UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA

PROGRAMA DE PÓS – GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

ESTUDOS COMPORTAMENTAIS E DO METABOLISMO ENERGÉTICO EM RATOS SUBMETIDOS A MODELOS DE ACIDÚRIA GLUTÁRICA TIPO I

GUSTAVO DA COSTA FERREIRA

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, Junho de 2009.

Livros Grátis

http://www.livrosgratis.com.br

Milhares de livros grátis para download.

Aos meus pais.

“O homem pode engrandecer o caminho; o caminho não pode engrandecer o homem...”

Confúcio

AGRADECIMENTOS

À UFRGS por me proporcionar uma formação de excelência e totalmente gratuita.

A todos os funcionários, colegas e professores do Departamento de Bioquímica pelos esforços incansáveis de manter este departamento dentre os melhores do país.

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

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

A todos os bolsistas de iniciação científica, Alana, Ângela, Anna Laura, Camila, Cris, Fernanda Hickmann, Fernanda Lopes, Karina Scussiato, Laís, Lisi, Luciana, Mateus, Rita e Vanessa, que estão ou estiveram alegrando o laboratório e ajudando a todos.

Aos colegas de pós-graduação que estão ou passaram pelo laboratório e deixaram saudade, Alexandre Amaral, Alexandre Solano, Ana Paula, Bianca, Carol Fernandes, César, Dênis, Estela, Fabrício, Giorgia, Guilhian, Karina Dalcin, Letícia, Lucila e Rafa por todo companheirismo, ajuda e amizade.

À Lali, por ser mais que uma amiga, pela excelente companhia e apoio em todos os momentos, inclusive os mais difíceis.

À Professora Carmen, por toda a amizade e tranquilidade para ensinar, pelo carinho e disposição constantes.

Ao Professor Gary Wenk e seus alunos, pela acolhida científica e paternal em terras frias e longínquas.

Aos Professores Piero Rinaldo e Dietrich Matern e a todos os colegas da Clínica Mayo, pelo exemplo de perfeccionismo e qualidade.

À Paulinha, pelo tempo em que brindou o laboratório com sua alegria, pela amizade e pela Coca-Cola com batatinhas.

Às minhas colegas e acima de tudo amigas Ane e Carol, por tudo que me ensinaram nesses anos, com trabalhos, sorrisos e amizade, e por tornar o laboratório um reduto quase familiar.

À Pati, por tudo que fez por mim desde que cheguei ao laboratório, pela paciência e o carinho, por ensinar que *“A alegria não é a estação final, e sim o caminho que escolhemos para chegar lá”* e por estar ao meu lado nesse caminho.

Ao Professor Moacir, exemplo maior que encontrei desde que ingressei na carreira de estudos, pela correção e rigidez de princípios e na hora de ensinar, e por ser mais do que meu orientador, a quem posso chamar de amigo.

Aos meu irmãos Raquel e Rafael, por todos os anos de convivência “pacífica”, pela amizade e alegrias compartilhadas.

Aos meus pais, por todo o carinho e cuidado com minha formação, por terem me concedido a “regalia” de uma vida de estudos e pelo amor com que fui criado e vivi cada dia de minha vida.

A Deus.

SUMÁRIO

PARTE I

Resumo 2

Abstract 4

Lista de Abreviaturas 6

I.1. INTRODUÇÃO 7

I.1.1. Erros Inatos do Metabolismo 7

I.1.2. Acidemias Orgânicas 8

I.1.3. Acidemia Glutárica Tipo I 9

I.1.3.1. Achados Clínicos 11

I.1.3.2. Diagnóstico 12

I.1.3.3. Achados Neuropatológicos 13

I.1.3.4. Tratamento 13

I.1.3.5. Modelos Animais de Acidemia Glutárica Tipo I 14

I.1.3.6. Fisiopatologia 16

I.1.4. Metabolismo energético no cérebro de mamíferos 21

I.1.5. Fosforilação oxidativa 23

I.1.6. Estudos comportamentais 26

I.1.6.1. Aprendizado e Memória no Labirinto Aquático de Morris 27

I.1.6.2 Campo aberto 28

I.1.6.3 Labirinto em cruz elevado 28

I.2. OBJETIVOS 30

I.2.1. Objetivo Geral 30

I.2.2. Objetivos Específicos 30

PARTE II

Capítulo I – *Chronic early postnatal glutaric acid administration causes cognitive deficits in the water maze* 33

Capítulo II – *Energy metabolism is compromised in skeletal muscle of rats chronically-treated with glutaric acid* 40

Capítulo III – *Evidence for a synergistic action of glutaric and 3-hydroxyglutaric acids disturbing rat brain energy metabolism* 54

PARTE III

III.1. DISCUSSÃO 64

III.2. CONCLUSÕES

III.2.1. Efeitos *in vivo* da administração crônica de AG sobre tarefas comportamentais 78

III.2.2. Efeitos *in vivo* da administração crônica de AG sobre parâmetros de metabolismo energético 78

III.2.3. Efeitos *in vitro* da co-incubação de AQ, AG e 3HG sobre parâmetros de metabolismo energético 79

III.3. PERSPECTIVAS 81

III.4. REFERÊNCIAS BIBLIOGRÁFICAS 82

PARTE I

Introdução e Objetivos

RESUMO

A acidemia glutárica tipo I (AG I) é um erro inato do metabolismo causado pela deficiência severa da atividade da enzima glutaril-CoA desidrogenase. Bioquimicamente, a AG I caracteriza-se por um aumento nas concentrações dos ácidos glutárico (AG) e 3-hidróxiglutárico (3HG) nos tecidos e líquidos corporais. Os pacientes afetados por essa doença apresentam macrocefalia ao nascimento e hipomielinização ou desmielinização progressiva do córtex cerebral. Crises de descompensação metabólica com encefalopatia aguda ocorrem principalmente entre 3 e 36 meses de vida, levando a uma marcada degeneração estriatal. Após as crises, os pacientes apresentam distonia e discinesia que progridem rapidamente até espasticidade. Apesar de diversos estudos apontarem para efeitos do AG e do 3HG induzindo disfunção energética, estresse oxidativo e excitotoxicidade, os mecanismos fisiopatológicos da AG I ainda são pouco conhecidos. Por outro lado, praticamente nada foi investigado sobre o comportamento de animais submetidos a modelos de AG I. Assim, os objetivos do presente trabalho foram estabelecer um modelo químico de AG I através de injeções subcutâneas de AG em ratos durante uma fase de intenso desenvolvimento do SNC, bem como investigar os efeitos deste modelo sobre o desempenho de ratos em tarefas comportamentais e sobre parâmetros de metabolismo energético em tecidos cerebrais (córtex cerebral e cérebro médio) e músculo esquelético. Observamos que esse tratamento não teve efeito sobre o peso corporal dos animais, bem como sobre a data de aparecimento dos pelos, abertura dos olhos, erupção dos dentes incisivos ou a tarefa do endireitamento em queda livre, indicando que o desenvolvimento físico e motor dos animais não foi alterado. Verificamos também que na tarefa do labirinto aquático de Morris os animais administrados com AG permaneceram por um período de tempo significativamente menor no quadrante alvo (onde a plataforma foi inicialmente colocada), além de permanecer por mais tempo no quadrante oposto ao quadrante alvo. Além disso, os animais administrados com AG também tiveram um menor número de passagens pelo local exato da plataforma e apresentaram uma maior latência para passar pela primeira vez sobre a posição da plataforma no dia do teste, em comparação aos animais controle (administrados com solução salina). Esses resultados indicam que a administração de AG provocou um déficit na memória e no aprendizado dos ratos. Por outro lado, observamos que o comportamento dos ratos na tarefa do labirinto em cruz elevado e no campo aberto não foi alterado pela administração do AG. Em relação aos parâmetros de metabolismo energético, observamos que a administração crônica do AG inibiu significativamente as atividades dos complexos I-III e II e aumentou a atividade do complexo IV da cadeia transportadora de elétrons em músculo esquelético, sem afetar essas atividades enzimáticas nas estruturas cerebrais estudadas. Observamos ainda que a atividade do ciclo de Krebs, medida pela produção de CO_2 a partir de acetato, não foi alterada pela administração crônica do AG, porém a atividade da enzima creatina quinase (CK) foi marcadamente reduzida apenas no músculo esquelético dos animais. Esses resultados indicam que a administração crônica do AG provocou um déficit energético em músculo

esquelético sem afetar as estruturas cerebrais, o que pode estar relacionado com as diferentes concentrações de AG atingidas nesses tecidos.

Outro objetivo deste trabalho foi investigar o efeito combinado *in vitro* do ácido quinolínico (AQ) que foi recentemente associado à fisiopatologia da AG I, com o AG ou o 3HG e do AG com o 3HG sobre vários parâmetros do metabolismo energético em córtex cerebral de ratos jovens. Observamos que, quando o AG, 3HG ou AQ foram testados isoladamente, ou quando AQ foi co-incubado com o AG ou o 3HG, não foram observadas alterações nos parâmetros de metabolismo energético examinados. Por outro lado, a combinação do AG com o 3HG provocou uma inibição da produção de CO_2 a partir de glicose, da atividade da enzima piruvato desidrogenase e a utilização de glicose em córtex cerebral de ratos, bem como um moderado aumento na produção de lactato a partir de glicose, porém de uma forma não significativa. Finalmente, observamos que a atividade da CK, particularmente a fração mitocondrial, foi significativamente inibida pela co-incubação do AG com o 3HG e que o GSH ou a combinação das enzimas catalase e superóxido dismutase preveniram totalmente a inibição dessa enzima.

Concluindo, demonstramos neste trabalho que a administração crônica de AG compromete o aprendizado/memória especial e inibe o metabolismo energético em ratos jovens. Mostramos também um efeito sinérgico *in vitro* do AG com o 3HG, alterando vários parâmetros do metabolismo energético.

ABSTRACT

Glutaric acidemia type I (GA I) is an inborn error of metabolism caused by a deficiency in the glutaryl-CoA dehydrogenase activity. Biochemically, GA I is characterized by the accumulation of glutaric (GA) and 3-hydroxyglutaric (3HG) acids in tissue and body fluids of affected patients, which present macrocephaly at birth and a progressive demyelination of cerebral cortex. Striatal degeneration following metabolic crises is the main neurological finding in this disease, occurring between 3 and 36 months of life. After crises, dystonia and diskinesia progress quickly. Although several studies suggest neurotoxic effects for GA and 3HG inducing energy dysfunction, oxidative stress and excitotoxicity, the pathophysiology of GA I is poorly unknown. However, practically nothing has been done to investigate whether GA, the most pronounced metabolite accumulating in GA I, could provoke deficit of performance in behavioral tasks. In this scenario, the aim of the present work was to establish chemically-induced animal model of GA I by subcutaneous injections of GA during a phase of rapid CNS development. We also aimed to investigate the effects of this model on rat performance in behavioral tasks and on energy metabolism in brain tissues (cerebral cortex and midbrain) and skeletal muscle of rats. It was observed that chronic GA administration did not change the animal body weight, the date of appearance of coat, eye opening or upper incisor eruption, nor the free-fall righting task, indicating that the physical and motor development was not altered. We also verified that GA-treated animals stayed for a significantly shorter time in the target quadrant, where the platform was formerly located, and spent significantly more time in the opposite quadrant as compared to controls (injected with saline). GA-treated rats also had a lower number of correct annulus crossings and presented a higher latency to cross over the platform position than saline-treated animals. These data suggest that early chronic postnatal GA administration caused a long-standing deficit in learning and memory processes of rats. On the other hand, we observed that rat behavior in the elevated plus maze and in the open field was not affected by GA administration. With regards to energy metabolism parameters, we observed that GA treatment significantly inhibited respiratory chain complexes I-III and II and increased complex IV enzyme activity in skeletal muscle, with no effects on these enzyme activities in brain tissues. We also observed that chronic GA treatment did not modify Krebs cycle activity, as assessed by CO_2 production from acetate, but markedly inhibited creatine kinase (CK) activity specifically in skeletal muscle. These data indicate that GA administration provoked energy deficit in rat skeletal muscle but not in brain structures. It is possible that this difference in GA effects is related to different GA levels reached in these tissues during the treatment.

We also aimed with this work to investigate the combined *in vitro* effect of quinolinic acid (QA), recently associated to GA I pathophysiology, with GA or 3HG and of GA with 3HG on various parameters of energy metabolism in brain of young rats. We found that when GA, 3HG or AQ were tested isolated, or when QA was co-incubated with GA or 3HG, no alterations were found in the examined parameters. On the other hand, the combination of GA with 3HG resulted in an inhibitition of CO_2 production from glucose, pyruvate dehydrogenase enzyme activity and glucose uptake from cerebral cortex, as well as in a mild increase in

the lactate production, although non-significantly. Finally, it was observed that CK activity, particularly the mitochondrial fraction, was significantly inhibited by the co-incubation of GA with 3HG and that GSH or the combination of catalase and superoxide dismutase enzymes were able to fully prevent this inhibition.

Concluding, we here demonstrated that chronic GA administration compromises the learning/memory processes and inhibits energy metabolism in young rats. We also showed a synergic *in vitro* effect between GA and 3HG, leading to alterations in various parameters of energy metabolism.

