Christensen E (1987) Glutaric aciduria type I: clinical heterogeneity and neuroradiologic features. Neurology 37:1654–1657.

Baran H, Staniek K, Kepplinger B, Stur J, Draxler M, Nohl H (2003) Kynurenines and the respiratory parameters on rat heart mitochondria. Life Sci 72:1103–1115.

Bjugstad KB, Zawada WM, Goodman S, Free CR (2001) IGF-1 and bFGF reduce glutaric acid and 3-hydroxyglutaric acid toxicity in striatal cultures. J Inherit Metab Dis 24:631–647.

Brismar J, Ozand PT (1995) CT and MR of the brain in glutaric acidemia type I: a review of 59 published cases and a report of 5 new patients. Am J Neuroradiol 16:675–683.

Cassina A, Radi R (1996) Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. Arch Biochem Biophys 328:309–316.

Chan KM, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca2+-stimulated ATPase activity. Anal Biochem 157:375–380.

Chow CW, Haan EA, Goodman SI, Anderson RM, Evans WA, Kleinschmidt-DeMasters BK, Wise G, McGill JJ, Danks DM (1988) Neuropathology in glutaric acidaemia type 1. Acta Neuropathol (Berl) 76:590–594.

da Silva CG, Bueno AR, Schuck PF, Leipnitz G, Ribeiro CA, Rosa RB, Dutra Filho CS, Wyse AT, Wannmacher CM, Wajner M (2004) Inhibition of creatine kinase activity from rat cerebral cortex by D-2-hydroxyglutaric acid in vitro. Neurochem Int 44:45–52.

da Silva CG, Ribeiro CA, Leipnitz G, Dutra-Filho CS, Wyse AA, Wannmacher CM, Sarkis JJ, Jakobs C, Wajner M (2002) Inhibition of cytochrome c oxidase activity in rat cerebral cortex and human skeletal muscle by D-2-hydroxyglutaric acid in vitro. Biochim Biophys Acta 1586:81–91.

Dalle-Donne I, Rossi R, Milzani A, Di Simplicio P, Colombo R (2001) The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. Free Radic Biol Med 31:1624–1632.

Das AM, Lucke T, Ullrich K (2003) Glutaric aciduria I: creatine supplementation restores creatine phosphate levels in mixed cortex cells from rat incubated with 3-hydroxyglutarate. Mol Genet Metab 78:108–111.

de Mello CF, Kölker S, Ahlemeyer B, de Souza FR, Fighera MR, Mayatepek E, Krieglstein J, Hoffmann GF, Wajner M (2001) Intrastriatal administration of 3-hydroxyglutaric acid induces convulsions and striatal lesions in rats. Brain Res 916:70–75.

de Oliveira Marques F, Hagen ME, Pederzolli CD, Sgaravatti AM, Durigon K, Testa CG, Wannmacher CM, de Souza Wyse AT, Wajner M, Dutra-Filho CS (2003) Glutaric acid induces oxidative stress in brain of young rats. Brain Res 964:153–158.

Elias PM, Goerke J, Friend DS, Brown BE (1978) Freeze-fracture identification of sterol-digitonin complexes in cell and liposome membranes. J Cell Biol 78:577–596.

Fischer JC, Ruitenbeek W, Berden JA, Trijbels JM, Veerkamp JH, Stadhouders AM, Sengers RC, Janssen AJ (1985) Differential investigation of the capacity of succinate oxidation in human skeletal muscle. Clin Chim Acta 153:23–36.

Floret D, Divry P, Dingeon N, Monnet P (1979) [Glutaric aciduria. 1 new case]. Arch Fr Pediatr 36:462–470.

Flott-Rahmel B, Falter C, Schluff P, Fingerhut R, Christensen E, Jakobs C, Musshoff U, Fautek JD, Deufel T, Ludolph A, Ullrich K (1997) Nerve cell lesions caused by 3-hydroxyglutaric acid: a possible mechanism for neurodegeneration in glutaric acidaemia I. J Inherit Metab Dis 20:387–390.

Frizzo ME, Schwarzbold C, Porciuncula LO, Dalcin KB, Rosa RB, Ribeiro CA, Souza DO, Wajner M (2004) 3-Hydroxyglutaric acid enhances glutamate uptake into astrocytes from cerebral cortex of young rats. Neurochem Int 44:345–353.