LISTA DE ABREVIATURAS

$\Delta\Psi m$ – potencial de membrana mitocondrial

ΔpH – potencial químico

3HG – ácido 3-hidroxiglutárico

AG – ácido glutárico

AG I – acidemia glutárica tipo I

AQ – ácido quinolínico

CAT – catalase

CK – creatina quinase

CoA – coenzima A

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

EIM – erros inatos do metabolismo

ETF – flavoproteína transferidora de elétrons

GABA – ácido γ-aminobutírico

GCDH – glutaril-coenzima A desidrogenase

GSH – glutationa reduzida

NMDA – N-metil-D-aspartato

Pi – fosfato inorgânico

SNC – sistema nervoso central

SOD – superóxido dismutase

I.1. INTRODUÇÃO

I.1.1. Erros Inatos do Metabolismo

Em 1908, Sir Archibald E. Garrod usou 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 frequência desta doença em indivíduos de uma mesma família e maior incidência de consanguinidade entre os pais dos pacientes. Baseando-se nas leis de Mendel e no fato de que os pais dos indivíduos afetados não apresentavam a doença, Garrod propôs um modelo de herança autossômica recessiva para este distúrbio. Através da observação de que o ácido homogentísico presente em excesso na urina dos pacientes era um metabólito normal da degradação protéica, ele relacionou este acúmulo a um bloqueio na rota de catabolismo da tirosina. 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, na maioria das vezes enzimáticas (Scriver et al., 2001). Presumiu-se, então, que em consequência deste bloqueio metabólico pode ocorrer o acúmulo de precursores tóxicos da reação catalisada pela enzima envolvida, com a formação de rotas metabólicas alternativas e a deficiência de produtos essenciais ao organismo (Bickel, 1987).

Até o momento foram caracterizados bioquimicamente 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 em seu conjunto afetam aproximadamente 1 a cada 500 a 2.000 recém nascidos vivos (Baric et al., 2001).

I.1.2. Acidemias Orgânicas

As acidemias ou acidúrias orgânicas constituem um grupo de EIM caracterizados pelo acúmulo de um ou mais ácidos orgânicos nos líquidos biológicos e tecidos dos pacientes afetados devido à deficiência da atividade de uma enzima 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 que requer equipamento sofisticado e de alto custo e/ou 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 a 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, dentre outros. As mais freqüentes manifestações laboratoriais são cetose, cetonúria, neutropenia, trombocitopenia, acidose metabólica, baixos níveis de bicarbonato, hiperglicinemia, hiperamonemia, hipo/hiperglicemia, acidose láctica, aumento dos níveis séricos de ácidos graxos livres e outros (Scriver et al., 2001; Saudubray et al., 2006). O uso da tomografia computadorizada revelou na maioria dos 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 (AG I, OMIM # 231670) é uma desordem neurometabólica autossômica recessiva, inicialmente descrita por Goodman e colaboradores em 1975 (Goodman et al., 1975). Bioquimicamente, a AG I é causada pela deficiência na atividade da enzima mitocondrial glutaril-CoA desidrogenase (GCDH, EC 1.3.99.7) (Goodman e Frerman, 2001) da via de catabolismo dos aminoácidos lisina, hidroxilisina e triptofano, que 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 R402W, mutação mais freqüente em caucasianos (Goodman et al., 1998; Zschocke et al., 2000). Existe uma grande heterogeneidade de mutações na deficiência da GCDH, porém, dentro de comunidades específicas o padrão pode ser mais homogêneo (Busquets et al., 2000). Apesar do conhecimento de diferentes mutações, não há correlação entre o genótipo, a atividade enzimática e o prognóstico dos pacientes (Goodman et al., 1998; Hoffmann e Zschocke, 1999; Kölker et al., 2006a). Com o bloqueio da atividade enzimática, formam-se rotas metabólicas alternativas que resultam na presença de concentrações elevadas dos ácidos glutárico (AG), 3-hidroxiglutárico (3HG) e, algumas vezes durante as crises, 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 AG e 40–200 μmol/L para o 3HG (Külkens et al., 2005; Sauer et al., 2006). Tais diferenças podem ser explicadas pelo fato de que o AG e o 3HG 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).

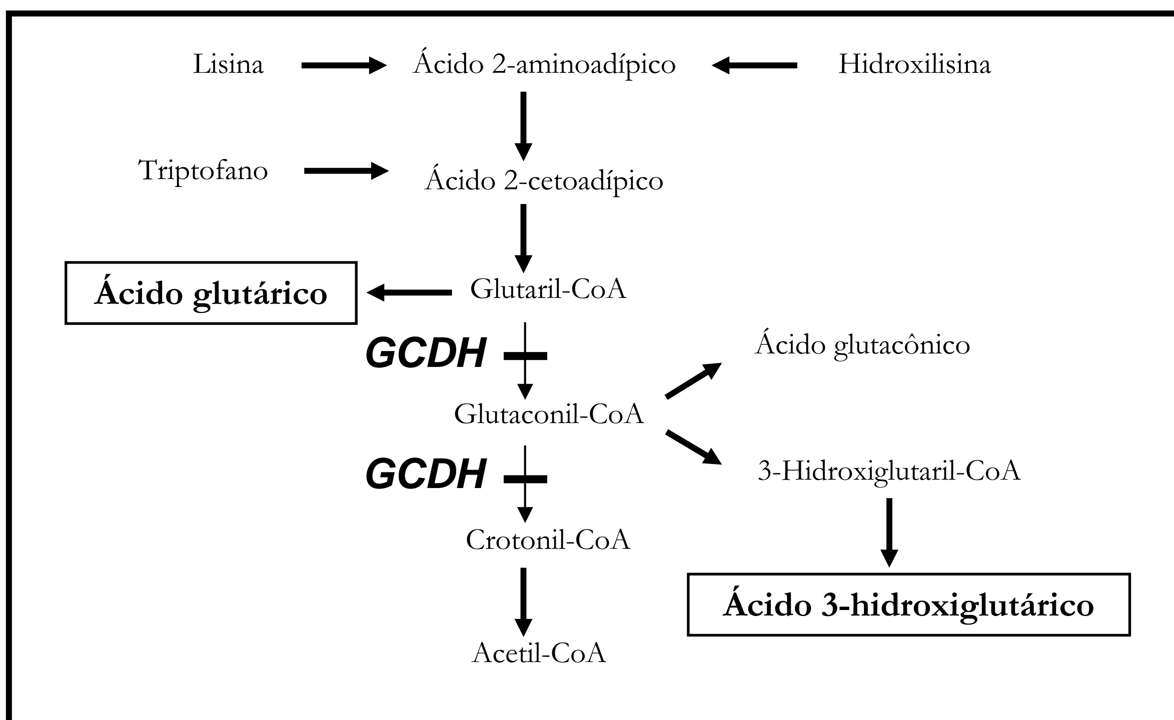

Figura 1. Deficiência da enzima glutaril-CoA desidrogenase (GCDH). (Adaptado de Goodman e Frerman, 2001)

A prevalência da AG I é estimada em 1: 30.000 a 1: 80.000 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 AG I (Külkens et al., 2005).

I.1.3.2. Diagnóstico

Apesar do desenvolvimento de diversas estratégias terapêuticas para o tratamento da AG I, o diagnóstico precoce continua sendo fator determinante para um melhor prognóstico para os pacientes afetados, uma vez que medidas preventivas podem ser tomadas. Usualmente, o marcador bioquímico da AG I é a presença de quantidades elevadas de AG e 3HG nos líquidos biológicos (especialmente na 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 massas (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 de algumas dessas doenças (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 ou no diagnóstico pré-natal (Busquets et al., 2000; Kölker et al., 2006a).

Alguns pacientes apresentam excreção pouco elevada, intermitente, ausente ou normal de AG (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 e a determinação de glutarilcarnitina por espectrometria de massas em tandem 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 cortical frontotemporal ao nascimento, formação espongiforme e diminuição de substância branca (leucoencefalopatia progressiva) 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) entre os primeiros 3 e 36 meses de vida (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 um melhor 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 et al., 2006).

Diversos medicamentos têm sido testados na terapia da AG 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 em um modelo animal de AG 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.

Convém enfatizar que as novas estratégias terapêuticas devem ser bem avaliadas, pois a utilização dessas terapias requer muito estudo e cautela quando de seu uso para os pacientes. Por outro lado, na medida em que se conheçam os mecanismos exatos do dano cerebral nos pacientes com AG I, melhores terapias se tornarão disponíveis.

I.1.3.5. Modelos Animais de Acidemia Glutárica Tipo I

Modelos animais que mimetizem as características metabólicas e neuropatológicas apresentadas pelos pacientes com AG I tem sido investigados. 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 AG, 3HG e

conjugados de glicina e carnitina, esse modelo não reproduz o fenótipo neurológico e a degeneração estriatal característica dos pacientes afetados. Neste modelo, foi estudado o desenvolvimento neuromotor dos camundongos geneticamente modificados, sendo observado que os animais apresentaram dificuldade de endireitar-se quando colocados de costas para o chão. Além disso, esses animais apresentaram um pequeno déficit motor que foi evidenciado por uma deficiência moderada ao caminhar sobre um plataforma estreita. Por outro lado, esses animais não puderam ser diferenciados de camundongos normais em relação às interações sociais ou ao comportamento na gaiola onde eles foram mantidos. 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 AG 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 do 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 AG I.

I.1.3.6. Fisiopatologia

A AG I é considerada uma acidemia orgânica "cerebral" pois os indivíduos afetados apresentam essencialmente sintomatologia neurológica. No intuito de explicar a fisiopatogenia do dano cerebral da AG I, distintos mecanismos têm sido propostos, envolvendo principalmente a toxicidade dos principais metabólitos acumulados nessa doença (AG e 3HG) sobre processos que levam a excitotoxicidade, estresse oxidativo e distúrbios de bioenergética.

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 e colaboradores (1976). Esse trabalho mostrou uma inibição competitiva da enzima glutamato descarboxilase (enzima que converte o glutamato a GABA) na presença dos ácidos AG, 3HG e glutacônico. Neste particular, concentrações estriatais reduzidas de GABA (caudato e putamen) foram encontradas em um paciente com AG I (Leibel et al., 1980). Outros estudos corroboraram esses achados, demonstrando que as convulsões decorrentes da administração intraestriatal de AG (Lima et al., 1998) e 3HG (de Mello et al., 2001) em ratos foram prevenidas por muscimol, um agonista de receptores $GABA_A$. Por outro lado, Ullrich e colaboradores (1999), contudo, não encontraram efeitos do 3HG sobre receptores de GABA.

Baseado no fato de que alguns autores relacionam as vacuolizações encontradas em estudos *postmortem* com cérebro de pacientes afetados pela deficiência da GCDH com a toxicidade glutamatérgica (Goodman et al., 1977; Forstner et al., 1999; Hoffmann e Zschocke, 1999), diversos trabalhos tentam

explicar a neurotoxicidade da AG I pela ação desses ácidos orgânicos sobre receptores e transportadores glutamatérgicos. Kölker e colaboradores (2000, 2002a) propuseram que o 3HG 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 3HG (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 3HG 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 3HG (Ullrich et al., 1999; Kölker et al., 2004b; Goodman, 2004). Para o AG, 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 AG 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 pela administração intraestriatal de AG 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 AG 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.

Por outro lado, vários trabalhos demonstraram a produção de radicais livres e a diminuição das defesas antioxidantes no cérebro de ratos na presença de AG e 3HG. Latini e colaboradores (2002, 2005b) mostraram que o 3HG 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. Uma produção aumentada de espécies reativas de oxigênio na presença de 3HG também foi evidenciada em culturas de neurônios de telencéfalos de embriões de pinto (Kölker et al., 2001). Com respeito ao AG, foi demonstrado um aumento na lipoperoxidação e uma diminuição das defesas antioxidantes, com diminuição da atividade da enzima glutationa peroxidase em cérebro de ratos (de Oliveira Marques et al., 2003). Além disso, Latini e colaboradores (2007) mostraram que tanto a administração aguda quanto a crônica de AG aumentam a lipoperoxidação e diminuem as defesas antioxidantes em diferentes estruturas cerebrais, fígado e eritrócitos de ratos.

Em relação aos distúrbios de bioenergética, estudos realizados em culturas de neurônios de ratos mostraram que o 3HG inibe os complexos II e V da cadeia transportadora de elétrons 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 transportadora de elétrons em homogeneizados de córtex cerebral e células C6 de glioma de ratos na presença desse metabólito. Além disso, o mesmo estudo demonstrou que o 3HG 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 3HG (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 AG 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 e II-III e da enzima creatina quinase em músculo esquelético e cérebro médio de ratos administrados agudamente com AG (Ferreira et al., 2005). Resultados semelhantes foram descritos também *in vitro* (da C. Ferreira et al., 2005).

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

Com base nesses resultados, alguns autores postularam o 3HG como a principal neurotoxina na AG I, atuando principalmente pela indução de excitotoxicidade (Ullrich et al., 1999; Kölker et al, 2002a). No entanto, outros estudos demonstram que o AG é igualmente neurotóxico (Lima et al., 1998; Kölker et al., 1999, 2001a,b, 2002; Ullrich et al., 1999; Porciuncula et al., 2000; Bjugstad et al., 2001; de Mello et al., 2001; de Oliveira et al., 2003; Rosa et al., 2004). Recentemente foi publicado um estudo por Zinnanti e colaboradores demonstrando que as concentrações de AG no cérebro de camundongos *Gcdh-/-* submetidos a uma sobrecarga de lisina aumentaram significativamente e que os mesmos apresentaram um padrão de neurodegeneração dependente do estágio de desenvolvimento, semelhante ao apresentado pelos pacientes afetados pela AG I (lesão estriatal), além de provocar a perda de seletividade da barreira hemato-encefálica (Zinnanti et al., 2006a). Além disso, os mesmos autores demonstraram que essa sobrecarga de lisina na dieta provoca uma disfunção mitocondrial idade-dependente no cérebro de camundongos *Gcdh-/-* (Zinnanti et al., 2007), fazendo com que o distúrbio bioenergético causado pelo AG possa ser um importante mecanismo fisiopatológico na AG I.

Por outro lado, apesar da intensa investigação e da severidade dos sintomas apresentados pelos pacientes afetados por essa doença, os mecanismos que levam à suscetibilidade frontotemporal cortical durante a

gestação e à janela de vulnerabilidade estriatal durante os primeiros anos de vida ainda não foram elucidados, constituindo-se nos principais desafios da pesquisa da patogênese da AG I (Goodman, 2004).

I.1.4. Metabolismo energético no cérebro de mamíferos

O principal substrato energético para o cérebro de mamíferos adultos é a glicose, porém o padrão de utilização deste nutriente varia conforme a etapa de desenvolvimento do sistema nervoso central (SNC), o estado nutricional do indivíduo e o destino de sua cadeia de átomos de carbono (Marks et al., 1996).

Nas primeiras horas após o nascimento, o lactato é a principal fonte de obtenção de energia cerebral em seres humanos (Vicario et al., 1991). Com o início do período de amamentação, os corpos cetônicos, formados a partir da oxidação dos ácidos graxos contidos no leite, são utilizados em grandes quantidades pelo cérebro. Assim, durante as primeiras semanas de desenvolvimento, os corpos cetônicos se constituem como uma das principais fontes para obtenção de energia pelo cérebro (Miller et al., 1982). Nesta fase, o consumo de glicose pelo cérebro é reduzido, aumentando gradativamente em poucas semanas. O equilíbrio entre o consumo de glicose e de corpos cetônicos é alcançado por volta dos 18 dias de vida, quando a utilização de glicose se torna preferencial em condições normais (Crone, 1965; Cremer et al., 1976).

O estado nutricional também dita o padrão de utilização de nutrientes pelo cérebro. Situações de jejum prolongado fazem com que o SNC passe a utilizar corpos cetônicos para obtenção de energia, a fim de poupar o organismo de um

catabolismo protéico exacerbado resultante da necessidade da manutenção da glicemia via gliconeogênese (Marks et al., 1996).

A glicose captada pelo cérebro é também fonte de carbono para a síntese de diversas outras biomoléculas essenciais (por exemplo, neurotransmissores), o que reforça a idéia de que a utilização de glicose não está atrelada somente à produção de energia.

O ciclo do ácido cítrico é a via comum de oxidação dos glicídios, aminoácidos e ácidos graxos (aproximadamente 95% do ATP sintetizado). O metabolismo energético cerebral se mostra essencialmente aeróbico, sendo a glicose o principal substrato utilizado (Clark et al., 1993), entrando no ciclo sob a forma de acetil-CoA que é então oxidado completamente a CO_2. As reações anapleróticas que alimentam o ciclo fornecendo diretamente seus intermediários também fornecem substratos para as reações de oxidação no cérebro**.** Quando não há hipóxia, a fosforilação oxidativa é dependente da concentração de ATP, ADP e fosfato inorgânico (Pi) e da razão mitocondrial de $NADH/NAD^+$ que é determinada pela atividade da cadeia transportadora de elétrons e pelo fornecimento de elétrons por enzimas mitocondriais. A cadeia transportadora de elétrons oxida o NADH e bombeia prótons para o espaço intermembrana da mitocôndria formando assim um gradiente de prótons que ativa a ATP sintase que produz ATP na fosforilação oxidativa (Erecinska e Silver, 1994).

A atividade do ciclo do ácido cítrico pode ser medida através da formação de CO_2. Neste particular, diversos substratos marcados por isótopos radioativos são oxidados *in vitro* pelo cérebro até CO_2. A utilização dessas substâncias tem

auxiliado no estudo do metabolismo energético cerebral a partir de diferentes pontos da rota aeróbica de oxidação, dependendo do substrato utilizado (por exemplo, glicose, acetato ou citrato) e da posição em que a molécula é marcada.

I.1.5. Fosforilação oxidativa

As coenzimas nucleotídicas reduzidas NADH e $FADH_2$, provenientes da oxidação de glicose, ácidos graxos, aminoácidos e intermediários do ciclo do ácido cítrico são moléculas ricas em energia, pois contêm elétrons com alto potencial energético que podem ser facilmente liberados (Nelson e Cox, 2000).

A fosforilação oxidativa é o processo pelo qual o O_2 é reduzido a H_2O, por elétrons doados pelo NADH e $FADH_2$ que fluem por vários pares de redução-oxidação (cadeia respiratória), ocorrendo concomitantemente a produção de ATP a partir de ADP e Pi (Nelson e Cox, 2000). Em eucariotos, a fosforilação oxidativa ocorre nas mitocôndrias, mais precisamente na cadeia transportadora de elétrons e é responsável pela maior parte da energia liberada pela célula (Figura 2).

As mitocôndrias são corpúsculos envolvidos por uma membrana externa, facilmente permeável a pequenas moléculas e íons e por uma membrana interna, impermeável à maioria das moléculas e íons, incluindo prótons H^+ (Nelson e Cox, 2000). A membrana interna é composta por aproximadamente 75% de proteínas. É livremente permeável somente a O_2, CO_2 e H_2O e contém, além das proteínas da cadeia transportadora de elétrons, várias proteínas de transporte que controlam a passagem de metabólitos, como piruvato, glicerolfosfato, malato, ácidos graxos, prótons e outras moléculas essenciais às funções mitocondriais (Abeles et al.,

1992). A seletividade da membrana mitocondrial interna para a maioria dos íons e metabólitos permite a formação de um gradiente iônico através dessa barreira e resulta na compartimentalização das funções metabólicas entre o citosol e a mitocôndria (Voet e Voet, 1990). O fluxo de elétrons a partir de NADH e $FADH_2$ até o O_2 (aceptor final de elétrons) se dá através de complexos enzimáticos ancorados na membrana mitocondrial interna com centros redox com afinidade crescente por elétrons. A seqüência de carreadores de elétrons, de um modo geral, reflete seus potenciais de redução relativos, sendo o processo global de transporte de elétrons exergônico.

A cadeia respiratória é composta por vários complexos enzimáticos e uma coenzima lipossolúvel, a coenzima Q ou ubiquinona (Di Donato, 2000). O complexo I, conhecido como NADH desidrogenase ou NADH: ubiquinona oxidorredutase, transfere os elétrons do NADH para a ubiquinona. O complexo II, sucinato desidrogenase, reduz a ubiquinona com elétrons do $FADH_2$ provenientes da oxidação do sucinato a fumarato no ciclo do ácido cítrico. O complexo III, citocromo bc_1 ou ubiquinona-citocromo *c* oxidorredutase, catalisa a redução do citocromo *c* a partir dos elétrons provenientes da ubiquinona reduzida. O complexo IV, conhecido como citocromo *c* oxidase, catalisa a transferência dos elétrons do citocromo *c* reduzido para o O_2, reduzindo-o a H_2O. Todos esses complexos possuem grupamentos prostéticos (grupamento heme, por exemplo) específicos para desempenharem o papel de aceptores e doadores de elétrons (Abeles et al., 1992).

O fluxo de elétrons através dos complexos da cadeia transportadora de elétrons é acompanhado pelo bombeamento de prótons da matriz mitocondrial para o espaço intermembranas. Com isso, cria-se um gradiente eletroquímico transmembrana que pode ser utilizado por um quinto complexo protéico, a ATP sintase, para a síntese de ATP. Dessa forma, a oxidação de substratos energéticos está acoplada ao processo de fosforilação do ADP, ou seja, quando o fluxo de prótons volta a favor do gradiente eletroquímico, a energia liberada é utilizada pela ATP sintase que funciona como uma bomba de prótons dependente de ATP trabalhando no sentido reverso, para liberar o ATP (Nelson e Cox, 2000).

Além do possível déficit na produção de ATP, que se constitui na principal moeda energética para os processos celulares, algumas doenças genéticas e outros distúrbios que interfiram na cadeia transportadora de elétrons aumentam os níveis de NADH, inibindo o ciclo do ácido cítrico e impedindo a entrada de piruvato neste. Como conseqüência, o piruvato é convertido a lactato, cujos níveis se elevam no sangue do indivíduo (Voet e Voet, 1990).

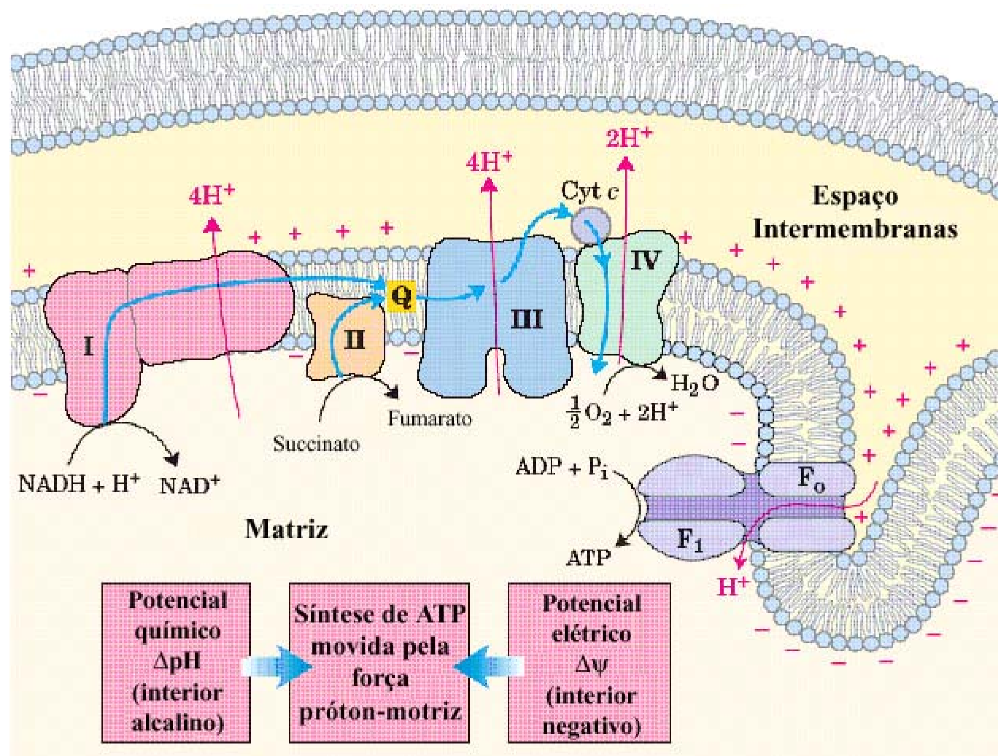

Figura 2. Fluxo de elétrons através dos quatro complexos da cadeia respiratória. O gradiente eletroquímico transmembrana formado possibilita a síntese de ATP pela ATP-sintase (adaptado de Nelson e Cox, 2004).

I.1.6. Estudos comportamentais

Uma abordagem importante para se avaliar dano funcional causado ao sistema nervoso central (SNC) por neurotoxinas é a utilização de tarefas comportamentais em animais. Neste particular, o agente potencialmente neurotóxico a ser testado pode causar déficit no desempenho dos animais nas tarefas selecionadas. No entanto, convém salientar que uma relação direta entre o grau da lesão cerebral e o déficit cognitivo nem sempre ocorre. Extensas lesões podem não causar déficit cognitivo, enquanto que pequenas lesões podem gerar

grandes conseqüências funcionais. Vários fatores podem contribuir para a dissociação entre o tamanho da lesão e o déficit funcional. Entre eles podemos citar a localização do dano cerebral, a habilidade prévia do animal, o tempo decorrido desde que ocorreu a lesão e as diferenças individuais. Independentemente destas observações, a realização e a análise do desempenho de animais submetidos a um tratamento com o agente potencialmente tóxico em tarefas comportamentais são importantes para avaliar as possíveis conseqüências funcionais de um dano neuronal (Olton e Markowska, 1994).

No modelo knockout de AG I foi apenas estudado o desenvolvimento motor dos camundongos, não tendo sido investigado o aprendizado e a memória dos animais (Koeller et al., 2002). No presente estudo, um dos objetivos foi verificar se a administração crônica com ácido glutárico poderia alterar a performance de ratos em várias tarefas que medem a cognição, atividade motora e emoção. As tarefas executadas foram como segue.

I.1.6.1 Aprendizado e Memória no Labirinto Aquático de Morris

Aprendizado e memória são funções básicas do SNC fundamentais para a adaptação de um organismo ao meio ambiente. O aprendizado pode ser definido como a aquisição de informações através da experiência e a memória, com o armazenamento de informações (Izquierdo, 1989). Ao escolhermos uma tarefa para avaliar o aprendizado e a memória de um animal, devemos levar em consideração o interesse e a capacidade do animal de aprender a tarefa e sua capacidade de executá-la. A tarefa do labirinto aquático de Morris é adequada para se avaliar cognição em ratos, uma vez que estes animais são bons

nadadores e apresentam uma boa capacidade de localização espacial que é requerida nesta tarefa. Por outro lado, a água é um meio aversivo para estes animais que procuraram escapar da mesma. Enfatize-se que a tarefa do labirinto aquático de Morris é adequada para a avaliação da integridade funcional de algumas estruturas do sistema nervoso central envolvidas com os processos envolvidos com aprendizado e memória e particularmente com o raciocínio espacial, como, por exemplo, o hipocampo e o estriado (Olton e Markowska, 1994; Save e Poucet, 2000).

I.1.6.2 Campo aberto

A atividade exploratória de animais de laboratório pode ser analisada pela tarefa do campo aberto que permite observar como o animal se comporta em um ambiente amplo (Ho et al., 2002).

O teste é realizado em uma caixa de madeira com dimensões estabelecidas, em que o fundo é dividido por linhas pretas em 12 quadrados iguais. Neste experimento é avaliada a movimentação espontânea entre as divisões da caixa que indica a atividade exploratória dos animais. Pode-se avaliar também o medo dos animais que se reflete pela imobilidade na caixa e a capacidade de exploração do ambiente e fuga que é avaliado através do número de *rearings* (Vianna, 2000).

I.1.6.3 Labirinto em cruz elevado

O labirinto em cruz elevado é um teste comportamental utilizado para avaliar ansiedade em roedores, principalmente por ser um modelo eficaz, simples,

de baixo custo e de fácil interpretação (Cruz-Morales et al., 2002). Para a realização do teste é utilizado um labirinto que consiste de dois braços fechados opostos, com paredes laterais e dois braços abertos opostos, ou seja, sem paredes. Os braços ficam dispostos em forma de uma cruz e os animais são inicialmente colocados na área central. Entre os parâmetros que podem ser analisados estão o número e o tempo de permanência nos braços abertos que refletem o nível de ansiedade dos animais.