Funchal C, Gottfried C, De Almeida LM, Wajner M, Pessoa-Pureur R (2004) Evidence that the branched-chain alpha-keto acids accumulating in maple syrup urine disease induce morphological alterations and death in cultured astrocytes from rat cerebral cortex. Glia 48:230–240.

Funk CB, Prasad AN, Frosk P, Sauer S, Kölker S, Greenberg CR, Del Bigio MR (2005) Neuropathological, biochemical and molecular findings in a glutaric acidemia type 1 cohort. Brain 128:711–722.

Goldberg ND, Passonneau JV, Lowry OH (1966) Effects of changes in brain metabolism on the levels of citric acid cycle intermediates. J Biol Chem 241:3997–4003.

Goodman SI, Norenberg MD, Shikes RH, Breslich DJ, Moe PG (1977) Glutaric aciduria: biochemical and morphologic considerations. J Pediatr 90:746–750.

Goodman SI, Frerman FE (2001) Organic acidemias due to defects in lysine oxidation: 2-ketoadipic academia and glutaric acidemia. In: The metabolic and molecular bases of inherited disease, 8th edition (Scriver CR, Beaudet AL, Sly WS, Walle D, eds), pp 2195–2204. New York: McGraw-Hill.

Goodman SI (2004) Development of pathogenic concepts in glutaryl-CoA dehydrogenase deficiency: the challenge. J Inherit Metab Dis 27:801–803.

Gregersen N, Brandt NJ (1979) Ketotic episodes in glutaryl-CoA dehydrogenase deficiency (glutaric aciduria). Pediatr Res 13:977–981.

Groen AK, Wanders RJ, Westerhoff HV, van der Meer R, Tager JM (1982) Quantification of the contribution of various steps to the control of mitochondrial respiration. J Biol Chem 257:2754–2757.

Heyes MP (1987) Hypothesis: a role for quinolinic acid in the neuropathology of glutaric aciduria type I. Can J Neurol Sci 14:441–443.

Hoffmann GF, Athanassopoulos S, Burlina AB, Duran M, de Klerk JB, Lehnert W, Leonard JV, Monavari AA, Muller E, Muntau AC, Naughten ER, Plecko-Starting B, Superti-Furga A, Zschocke J, Christensen E (1996) Clinical course, early diagnosis, treatment, and prevention of disease in glutaryl-CoA dehydrogenase deficiency. Neuropediatrics 27:115–123.

Hoffmann GF, Zschocke J (1999) Glutaric aciduria type I: from clinical, biochemical and molecular diversity to successful therapy. J Inherit Metab Dis 22:381–391.

Hughes BP (1962) A method for the estimation of serum creatine kinase and its use in comparing creatine kinase and aldolase activity in normal and pathological sera. Clin Chim Acta 7:597–603.

Jones DH, Matus AI (1974) Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. Biochim Biophys Acta 356:276–287.

Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF (1999) 3-Hydroxyglutaric and glutaric acids are neurotoxic through NMDA receptors in vitro. J Inherit Metab Dis 22:259–262.

Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF (2000) Cerebral organic acid disorders induce neuronal damage via excitotoxic organic acids in vitro. Amino Acids 18:31–40.

Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF (2001a) Contribution of reactive oxygen species to 3-hydroxyglutarate neurotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr Res 50:76–82.

Kölker S, Ahlemeyer B, Huhne R, Mayatepek E, Krieglstein J, Hoffmann GF (2001b) Potentiation of 3-hydroxyglutarate neurotoxicity following induction of astrocytic iNOS in neonatal rat hippocampal cultures. Eur J Neurosci 13:2115–2122.

Kölker S, Kohr G, Ahlemeyer B, Okun JG, Pawlak V, Horster F, Mayatepek E, Krieglstein J, Hoffmann GF (2002) Ca(2+) and Na(+) dependence of 3-hydroxyglutarate-induced excitotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr Res 52:199–206.