I.2. OBJETIVOS

I.2.1. Objetivo Geral

O objetivo geral deste trabalho foi investigar os efeitos da administração crônica de ácido glutárico sobre tarefas comportamentais e sobre parâmetros de metabolismo energético em córtex cerebral, cérebro médio e músculo esquelético de ratos jovens. Avaliamos também os efeitos *in vitro* da incubação simultânea dos ácidos glutárico, 3-hidroxiglutárico e quinolínico sobre parâmetros de metabolismo energético em córtex cerebral de ratos jovens, na tentativa de identificar efeitos sinérgicos dos metabólitos acumulados na AG I e, portanto melhor compreender os mecanismos fisiopatológicos do dano tecidual característico desta doença.

I.2.2. Objetivos Específicos

- Estabelecer um modelo crônico de acidemia glutárica tipo I induzido quimicamente pela administração subcutânea de ácido glutárico (AG) em ratos, mimetizando o principal achado bioquímico desta doença;
- Investigar os efeitos da administração crônica subcutânea de ácido glutárico (AG) em ratos sobre a performance de ratos nas tarefas comportamentais labirinto aquático de Morris, labirinto em cruz elevado e campo aberto;
- Investigar os efeitos da administração crônica subcutânea de ácido glutárico (AG) sobre a produção de CO_2 a partir de [1-^{14}C]acetato, bem como sobre as atividades dos complexos da cadeia transportadora de elétrons I-IV e da enzima

creatina quinase em córtex cerebral, cérebro médio e músculo esquelético de ratos;

- Investigar os efeitos *in vitro* da co-incubação dos ácidos glutárico (AG), 3-hidroxiglutárico (3HG) e quinolínico (AQ) sobre a utilização de glicose, liberação de lactato, produção de CO_2 a partir de [U-^{14}C]glicose e [1-^{14}C]acetato e sobre a atividade da enzima creatina quinase em córtex cerebral de ratos;
- Investigar os efeitos *in vitro* da co-incubação dos ácidos glutárico (AG) e 3-hidroxiglutárico (3HG) sobre a utilização de glicose, liberação de lactato, produção de CO_2 a partir de [U-^{14}C]glicose e [1-^{14}C]acetato e sobre as atividades das enzimas piruvato desidrogenase e creatina quinase em córtex cerebral de ratos.

PARTE II

Artigos Científicos

Capítulo I

Chronic early postnatal glutaric acid administration causes cognitive deficits in the water maze

Gustavo da C. Ferreira, Patrícia F. Schuck, Carolina M. Viegas, Anelise Tonin, César A.J. Ribeiro, Leticia F. Pettenuzzo, Lenir O. Pereira, Carlos A. Netto, Moacir Wajner

Artigo científico publicado no periódico

Behavioural Brain Research 187: 411–416, 2008

Available online at www.sciencedirect.com
ScienceDirect
Behavioural Brain Research 187 (2008) 411–416
BEHAVIOURAL BRAIN RESEARCH
www.elsevier.com/locate/bbr

Research report

Chronic early postnatal glutaric acid administration causes cognitive deficits in the water maze

Gustavo da Costa Ferreira [a], Patrícia Fernanda Schuck [a], Carolina Maso Viegas [a], Anelise Tonin [a], César Augusto João Ribeiro [a], Leticia Ferreira Pettenuzzo [a], Lenir Orlandi Pereira [a], Carlos Alexandre Netto [a], Moacir Wajner [a,b,c,*]

[a] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, 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 30 July 2007; received in revised form 1 October 2007; accepted 7 October 2007
Available online 11 October 2007

Abstract

Glutaric acidemia type I (GA I) is an autosomal recessive metabolic disorder caused by glutaryl-CoA dehydrogenase deficiency leading to predominant accumulation of glutaric acid (GA), and to a lesser extent of 3-hydroxyglutaric acid (3HG) in body fluids and tissues. The clinical manifestations of GA I are predominantly neurological. Although the pathophysiological mechanisms responsible for the brain damage of this disease are virtually unknown, they are thought to be due to the neurotoxic actions of GA and 3HG. Therefore, in the present work we investigated whether chronic exposure of GA (5 μmol g of body weight^{-1}, twice per day), the major metabolite accumulating in GA I, during early development (from the 5th to the 28th day of life) could alter the cognitive performance of adult rats in the Morris water maze, open field and elevated plus maze tasks. Control rats were treated with saline in the same volumes. GA administration provoked an impairment of spatial performance in the water maze since adult rats pretreated with GA were not able to remember the previous location of the platform spending significantly less time in the training quadrant. In contrast, GA chronic administration did not affect rat performance in the open field and elevated plus maze tasks, indicating that motor activity and anxiety was not changed by GA. The results provide evidence that early chronic GA treatment induces long-lasting spatial behavioral deficit.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Glutaric acidemia type I; Glutaric acid; Memory; Water maze; Rat

1. Introduction

Glutaric acidemia type I (GA I, McKusick 23167; OMIM #231670) is an autosomal recessive disease caused by deficiency of the activity of the mitochondrial enzyme glutaryl-CoA dehydrogenase (EC 1.3.99.7), which is involved in the catabolic pathway of lysine, hydroxylysine and tryptophan [1]. Increased concentrations of glutaric acid (GA), as well as 3-hydroxyglutaric (3HG), but in lesser amounts, are found in the body fluids and brain tissue of GA I patients [2,3]. Clinical manifestations of GA I are predominantly neurological, occurring especially after encephalopathic crises, which are accompanied by bilateral destruction of caudate and putamen with severe loss of medium-sized GABAergic neurons [3,4]. Fronto-operculo-temporal hypoplasia frequently detected at birth is a distinctive radiological appearance that may be pathognomonic for GA I. Over subsequent years, progressive involvement of the cortical white matter with vacuolization of cerebral structures (cerebral atrophy) occurs leading to cognitive impairment [5]. Although the neuropathological findings are pronounced in this disease, the mechanisms underlying the acute and chronic progressive brain damage of GA I are not completely defined to date. However, various *in vivo* and *in vitro* studies have shown neurotoxic effects of GA and 3HG, including excitotoxicity [6–10],

* Corresponding author at: Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Tel.: +55 51 3308 5571; fax: +55 51 3308 5539.
E-mail address: mwajner@ufrgs.br (M. Wajner).

0166-4328/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbr.2007.10.003

oxidative damage [11–15] and disruption of energy metabolism [16–21].

We have recently induced in our laboratory high sustained brain GA concentrations similar to those found in GA I patients by injecting GA subcutaneously to developing rats [20]. In the present study, we tested this chemically-induced animal model during early development (5th to the 28th day of life) on the cognitive performance of adult rats in various behavioral tasks in order to test whether chronic GA administration to infantile rats could provoke long-standing or permanent brain damage involving cerebral structures involved in learning/memory.

2. Material and methods

2.1. Chemicals

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

2.2. Subjects

A total of 16 male Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS, were used. Pregnant rats were housed in individual cages and left undisturbed during gestation. Forty-eight hours after delivery, litters were culled to eight male pups; rats were weaned at 21 days of life. The animals were divided so that in each cage there was the same number of rats for each treatment. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature ($22 \pm 1\,^{\circ}C$) colony room. The "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

2.3. In vivo treatment

Saline-buffered GA, pH 7.4 (5 μmol g of body weight^{-1}) was administered subcutaneously, twice a day, from the 5th to the 28th day of life to produce brain concentrations of GA of around 0.6 μmol g^{-1}, ~0.72 mM [20]. Control animals received saline subcutaneously in the same volumes and frequency. All solutions were prepared so that each animal received 10 μL solution. g of body weight^{-1}.

2.4. Physical development

Sixteen male rats were used for the neurobehavioral development studies. Maturation of physical characteristics was determined daily at the appropriate ages by one experimenter that was not aware of the subject condition. Litters were inspected between 12:00 and 15:00 h, and progress of physical development was followed throughout the experiment. The date of appearance of hair, eruption of upper incisors, and eye opening was recorded using previously reported criteria [22,23] described in detail by Smart and Dobbing [24].

2.5. Free-fall righting task

On postnatal day 14, animal ability to turn in midair to land on all fours after being dropped back downwards from 35 cm onto a cotton wool pad was measured. Each animal was tested in three consecutive trials spaced 15 s apart, and scored one point if it landed ventrally, with all legs distant from the body in each trial [22]. Therefore, a maximum of three points could be assigned to each animal.

2.6. Cognitive tasks

All behavioral tests were performed between 14:00 and 17:00 h. Each experimental group was tested in the different cognitive tasks with a two-day interval in between. Animals were then tested in the elevated plus maze on the 60th and 61st postnatal day, and on the open field task on postnatal days 64 and 65. Finally, behavior of rats in the Morris water maze was assessed from the 68th to the 77th postnatal day.

2.7. Morris water maze task

The experimental procedure was carried out as previously described [25]. Briefly, the animals were left to recover for approximately one month after GA treatment. Spatial learning/memory was tested in the Morris water maze [26], which consisted of a black circular pool (200 cm in diameter, 100 cm high), theoretically divided into four equal quadrants for the purpose of analysis. The pool was filled to a depth of 50 cm with water ($23 \pm 1\,^{\circ}C$) made opaque by the addition of milk. The escape platform was transparent, had a diameter of 10 cm and was placed 2 cm below the water surface. The experimenter remained at the same location on each trial, corresponding to the adjacent target quadrant, approximately 50 cm from the outside edge of the tank, on each trial. A video camera was mounted above the center of the tank and all trials were recorded. The room was dimly illuminated in order to provide extra-maze clues so allowing rats to develop a spatial map strategy. Two black and white large cartoons were hanged on the walls.

2.7.1. Reference memory test

Rats had daily sessions of 4 trials per day for 5 days to find the submerged platform that was located in the center of a quadrant of the tank and remained there throughout training. We observed that all animals of each group were able to swim in a normal way during all trials. On each trial the rat was placed in the water, facing the edge of the tank, in one of the four standard start locations (N, S, W and E). The order of the start locations was varied in a quasi-random sequence so that, for each block of four trials, any given sequence was not repeated on consecutive days. The rat was then allowed 60 s to search for the platform. Latency to find the platform (escape latency) and swimming speed were measured in each trial. Once the rat located the platform, it was permitted to remain on it for 10 s. If it did not find the platform within this time, it was guided to it and allowed to remain on it for 10 s. After each trial, the rats were removed, dried in a towel and put back in their home cages. The interval between trials was 15–20 min [27].

2.7.2. Probe trial

One day after the last training session, each rat was subjected to a probe trial (60 s) in which there was no platform present. The time spent in the quadrant of the former platform position, the time spent in the opposite quadrant, the correct annulus crossing, i.e., the number of times animals passed through the circular area that formerly contained the submerged platform during acquisition, and the latency to cross over the platform place for the first time were taken as a measure for spatial memory.

2.7.3. Working memory (repeated acquisition) test

Working memory test was tested a week after the probe test, consisting of four trials per day during four consecutive days. The working memory test was procedurally similar to reference memory test except that the platform location was changed daily. The first trial of the day was an informative sample trial in which the rat was allowed to swim to the platform in its new location. Spatial working memory was regarded as the mean escape latency of the second until the fourth trial.

2.8. Open field task

The apparatus consisted of a wooden box measuring 60 cm × 40 cm × 50 cm with a glass front wall, whose floor was divided by black lines into 12 equal squares. The animals were gently placed facing the rear left corner of the arena and the number of squares crossed with the four paws recorded for 5 min to eval-

uate motor activity [28]. The testing room was dimly illuminated with indirect white lighting.

2.9. Elevated plus maze task

Based on the design of Lister [29], the maze consisted of two opposite closed arms (30 cm × 5 cm) enclosed with walls (15 cm in height) and two opposite open arms (also 30 cm × 5 cm, without edges), forming a plus shape. The whole apparatus had a central arena (5 cm × 5 cm) and was elevated to 80 cm above the floor by a tripod. Each rat was placed in the central arena of the maze facing an open arm, and observed for 5 min. The number of entries into the open arms and the time spent in the open and in the enclosed arms were measured during two trail sessions. The maze was cleaned with water after each rat performance. The testing room was illuminated only by a dim red light (25 W).

2.10. Statistical analysis

The evaluation of reference and working memory in the water maze and anxiety in the elevated plus maze was carried out by two-way ANOVA, considering the factors: GA pretreatment × days of training. Data of probe trial and open field were analyzed by the Student's *t*-test. Values of $P < 0.05$ were considered significant. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

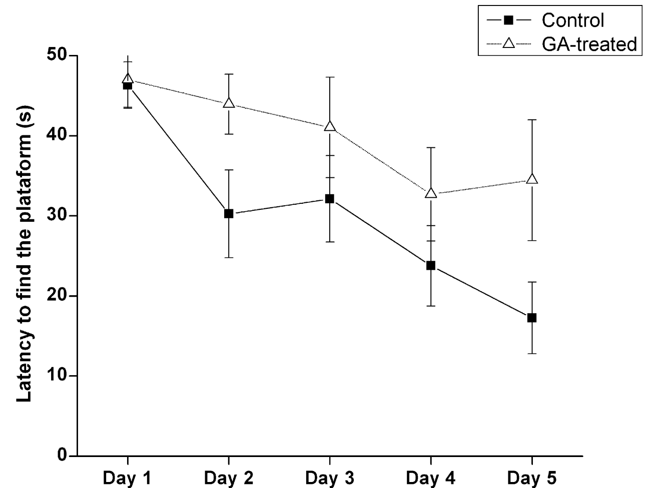

Fig. 1. Effect of early postnatal chronic glutaric acid (GA) administration on acquisition learning in the Morris water maze task. Data represent means ± S.E.M. of latency to find the platform across blocks of four trials on each day for eight animals per group (control and GA). No significant differences between groups were detected (two-way ANOVA).

3. Results

We initially observed that approximately one fifth of rats treated with GA had convulsions immediately after the injections. These animals were discarded from our study. This is in agreement with previous findings showing that GA intrastriatal administration caused seizures in adult rats [7,30]. On the other hand, body weight of rats submitted to chronic GA-treatment was similar to that of control rats (data not shown) indicating that chronic postnatal administration of GA does not alter their appetite or provoke malnutrition.

We also assessed the effect of chronic GA administration on the date of appearance of certain physical landmarks and reflexes. In this context, we did not observe any delay in the date of appearance of coat, eye opening and upper incisor eruption, neither impairment of the free-fall righting task GA treated rats (data not shown).

As regards to rat behavior in the Morris water maze task, all groups of animals improved the acquisition performance, i.e., decreased the latency to find the platform from the first to the last day of training (day 5) [$F(4,52) = 7.92$; $P < 0.001$] (Fig. 1). However, it can be seen that GA-treated animals presented a higher but not significant latency to find the platform than saline-treated rats along time [$F(4,52) = 0.30$; $P > 0.05$]. Furthermore, on the probe trial, with the platform removed,

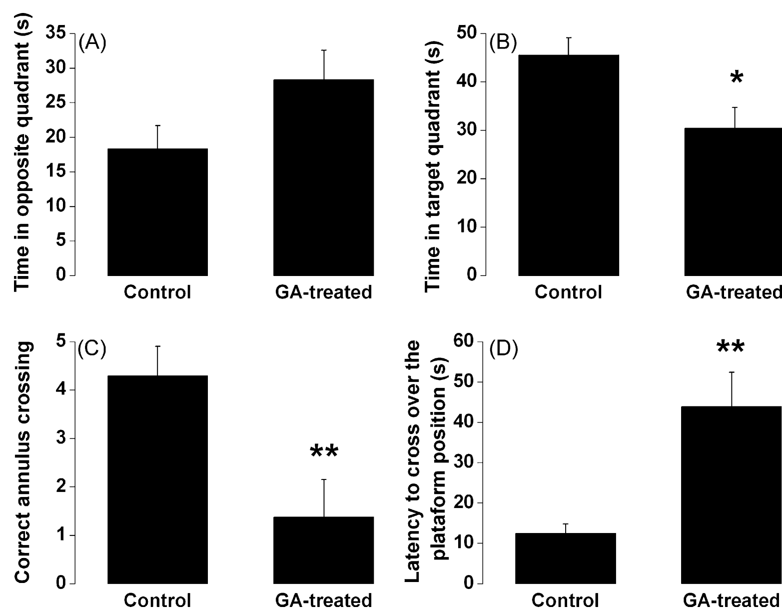

Fig. 2. Effect of early postnatal chronic glutaric acid (GA) administration on probe trial. Time spent in the training quadrant (A); time spent in the opposite quadrant (B); correct annulus crossings (C) and latency to cross over the platform position (D). Data represent means ± S.E.M. for eight animals per group (control and GA). Results in (A) and (B) represent percentage of total time. $^{*}P < 0.05$; $^{**}P < 0.01$ compared to control group (Student's *t*-test for independent samples).

Table 1
Effect of early postnatal chronic glutaric acid (GA) administration on rat performance in the open field task

	Trial 1		Trial 2	
	Saline	GA	Saline	GA
Number of crossings	95.1 ± 15.1	91.51 ± 12.0	76.1 ± 40.9	74.2 ± 41.8
Number of fecal *bolli*	1.14 ± 0.99	3.00 ± 1.45	1.28 ± 1.97	1.62 ± 2.56
Number of rearings	38.9 ± 7.07	33.5 ± 15.0	26.7 ± 15.4	25.5 ± 14.8
Latency to leave the first square	3.29 ± 0.75	2.50 ± 0.42	2.71 ± 1.60	2.00 ± 0.53

Data represent means ± S.E.M. for eight animals per group. Latency to leave the first square is expressed as seconds. No significant differences between groups were detected (Student's *t*-test for independent samples).

GA-treated rats were not able to remember the location of the platform, spending significantly less time in the training quadrant (GA-treated: 30.4% ± 12.2) than the saline-injected animals (controls: 45.5% ± 9.5) [$t(15) = 2.64$, $P < 0.05$] and apparently more time in the opposite quadrant (GA-treated: 28.3% ± 12.1; controls:18% ± 8.9) [$t(15) = 1.84$, $P = 0.089$]. GA-treated rats also had a lower number of correct annulus crossings than the control group (GA-treated: 1.37 ± 2.19; controls: 4.3 ± 1.6) [$t(15) = 2.89$, $P < 0.05$] and presented a higher latency to cross over the platform position (GA: 43.8 ± 8.46 s; controls: 12.4 ± 2.40 s) [$t(15) = -3.36$; $P < 0.01$] (Fig. 2).

We also verified that rats submitted to the water maze task presented no motor deficits, as assessed by swimming speed (GA-treated: 19.4 ± 2.97 cm s^{-1}; controls: 18.2 ± 2.88 cm s^{-1}) [$t(15) = 0.46$; $P > 0.05$].

On the other hand we observed that GA treatment did not alter the number of crossings [$t(15) = 0.19$; $P > 0.05$], and fecal *bolli* [$t(15) = 1.03$; $P > 0.05$], neither the latency to leave the first square [$t(15) = 0.95$; $P > 0.05$]) at training in the open field task, when compared to the control group (Table 1). However, both groups decreased numbers of rearings [$F(1,14) = 21.8$; $P < 0.001$] and crossings [$F(1,14) = 9.72$; $P < 0.001$] at the testing session.

Fig. 3 provides a cartoon with the swim paths taken by a representative animal from each group in the probe trial. The paths taken by controls were largely confined to the latter training quadrant, whereas the paths taken by GA-treated rats appeared as circular patterns that were more equally distributed within the quadrants where the platform was in the acquisition phase.

Fig. 4 shows that all experimental groups improved water maze performance in the working memory evaluation, i.e., the latency to find the platform decreased from the first to the last trial of each training day [$F(4,52) = 6.65$; $P < 0.05$], and there was no difference between saline- and GA-treated rats [$F(4,52) = 1.09$; $P > 0.05$].

Finally, we submitted saline- and GA-treated rats to the elevated plus maze task. Table 2 shows that GA treatment did not alter rat performance in the plus maze task since there was no difference between groups in the number of entries [$t(15) = 0.96$; $P > 0.05$] and time spent into the open arms [$t(15) = 0.01$; $P > 0.05$] and in the number of entries into the enclosed arms [$t(15) = 0.70$; $P > 0.05$].

4. Discussion

To our knowledge practically nothing has been done to investigate whether GA, the most pronounced metabolite accumulating in GA I, could provoke deficit of performance in behavioral tasks. Therefore, in the present study, we produced high sustained levels of GA in the brain (0.72 mM) of developing rats similar to those found in human GA I by using a previously described chemically induced model [20]. This model does not exactly mimic human GA I, in which, besides GA, other metabolites accumulate in lesser amounts. However, it reproduces the main biochemical feature of this disorder, which is

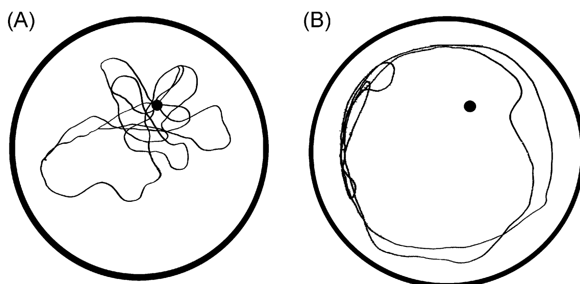

Fig. 3. Swim paths taken by a representative rat of each group with respect to time spent in the former location of the platform during the probe trial: saline-treated rats (A) and glutaric acid-treated rats (B).

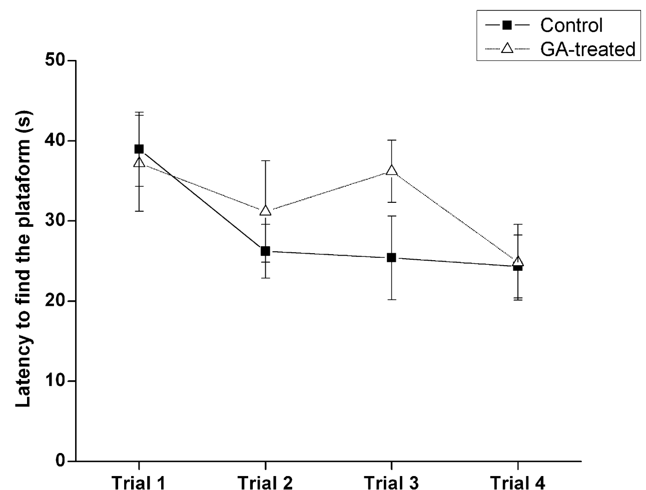

Fig. 4. Effect of early postnatal chronic glutaric acid (GA) administration on working memory in the Morris water maze task. Data represent means ± S.E.M. of latency to find the platform across blocks of four trials per day for eight animals per group (control and GA). No significant difference between groups was detected (two-way ANOVA).

Table 2
Effect of early postnatal chronic glutaric acid (GA) administration on rat performance in the elevated plus maze task

	Trial 1		Trial 2	
	Saline	GA	Saline	GA
Open arm entries	3.42 ± 0.61	4.67 ± 1.20	2.57 ± 0.87	4.17 ± 1.72
Enclosed arm entries	6.87 ± 0.74	8.33 ± 0.95	8.43 ± 1.39	8.50 ± 1.18
Time in open arm[a]	57.9 ± 12.2	53.5 ± 12.2	43.0 ± 15.5	36.0 ± 16.9

Data represent means ± S.E.M. for eight animals per group.
[a] Time in open arm is expressed as percentage of total time. No significant differences between groups were detected (two-way ANOVA).

high sustained tissue levels of GA. GA was administered during a period (5th to 28th day of life) of great cellular proliferation and synaptogenesis in various cerebral structures involved in learning/memory in rats [31–34]. The animals were allowed to recover for 30–45 days, and, thereafter, their behavioral performance was tested in the Morris water maze, elevated plus maze and open field tasks.

We first observed that chronic administration had no effect on body weight, implying that chronic GA injection did not cause malnutrition in the animals. This observation is important since malnourished animals may behave differently in neurobehavioral tests [24,35] that so this undesirable effect can be ruled out as for the interpretation of behavioral alterations observed in GA chronically treated rats. Moreover, chronic GA administration did not change the date of appearance of coat, eye opening or upper incisor eruption, nor the free-fall righting task, indicating that the physical and motor development was not substantially modified by high sustained levels of GA.

As regards to rat behavior, we observed that saline- and GA-injected rats improved water maze acquisition performance, i.e., decreased the latency to find the platform from the first to the last day of training. However, we verified that GA-treated animals presented a clear trend to higher latency to find the platform during the training in the Morris water maze when compared to controls. Furthermore, rats receiving chronic GA administration stayed for a significantly shorter time in the quadrant where the platform was formerly located and spent significantly more time in the opposite quadrant as compared to controls. GA-treated rats also had a lower number of correct annulus crossings and presented a higher latency to cross over the platform position than saline-treated animals. These data suggest that early chronic postnatal administration of GA caused a long-standing deficit in spatial learning probably secondary to GA-induced brain damage.

It should be emphasized that the deficit in spatial navigation shown in GA-treated rats cannot be attributed to a decreased motor activity since the number of crossings responses (open field) and the swimming speed (water maze) revealed that both groups of animals had the same locomotor activity. Furthermore, by looking at the paths that the animal groups took, we observed that GA-treated animals spent similar time in the four quadrants of the swimming pool and presented a circular path, suggesting that these rats used a circular pattern as strategy in order to find the platform.

Therefore, it can be concluded that early chronic postnatal administration of GA to rats caused a deficit of adult rat performance in the water maze task, indicating a deficit in spatial learning. In contrast, working memory was not affected in GA-injected rats. Similarly, rat behavior in the elevated plus maze task was not changed by GA, indicating that GA-treatment was not anxiogenic. On the other hand, GA-treatment did not change rat performance in the open field task, as observed by the significant decrease of number of rearings and crossings at the testing session.

We therefore demonstrated here that GA impaired the reference memory in the water maze task without affecting working memory. These data support the view that different mechanisms are probably involved in the acquisition of these memories. In this scenario, the reference memory task is basically processed in the hippocampus [36], while the working memory establishment depends mainly on the striatum and prefrontal cortex [37]. Taken together, it may be presumed that hippocampus could be damaged by GA early post-natal administration.

The exact mechanisms through which GA impairs rat learning/memory are still unknown. However, it is possible that the brain GA concentrations achieved in our chemical model (0.72 mM) may be high enough to induce brain damage during a phase of rapid CNS maturation and particularly in cerebral structures involved in learning, memory and interaction with the environment. In this context, it should be stressed that oxidative stress is associated with memory deficits [38–40], energetic substrates such as creatine and succinate are capable to prevent learning/memory deficit [41] and that excitotoxicity may impair learning/memory [42]. Since GA has been shown to elicit oxidative stress [15,43], excitotoxicity [7,9], and compromise brain energy metabolism [16–21], it could be presumed that GA may act in one or more of these processes, leading to learning/memory impairment.

In conclusion, the present study shows for the first time that high sustained brain concentrations of GA during early development compromise biochemical pathways involved in learning/memory processes. Even though it is difficult to extrapolate our findings to the human condition, the results of the present study indicate that GA may lead to learning/memory deficits.