Kölker S, Hoffmann GF, Schor DS, Feyh P, Wagner L, Jeffrey I, Pourfarzam M, Okun JG, Zschocke J, Baric I, Bain MD, Jakobs C, Chalmers RA (2003) Glutaryl-CoA dehydrogenase deficiency: region-specific analysis of organic acids and acyl-

carnitines in postmortem brain predicts vulnerability of the putamen. Neuropediatrics 34:253–260.
Kölker S, Koeller DM, Okun JG, Hoffmann GF (2004) Pathomechanisms of neurodegeneration in glutaryl-CoA dehydrogenase deficiency. Ann Neurol 55:7–12.
Latini A, Borba Rosa R, Scussiato K, Llesuy S, Bello-Klein A, Wajner M (2002) 3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats. Brain Res 956:367–373.
Latini A, Scussiato K, Leipnitz G, Dutra-Filho CS, Wajner M (2005) Promotion of oxidative stress by 3-hydroxyglutaric acid in rat striatum. J Inherit Metab Dis 28:57–67.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.
Moncada S, Higgs A (1993) The L-arginine-nitric oxide pathway. N Engl J Med 329:2002–2012.
Okuda S, Nishiyama N, Saito H, Katsuki H (1998) 3-Hydroxykynurenine, an endogenous oxidative stress generator, causes neuronal cell death with apoptotic features and region selectivity. J Neurochem 70:299–307.
Radi R, Cassina A, Hodara R (2002) Nitric oxide and peroxynitrite interactions with mitochondria. Biol Chem 383:401–409.
Rosa RB, Schwarzbold C, Dalcin KB, Ghisleni GC, Ribeiro CA, Moretto MB, Frizzo ME, Hoffmann GF, Souza DO, Wajner M (2004) Evidence that 3-hydroxyglutaric acid interacts with NMDA receptors in synaptic plasma membranes from cerebral cortex of young rats. Neurochem Int 45:1087–1094.
Rustin P, Chretien D, Bourgeron T, Gerard B, Rötig A, Saudubray JM, Munnich A (1994) Biochemical and molecular investigations in respiratory chain deficiencies. Clin Chim Acta 228:35–51.
Rustin P, Rötig A (2002) Inborn errors of complex II-unusual human mitochondrial diseases. Biochim Biophys Acta 1553:117–122.
Sauer SW, Okun JG, Schwab MA, Crnic LR, Hoffmann GF, Goodmann SI, Koeller DM, Kölker S (2005) Bioenergetics in glutaryl-coenzyme A dehydrogenase deficiency, a role for glutaryl-coenzyme A. J Biol Chem (http://www.jbc.org/cgi/content/full/280/23/21830).
Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD (1990) Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 54:823–827.
Silva CG, Silva AR, Ruschel C, Helegda C, Wyse AT, Wannmacher CM, Dutra-Filho CS, Wajner M (2000) Inhibition of energy production in vitro by glutaric acid in cerebral cortex of young rats. Metab Brain Dis 15:123–131.
Stokke O, Goodman SI, Moe PG (1976) Inhibition of brain glutamate decarboxylase by glutarate, glutaconate, and beta-hydroxyglutarate: explanation of the symptoms in glutaric aciduria? Clin Chim Acta 66:411–415.
Strauss KA, Morton DH (2003) Type I glutaric aciduria, part 2: a model of acute striatal necrosis. Am J Med Genet C Semin Med Genet 121:53–70.
Strauss KA, Puffenberger EG, Robinson DL, Morton DH (2003) Type I glutaric aciduria, part 1: natural history of 77 patients. Am J Med Genet C Semin Med Genet 121:38–52.
Tsakiris S, Deliconstantinos G (1984) Influence of phosphatidylserine on ($Na^+ + K^+$)-stimulated ATPase and acetylcholinesterase activities of dog brain synaptosomal plasma membranes. Biochem J 220:301–307.
Ullrich K, Flott-Rahmel B, Schluff P, Musshoff U, Das A, Lucke T, Steinfeld R, Christensen E, Jakobs C, Ludolph A, Neu A, Roper R (1999) Glutaric aciduria type I: pathomechanisms of neurodegeneration. J Inherit Metab Dis 22:392–403.
Vander Heiden MG, Chandel NS, Li XX, Schumacker PT, Colombini M, Thompson CB (2000) Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival. Proc Natl Acad Sci U S A 97:4666–4671.
Varadkar S, Surtees R (2004) Glutaric aciduria type I and kynurenine pathway metabolites: a modified hypothesis. J Inherit Metab Dis 27:835–842.

(Accepted 9 May 2005)
(Available online 19 August 2005)