Acknowledgements

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

References

[1] Goodman SI, Frerman FE. Organic acidemias due to defects in lysine oxidation: 2-ketoadipic academia and glutaric acidemia. In: Scriver CR, Beaudet AL, Sly WS, Walle D, editors. The metabolic and molecular bases of inherited disease. 8th ed. New York: McGraw-Hill; 2001. p. 2195–204.

[2] Goodman SI, Norenberg MD, Shikes RH, Breslich DJ, Moe PG. Glutaric aciduria: biochemical and morphologic considerations. J Pediatr 1977;90:746–50.

[3] Funk CB, Prasad AN, Frosk P, Sauer S, Kölker S, Greenberg CR, et al. Neuropathological, biochemical and molecular findings in a glutaric academia type 1 cohort. Brain 2005;128:711–22.

[4] Hoffmann GF, Zschocke J. Glutaric aciduria type I: from clinical, biochemical and molecular diversity to successful therapy. J Inherit Metab Dis 1999;22:381–91.

[5] Strauss KA, Morton DH. Type I glutaric aciduria. Part 2. A model of acute striatal necrosis. Am J Med Genet 2003;121:53–70.

[6] Flott-Rahmel B, Falter C, Schluff P, Fingerhut R, Christensen E, Jakobs C, et al. Nerve cell lesions caused by 3-hydroxyglutaric acid: a possible mechanism for neurodegeneration in glutaric acidaemia I. J Inherit Metab Dis 1997;20:387–90.

[7] Lima TT, Begnini J, de Bastiani J, Fialho DB, Jurach A, Ribeiro MC, et al. Pharmacological evidence for GABAergic and glutamatergic involvement in the convulsant and behavioral effects of glutaric acid. Brain Res 1998;802:55–60.

[8] Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF. 3-Hydroxyglutaric and glutaric acids are neurotoxic through NMDA receptors in vitro. J Inherit Metab Dis 1999;22:259–62.

[9] Wajner M, Kolker S, Souza DO, Hoffman GF, de Mello CF. Modulation of glutamatergic and GABAergic neurotransmission in glutaryl-CoA dehydrogenase deficiency. J Inherit Metab Dis 2004;27(6):825–8.

[10] Rosa RB, Schwarzbold C, Dalcin KB, Ghisleni GC, Ribeiro CA, Moretto MB, et al. Evidence that 3-hydroxyglutaric acid interacts with NMDA receptors in synaptic plasma membranes from cerebral cortex of young rats. Neurochem Int 2004;45:1087–94.

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

[12] Kölker S, Ahlemeyer B, Huhne R, Mayatepek E, Krieglstein J, Hoffmann GF. Potentiation of 3-hydroxyglutarate neurotoxicity following induction of astrocytic iNOS in neonatal rat hippocampal cultures. Eur J Neurosci 2001;13:2115–22.

[13] Latini A, Borba Rosa R, 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 2002;956:367–73.

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

[15] de Oliveira Marques F, Hagen ME, Pederzolli CD, Sgaravatti AM, Durigon K, Testa CG, et al. Glutaric acid induces oxidative stress in brain of young rats. Brain Res 2003;964:153–8.

[16] Ullrich K, Flott-Rahmel B, Schluff P, Musshoff U, Das A, Lucke T, et al. Glutaric aciduria type I: pathomechanisms of neurodegeneration. J Inherit Metab Dis 1999;22:392–403.

[17] Silva CG, Silva AR, Ruschel C, Helegda C, Wyse AT, Wannmacher CM, et al. Inhibition of energy production in vitro by glutaric acid in cerebral cortex of young rats. Metab Brain Dis 2000;15:123–31.

[18] Das AM, Lucke 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 2003;78:108–11.

[19] Latini A, Rodriguez M, Rosa RB, Scussiato K, Leipnitz G, Reis de Assis D, et al. In vitro effect of 3-hydroxyglutaric acid on energy metabolism in brain of young rats. Neuroscience 2005;135:111–20.

[20] Ferreira GC, Viegas CM, Schuck PF, Tonin A, Ribeiro CAJ, Coelho DM, et al. Glutaric acid administration impairs energy metabolism in midbrain and skeletal muscle of young rats. Neurochem Res 2005;30(9): 1123–31.

[21] da C, Ferreira G, Viegas CM, Schuck PF, Latini A, Dutra-Filho CS, et al. Glutaric acid moderately compromises energy metabolism in rat brain. Int J Dev Neurosci 2005;23:687–93.

[22] Da Silva VA, Malheiros LR, Bueno FMR. Effect of toluene exposure during gestation on neurobehavioral development of rats and hamsters. Braz J Med Biol Res 1990;23:533–7.

[23] Mello CF, Somer JE, Tavaroni V, Graciolli R, Wu V, Torres G, et al. Effects of postnatal methylmalonate administration on neurobehavioral development of rats. Braz J Med Biol Res 1994;27(3):655–61.

[24] Smart JL, Dobbing J. Vulnerability of developing brain. II. Effect of early nutritional deprivation on reflex ontogeny and development of behavior in the rat. Brain Res 1971;28:85–95.

[25] Pettenuzzo LF, Schuck PF, Fontella F, Wannmacher CMD, Wyse AT, Dutra-Filho CS, et al. Ascorbic acid prevents cognitive deficits caused by chronic administration of propionic acid to rats in the water maze. Pharmacol Biochem Behav 2002;73:623–9.

[26] Morris RGM, Garrud P, Rawlins JNP, O'Keefe J. Place navigation impaired in rats with hippocampal lesions. Nature 1982;297:681–3.

[27] Warren SG, Juraska JM. Sex differences and estropausal phase effects on water maze performance in aged rats. Neurol Learn Mem 2000;74:229–40.

[28] Walsh RN, Cummins RA. The open-field test: a critical review. Psychol Bull 1976;83:482–504.

[29] Lister RG. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 1987;92(2):180–5.

[30] Fighera MR, Royes LF, Furian AF, Oliveira MS, Fiorenza NG, Frussa-Filho R, et al. GM1 ganglioside prevents seizures, Na^+,K^+-ATPase activity inhibition and oxidative stress induced by glutaric acid and pentylenetetrazole. Neurobiol Dis 2006;22(3):611–23.

[31] Winick M, Noble A. Quantitative changes in DNA, RNA and protein during prenatal and postnatal growth in rat. Dev Biol 1965;12:451–66.

[32] Roisen FJ, Bartfeld H, Nagele R, Yorke G. Gangliosides stimulation of axonal sprouting in vivo. Science 1981;214:577–8.

[33] Dreyfus H, Ferret B, Harth S, Giorgio A, Durand M, Freysz L, et al. Metabolism and function of gangliosides in developing neurons. J Neurosci Res 1984;12:311–22.

[34] Dutra JC, Wajner M, Wannmacher CMD, Dutra-Filho CS, Rache E. Inhibition of succinate dehydrogenase and beta-hydroxybutyrate dehydrogenase activities by methylmalonate in brain and liver of developing rats. J Inherited Metab Dis 1993;16:147–53.

[35] Davis PH, Squire LR. Protein synthesis and memory: a review. Psychol Bull 1984;96:518–59.

[36] Arteni NS, Salgueiro J, Torres I, Achaval M, Netto CA. Neonatal cerebral hypoxia–ischemia causes lateralized memory impairments in the adult rat. Brain Res 2003;973:171–8.

[37] Goldman-Rakic PS. The 'psychic' neuron of the cerebral cortex. Ann N Y Acad Sci 1999;868:13–26.

[38] Bickford PC, Shukitt-Hale B, Joseph J. Effects of aging on cerebellar noradrenergic function and motor learning: nutritional interventions. Mech Ageing Dev 1999;111:141–54.

[39] Serrano F, Klann E. Reactive oxygen species and synaptic plasticity in the aging hippocampus. Aging Res Ver 2004;3(4):431–43.

[40] da Silva AL, Piato AL, Bardini S, Netto CA, Nunes DS, Elisabetsky E. Memory retrieval improvement by *Ptychopetalum olacoides* in young and aging mice. J Ethnopharmacol 2004;95(2–3):199–203.

[41] Vasques VC, Brinco F, Wajner M. Intrahippocampal administration of the branched-chain alpha-hydroxy acids accumulating in maple syrup urine disease compromises rat performance in aversive and non-aversive behavioral tasks. J Neurol Sci 2005;232(1–2):11–21.

[42] Leke R, Oliveira DL, Schmidt AP, Avila TT, Jorge RS, Fisher A, et al. Methotrexate induces seizure and decreases glutamate uptake in brain slices: prevention by ionotropic glutamate receptors antagonists and adenosine. Life Sci 2006;80(1):1–8.

[43] Latini A, Ferreira GC, Scussiato K, Schuck PF, Solano AF, Dutra-Filho CS, et al. Induction of oxidative stress by chronic and acute glutaric Acid administration to rats. Cell Mol Neurobiol 2007;27(4):4234–8.

Capítulo II

Energy metabolism is compromised in skeletal muscle of rats chronically-treated with glutaric acid

Gustavo da C. Ferreira, Patrícia F. Schuck, Carolina M. Viegas, Anelise Tonin, Alexandra Latini, Carlos S. Dutra-Filho, Angela T.S. Wyse, Clóvis M.D. Wannmacher, Carmen R. Vargas, MoacirWajner

Artigo científico publicado no periódico

Metabolic Brain Disease 22:111–123, 2007

Metab Brain Dis (2007) 22:111–123
DOI 10.1007/s11011-006-9043-0

ORIGINAL PAPER

Energy Metabolism is Compromised in Skeletal Muscle of Rats Chronically-Treated with Glutaric Acid

Gustavo da C. Ferreira · Patrícia F. Schuck · Carolina M. Viegas · Anelise Tonin · Alexandra Latini · Carlos S. Dutra-Filho · Angela T. S. Wyse · Clóvis M. D. Wannmacher · Carmen R. Vargas · Moacir Wajner

Received: 19 June 2006 / Accepted: 28 November 2006 /
Published online: 13 January 2007
© Springer Science+Business Media, LLC 2007

Abstract Glutaric acidemia type I (GA I) (GA I, McKusick 23167; OMIM # 231670) is an autosomal recessive metabolic disorder caused by glutaryl-CoA dehydrogenase deficiency (EC 1.3.99.7). Clinically, the disease is characterized by macrocephaly, hypotonia, dystonia and diskinesia. Since the pathophysiology of this disorder is not yet well established, in the present investigation we determined a number of energy metabolism parameters, namely $^{14}CO_2$ production, the activities of the respiratory chain complexes I–IV and of creatine kinase, in tissues of rats chronically exposed to glutaric acid (GA). High tissue GA concentrations (0.6 mM in the brain, 4 mM in skeletal muscle and 6 mM in plasma) were induced by three daily subcutaneous injections of saline-buffered GA (5 μmol $\cdot$ g^{-1} body weight) to Wistar rats from the 5th to the 21st day of life. The parameters were assessed 12 h after the last GA injection in cerebral cortex and middle brain, as well as in skeletal muscle homogenates of GA-treated rats. GA administration significantly inhibited the activities of the respiratory chain complexes I-III and II and induced a significant increase of complex IV activity in skeletal muscle of rats. Furthermore, creatine kinase activity was also inhibited by GA treatment in skeletal muscle. In contrast, these measurements were not altered by GA administration in the brain structures studied. Taken together, it was demonstrated that chronic GA administration induced an impairment of energy metabolism in rat skeletal

G. C. Ferreira · P. F. Schuck · C. M. Viegas · A. Tonin · A. Latini · C. S. Dutra-Filho · A. T. S. Wyse · C. M. D. Wannmacher · C. R. Vargas · M. Wajner (✉)
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600, Anexo, Porto Alegre, RS, Brazil
e-mail: mwajner@ufrgs.br

A. Latini · C. R. Vargas · M. Wajner
Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

C. R. Vargas
Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

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

muscle probably due to a higher tissue concentration of this organic acid that may be possibly associated to the muscle weakness occurring in glutaric acidemic patients.

Keywords Glutaric acid · Energy metabolism · Respiratory chain · Creatine kinase · Skeletal muscle

Introduction

Glutaric acidemia type I (GA I) is an autosomal recessive metabolic disorder caused by the deficiency of the enzyme glutaryl-CoA dehydrogenase (GCDH) (Goodman et al., 1975). Glutaric acid (GA) is the major metabolite accumulated in the body fluids and in brain tissue of GA I patients, but 3-hydroxyglutaric (3HGA) and *trans*-glutaconic (*t*GA) acids are also found at lower concentrations (Goodman and Frerman, 2001; Strauss and Morton, 2003). Clinically, the disease is characterized by macrocephaly at birth and by a marked dystonia and diskinesia, sometimes associated to extreme hypotonia. The onset of symptoms may be gradual or occur suddenly after an acute metabolic crisis with encephalopathy, when the accumulating metabolites can reach millimolar concentrations (Goodman et al., 1977; Hoffmann and Zchoscke, 1999; Kölker et al., 2006).

The mechanisms leading to tissue damage in GA I are not fully established. However, accumulating evidence from several *in vitro* and *in vivo* animal model studies suggested that excitotoxicity, oxidative stress and energy impairment are possibly involved in the brain abnormalities of these patients (Flott-Rahmel et al., 1997; Lima et al., 1998; Kölker et al., 1999, 2001a,b, 2002; Ullrich et al., 1999; Porciuncula et al., 2000; Bjugstad et al., 2001; de Mello et al., 2001; Latini et al., 2002, 2005; de Oliveira et al., 2003; Frizzo et al., 2004; Rosa et al., 2004). However, excitotoxicity as a major underlying mechanism of brain injury in GA I was recently disputed (Silva et al., 2000; Bjugstad et al., 2001; Kölker et al., 2002; Das et al., 2003; Freudenberg et al., 2004; Lund et al., 2004; Sauer et al., 2005; da C. Ferreira et al., 2005; Ferreira et al., 2005).

On the other hand, a number of GA I patients excrete increased concentrations of lactate, 3-hydroxybutyrate, acetoacetate and dicarboxylic acids, possibly reflecting a mitochondrial dysfunction (Gregersen and Brandt, 1979; Floret et al., 1979). In this scenario, it has been recently postulated that the nature of the striatal damage in GA I is similar to the brain injury that occurs in infants after hypoxia-ischemia or systemic intoxication with 3-nitropropionic acid (Brouillet et al., 1998), in which complex II activity is irreversible blocked (Strauss and Morton, 2003).

We have recently reported that acute administration of GA, the major metabolite accumulating in GA I, to developing rats induced brain concentrations similar to those found in brain of GA I patients (Ferreira et al., 2005). Although, we have demonstrated a moderate inhibition of complex I–III activity of the respiratory chain in middle brain and of complexes I–III and II–III in skeletal muscle, this acute model does not give information about the disease course under GA chronic exposure, indicating that a more representative model of this disorder is needed to clarify the pathogenesis of GA I.

In the present investigation we report the effects of chronic subcutaneous administration of GA to developing rats (from the 5th to the 21st day of post-natal life) on critical enzyme activities of energy metabolism, including those of the respiratory chain complexes I–IV and of creatine kinase (CK), as well as on $^{14}CO_2$ production from acetate in cerebral cortex, middle brain and skeletal muscle of developing rats.

Experimental procedure

Subjects and reagents

Wistar rats from our breeding stock were used. The animals were maintained on a 12-h light/dark cycle (lights on at 7:00) in a constant-temperature colony room (25 ± 2°C) with water and standard lab chow (Supra, Porto Alegre) *ad libitum*. Pregnant rats were housed in individual cages and left undisturbed throughout gestation. Twenty-four hours after delivery all rats were placed together and eight male rats were randomly assigned to each dam. The "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All chemicals were purchased from Sigma Chemical Co (St Louis, USA), except for [1-^{14}C] acetate which was purchased from Amersham International plc, UK.

Chronic glutaric acid (GA) treatment

Wistar rats were subcutaneously administered three times a day with a GA solution (5 μmol $\cdot$ g^{-1} body weight) buffered with NaOH to pH 7.4 from the 5th to the 21st day of life. The administered dose was based on a previous report from our laboratory, which resulted in brain GA concentrations similar to those found in GA I patients (Ferreira et al., 2005). Control group corresponded to animals treated with saline solution at the same volumes.

Tissue preparation

Animals were killed by decapitation 12 h after the last injection (GA concentrations returned to normal undetected levels) and the structures (cerebral cortex, middle brain consisting of thalamus, hypothalamus and striatum and skeletal muscle) were immediately dissected onto a Petri dish placed on ice. The tissues were rapidly homogenized in the specific buffer used for each technique using a ground glass type Potter-Elvejhem homogenizer and the biochemical parameters finally determined. For $^{14}CO_2$ production, rats were sacrificed by decapitation, the brain was rapidly removed and the structures were isolated and homogenized (1:10, w/v) in Krebs-Ringer bicarbonate buffer, pH 7.4. For the determination of the mitochondrial complexes I–III, II, II–III and IV activities, structures were homogenized (1:20, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 UI.mL^{-1} heparin). The homogenates were centrifuged at 800 $\times$ g for 10 min and the supernatants were kept at -70°C until be used for enzyme activity determination. For total CK activity determination, the structures were homogenized (1:1000 w/v) in isosmotic saline solution. The period between tissue preparations and enzyme analysis was always less than 5 days.

Determination of the respiratory chain complex activities

The activities of the respiratory chain enzyme complexes were measured in cerebral cortex, middle brain and skeletal muscle supernatants of 22-day-old rats. Succinate–2,6-dichlorophenolindophenol (DCIP)–oxidoreductase (complex II) and succinate:cytochrome *c* oxidoreductase (complex II–III) activities were determined according to the method of Fischer et al. (1985). The activity of cytochrome *c* oxidase (COX, complex IV) was

measured according to Rustin et al. (1994), whereas that of NADH:cytochrome *c* oxidoreductase (complex I–III) was assayed according to the method described by Schapira et al. (1990). The methods to measure these activities were slightly modified, as described in detail in a previous report (da Silva et al., 2002). Results were expressed as nmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1}.

$^{14}CO_2$ Production

$^{14}CO_2$ production from acetate was evaluated in cerebral cortex, middle brain and skeletal muscle of 22-day-old rats. Homogenates were added to small flasks (11 cm^3) in a volume of 0.45 mL. Flasks were first pre-incubated in a metabolic shaker at 35°C for 15 min (90 oscillations min^{-1}). We then added [1-^{14}C] acetate (0.055 μCi) plus 1.0 mM of unlabeled acetate. The flasks were gassed with an O_2/CO_2 (95:5) mixture and sealed with rubber stoppers and parafilm M. Glass center wells containing a folded 65/5 mm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min of incubation, 0.2 mL of 50% trichloroacetic acid was added to the medium and 0.1 mL of benzethonium hydroxide was supplemented to the center of the wells with needles introduced through the rubber stopper. The flasks were left to stand for a further 30 min to complete $^{14}CO_2$ trapping and then opened. The filter papers were removed and added to vials containing POP-POPOP-toluene scintillation fluid, and radioactivity was counted (da C. Ferreira et al., 2005). Results were expressed as pmol substrate converted to $^{14}CO_2 \cdot h^{-1} \cdot g \cdot tissue^{-1}$.

Creatine kinase (CK) activity

CK activity was measured in total homogenates from tissues of 22-day-old rats as previously described (Schuck et al., 2002). The reaction mixture consisted of the following medium: 60 mM Tris-HCl buffer pH 7.5, containing 7 mM phosphocreatine, 9 mM $MgSO_4$, 0.625 mM *n*-dodecyl-b-D-maltoside and approximately 0.4–1.2 μg protein in a final volume of 0.1 mL. After 15 min of pre-incubation at 37°C, the reaction was started by the addition of 3.2 mM ADP plus 0.8 mM reduced glutathione (GSH). The reaction was stopped after 10 min by the addition of 1 μmol *p*-hydroxymercuribenzoic acid. The creatine formed was measured according to Hughes (1962). The color was developed by the addition of 0.1 mL 20% α-naphthol and 0.1 mL 20% diacetyl in a final volume of 1 mL and read after 20 min at 540 nm. Results were expressed as μmol creatine $\cdot$ min^{-1} $\cdot$ mg protein^{-1}.

Protein determination

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

Statistics

Data are presented as mean $\pm$ S.E.M. and were analyzed by the Student *t*-test for unpaired samples. Only significant values are shown in the text. Values of $p < 0.05$ were considered to be significant. The statistical analysis was performed on an IBM PC-compatible computer using the SPSS (Statistical Package for the Social Sciences) software.

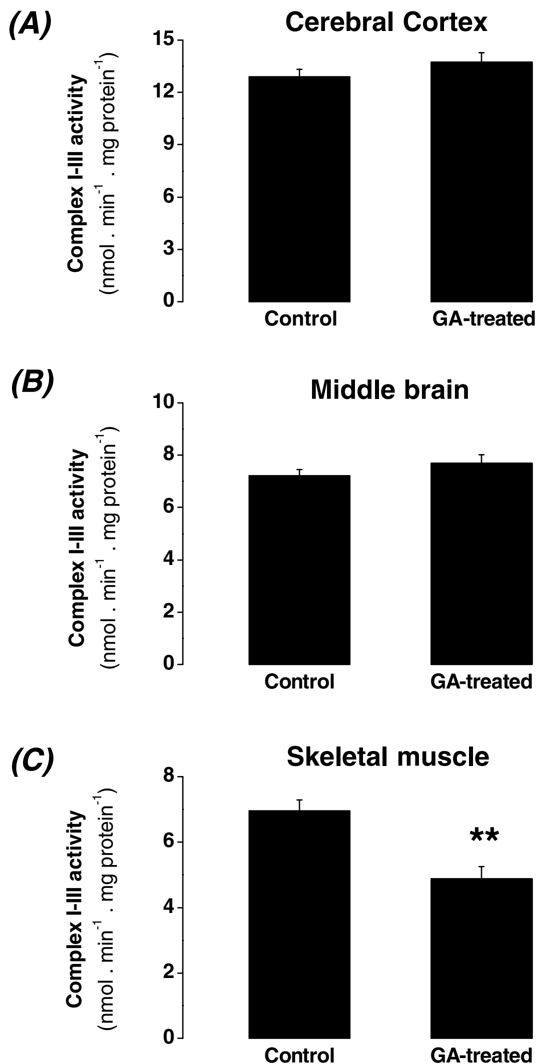

Fig. 1 *In vivo* effect of chronic glutaric acid (GA) administration on the activity of complex I-III of the respiratory chain in cerebral cortex (**A**), middle brain (**B**) and skeletal muscle (**C**) of 22-day-old rats. Data are represented as mean ± S.E.M. for 5–7 experiments (animals) performed in duplicate and expressed as $nmol \cdot min^{-1} \cdot mg^{-1}$ of protein.$^{**}p < 0.01$, compared to controls (Student's *t*-test for unpaired samples)

Results

First, we observed that there were no significant differences between the body weight of GA- and saline-treated animals (data not shown).

We investigated the effect of chronic GA subcutaneous administration on the respiratory chain complexes and CK activities, as well as on $^{14}CO_2$ production, in cerebral cortex, middle brain and skeletal muscle from developing rats. Figures 1 and 2 show that GA administration significantly inhibited the respiratory chain complexes I–III (up to 30%) [$t_{(5)} = 3.559$; $P < 0.01$] and II (up to 20%) [$t_{(5)} = 2.466$; $P < 0.05$] activities in skeletal muscle, but not in the cerebral structures. Furthermore, complex II–III activity was not altered in all tissues examined (data not shown), whereas the activity of complex IV was increased (up to 30%) by GA treatment [$t_{(4)} = -4.867$; $P < 0.05$] only in skeletal muscle (Fig. 3).

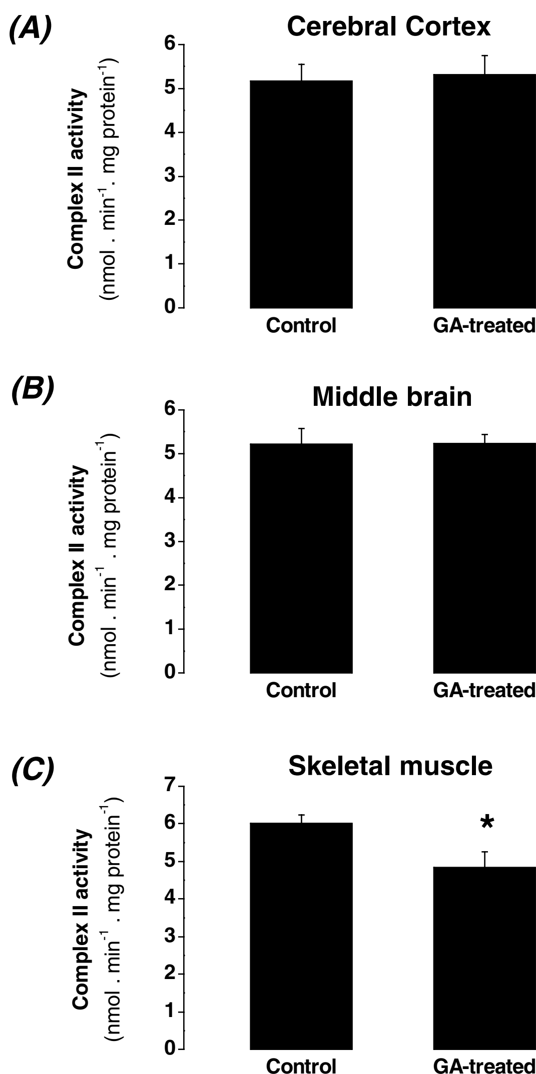

Fig. 2 *In vivo* effect of chronic glutaric acid (GA) administration on the activity of complex II of the respiratory chain in cerebral cortex (**A**), middle brain (**B**) and skeletal muscle (**C**) of 22-day-old rats. Data are represented as mean ± S.E.M. for 5–7 independent experiments (animals) performed in duplicate and expressed as $nmol \cdot min^{-1} \cdot mg^{-1}$ of protein. $^{*}p < 0.05$, compared to controls (Student's *t*-test for unpaired samples)

We also evaluated the influence of chronic GA exposition on the activity of the citric acid cycle by measuring $^{14}CO_2$ production using acetate as substrate. Table 1 shows that acetate utilization was not altered in all structures (cerebral cortex, middle brain and skeletal muscle) studied.

We finally observed that the administration of GA significantly reduced (up to 48%) the activity of CK in skeletal muscle [$t_{(4)} = 2.804$; $P < 0.05$], with no alteration in the cerebral structures (Fig. 4).

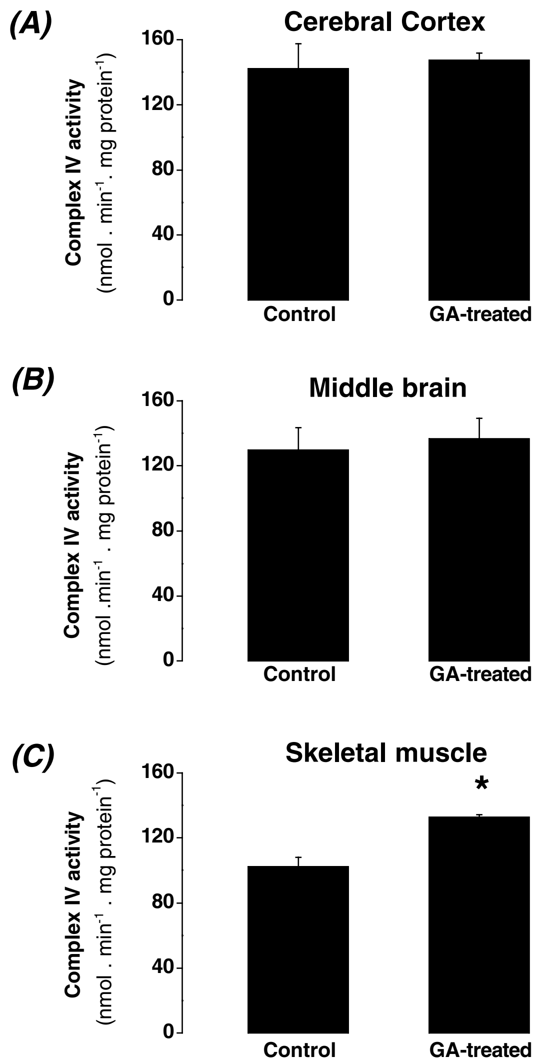

Fig. 3 *In vivo* effect of chronic glutaric acid (GA) administration on the activity of complex IV of the respiratory chain in cerebral cortex (**A**), middle brain (**B**) and skeletal muscle (**C**) of 22-day-old rats. Data are represented as mean ± S.E.M. for 4–7 independent experiments (animals) performed in duplicate and expressed as $nmol \cdot min^{-1} \cdot mg^{-1}$ of protein. $^{*}p < 0.05$, compared to controls (Student's *t*-test for unpaired samples)

Discussion

Although animal models cannot exactly mimic human diseases, they are necessary to investigate the mechanisms of tissue damage in various inborn errors of metabolism. In this scenario, a genetic mouse model of GA I has been developed with a biochemical phenotype similar to that of affected patients with GA I, i.e. high urinary levels of GA and 3HGA (Koeller et al., 2002). However, this model does not lead to the characteristic pathological findings of GA I, indicating that a more representative model of this disease is necessary to clarify its pathogenesis. Another disadvantage of this genetic animal model is that GA and 3HGA accumulate simultaneously, impairing the evaluation of the extent of the contribution or each metabolite to the pathology of the disorder.

Table 1 *In vivo* effect of chronic administration of glutaric acid (GA) on $^{14}CO_2$ production from acetate in cerebral cortex, middle brain and skeletal muscle of 22-day-old rats

Animal treatment	Cerebral cortex	Middle brain	Skeletal muscle
Control	1453 ± 179	1504 ± 111	782.7 ± 48.3
GA-treated	1480 ± 59.2	1334 ± 68.8	928.8 ± 108

Note. Data are represented as mean ± S.E.M. for five independent experiments (animals) performed in duplicate and expressed as pmol · min^{-1} · mg of $protein^{-1}$. No significant differences between saline- (controls) and GA-treated rats were found (Student *t*-test for unpaired samples).

We have recently produced high GA levels in the brain (0.6 mM) and skeletal muscle (4.0 mM) of young rats by acute subcutaneous administration of GA, the major metabolite accumulating in GA I and observed that GA acute treatment provoked a moderate inhibition of complex I–III activity of the respiratory chain in middle brain (25%) and of complexes I–III (25%) and II–III (15%) activities in skeletal muscle (Ferreira et al., 2005). Apart from the fact that GA brain concentrations observed in our chemical model are comparable to those found in *post mortem* brain of human GA I patients (Goodman et al., 1975), it does not mimic the biochemical phenotype of the human condition since plasma GA concentrations are much higher. GA levels were approximately 10-fold higher than in the brain and twice that in skeletal muscle and heart indicating that the brain is less permeable to GA than the peripheral organs. In this context, lower permeability of the blood brain barrier to GA administration has been recently reported (Sauer et al., 2006).

In the present study we utilized the same doses of GA (5 μmol per gram weight) used to develop acute increase of GA tissue concentrations (Ferreira et al., 2005) and induced a chronic model through three subcutaneous injections of GA per day during the first weeks of life in the rat. The major aim of this investigation was to examine whether high sustained concentrations of GA in tissues of young rats provoked by subcutaneous administration of this organic acid to rats from the 5th to the 21st day of life could disrupt energy metabolism *in vivo*, by evaluating critical enzyme activities responsible for most of ATP production and transfer within the cell. We determined the respiratory chain complexes and CK activities in the brain and skeletal muscle of young rats, because affected patients have predominantly neurologic symptoms associated to hypotonia during crises of metabolic decompensation.

We observed that chronic GA administration provoked a significant inhibition of the activity of complexes I–III and II in skeletal muscle, whereas no inhibition of the respiratory chain activities could be detected in the brain structures. These data differ to a certain extent from previous *in vivo* experiments showing that acute GA administration inhibits complex I–III in the brain and complexes I–III and II–III in skeletal muscle (Ferreira et al., 2005) and *in vitro* findings demonstrating that GA significantly inhibits *in vitro* the rate of ATP synthesis and complex I–III and II–III activities in rat cerebral cortex (Silva et al., 2000), as well as CK, succinate dehydrogenase and complex I–III activity in middle brain (da Costa Ferreira et al., 2005). We have to consider that the inhibition of complex I–III activity caused by acute *in vivo* administration of GA and by the addition of this organic acid (5.0 mM) to the assays (*in vitro* experiments) performed with brain homogenates may be possibly due to the presence of high concentrations of this metabolite when the respiratory chain activities were determined. In the present study, the enzymatic assays were performed 12 h after the last injection, when GA concentrations were undetectable (results not shown).

Ullrich and colleagues (1999) also found a very weak inhibition of complex II–III of the respiratory chain by high doses of GA (4 mM), with no alteration of other parameters

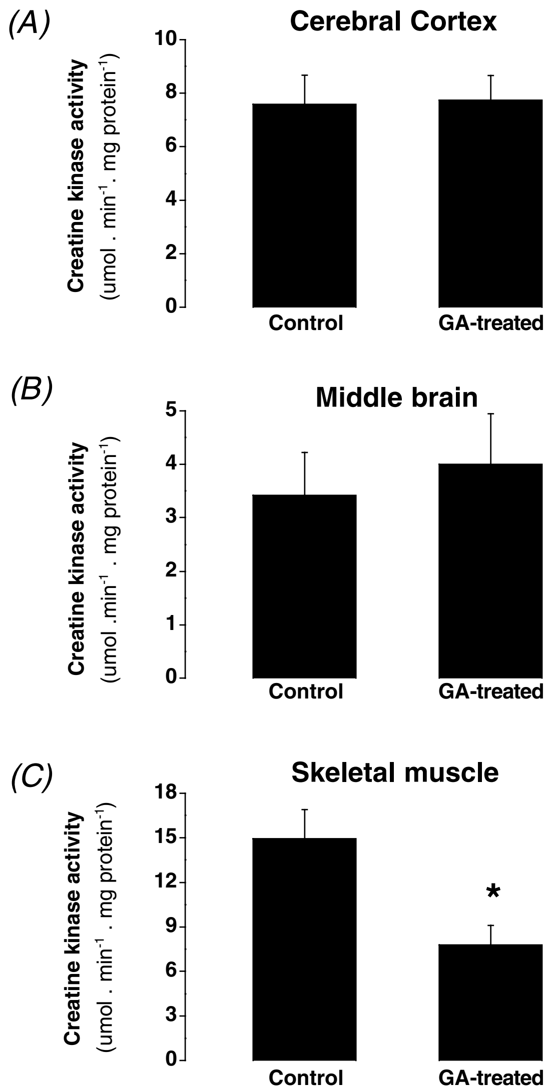

Fig. 4 *In vivo* effect of chronic glutaric acid (GA) administration on creatine kinase activity in cerebral cortex (**A**), middle brain (**B**) and skeletal muscle (**C**) of 22-day-old rats. Data are represented as mean ± S.E.M. for 4–7 four independent experiments (animals) performed in triplicate and expressed as μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} of protein. $^{*}p < 0.05$, compared to controls (Student's *t*-test for unpaired samples)

of energy metabolism in corticostriatal neuronal cultures from rat embryos, whereas other investigators did not find any inhibition of the single respiratory chain complex activities in submitochondrial particles from bovine heart and postulated that GA does not directly inhibit the respiratory chain (Kölker et al., 2002).

It should be also noted that GA elicits reactive species generation (de Oliveira Marques et al., 2003) that may inactivate some of the respiratory chain complexes which are vulnerable to free radical attack (Hillered and Ernester, 1983; Zhang et al., 1990; Melov et al., 1999; Rafique et al., 2001) and therefore provoke an indirect action on energy metabolism. In this context, our assays were performed in tissue homogenates that contain the whole cell machinery necessary to generate free radicals, whereas the studies by Kölker and colleagues (2002) were performed in purified submitochondrial membranes. Therefore, these

apparent conflicting results may be because of distinct methodological approaches, tissues, organ preparations and concentrations of the metabolite utilized in the various experiments, which may not detect a mild indirect inhibitory action of GA. Taken together, it may be presumed that GA has a mild inhibitory action on energy metabolism which depends on the experimental conditions utilized.

We also observed that the respiratory chain complex IV activity was increased in skeletal muscle of rats submitted to GA chronic treatment. Although at the present we cannot explain these findings, increased activity of complex IV has already been observed in common neurodegenerative diseases in which energy impairment is thought to play a relevant role as a result of a functional compensation of the surviving cells (Nagy et al., 1999; Strazielle et al., 2003).

On the other hand, if the inhibition provoked by GA on the respiratory chain (complexes I–III and II) in the skeletal muscle was physiologically marked, then an interruption of the tricarboxylic acid cycle manifested by a reduction of $^{14}CO_2$ production from acetate due to a blockage of the respiratory chain would be expected, but this was not the case. It can be therefore concluded that GA-mediated inhibitory effect on the respiratory chain in skeletal muscle was mild.

We also observed that CK activity was markedly reduced (48% inhibition) by GA chronic administration in the skeletal muscle, but not in cerebral cortex and middle brain.

Taken together, the inhibitory effects of GA observed in complexes I–III and II of the respiratory chain and especially on CK activity in skeletal muscle indicate that energy transfer and production (inhibition of the respiratory chain) are disturbed in this tissue following chronic GA treatment. On the other hand, the reason by which GA chronic treatment provoked energy disturbance in skeletal muscle but not in the brain structures may be due to the quantity of this organic acid in the tissues examined since brain GA concentrations were about 5-fold lower than in the skeletal muscle (Ferreira et al., 2005). It should be emphasized that the brain GA concentrations achieved in our model (0.6 mM) were similar to those previously described in *post mortem* brain of GA I patients (Goodman et al., 1975; Kölker et al., 2003), although recently higher GA levels (up to 5.0 mM) have been reported in brain biopsy during metabolic crises (Külkens et al., 2005). Therefore, we cannot exclude the possibility that higher brain GA levels would lead to alterations of energy metabolism.

Furthermore, the pathophysiological relevance of these findings to human GAI pathology are unknown since GA levels in the peripheral tissues of GA I patients are still not established. Interestingly, it should be noted that GA concentrations found in peripheral tissues in our chemically-induced *in vivo* model (Ferreira et al., 2005) are similar to those described in the knock out murine model of GA I (Köeller et al., 2002; Sauer et al., 2006). It is therefore conceivable that high concentrations of GA may be achieved in the peripheral tissues of glutaric acidemic patients. If this is the case, it may be presumed that alterations of muscle bioenergetics may be associated to the hypotonia occurring in these patients especially during metabolic crises when the accumulating metabolites dramatically increase, as occurs in patients affected by primary mitochondrial disorders (Mordekar et al., 2006).

In conclusion, we showed in the present work that GA chronically administered to rats caused impairment of energy metabolism in skeletal muscle of young rats, reflected by a marked inhibition of CK and a mild blockage of the respiratory chain at complexes I–III and II. Although it is difficult to extrapolate our data to the human condition, if that is the case, it may be presumed that our present findings could be, at least in part, related to the hypotonia found in GA I affected patients.

Acknowledgments We are grateful to the financial support of CNPq, PRONEX, FAPERGS and PROPESQ/UFRGS.

References

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

Brouillet E, Guyot MC, Mittoux V, Altairac S, Conde F, Palfi S, Hantraye P (1998) Partial inhibition of brain succinate dehydrogenase by 3-nitropropionic acid is sufficient to initiate striatal degeneration in rat. J Neurochem 70:794–805

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

da Silva CG, Ribeiro CA, Leipnitz G, Dutra-Filho CS, Wyse AT, 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

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

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

Fischer JC, Ruitenbeek W, Berden JA, Trijbels JM, Veerkamp JH, Stadhouders M, 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) Acidurie glutarique. 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

Freudenberg F, Lukacs Z, Ullrich K (2004) 3-Hydroxyglutaric acid fail 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, Childs B, Kinzler KW, Vogelstein B (eds) The metabolic and molecular bases of inherited diseas, 8th edn. McGraw-Hill, New York, pp 2195–2204

Goodman SI, Markey SP, Moe 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: Biochemical and morphologic considerations. J Pediatr 90:746–750

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

Hillered L, Ernster L (1983) Respiratory activity of isolated rat brain mitochondria following *in vitro* exposure to oxygen radicals. J Cereb Blood Flow Metab 3:207–214

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

Hughes BP (1962) A method for the estimation of serum CK and its use in comparing creatine kinase and aldolase activity in normal and pathological sera. Clin Chim Acta 7:597–603

Koeller DM, Woontner M, Crnic LS, Kleinschmidt-DeMasters B, Stephens J, Hunt EL, Goodman SI (2002) Biochemical, pathologic and behavioral analysis of a mouse model of glutaric acidemia type I. Hum Mol Genet 11:347–357

Kölker S, Garbade SF, Greenberg CR, Leonard JV, Saudubray JM, Ribes A, Kalkanoglu HS, Lund AM, Merinero B, Wajner M, Troncoso M, Williams M, Walter JH, Campistol J, Marti-Herrero M, Caswill M, Burlina AB, Lagler F, Maier EM, Schwahn B, Tokatli A, Dursun A, Coskun T, Chalmers RA, Koeller DM, Zschocke J, Christensen E, Burgard P, Hoffmann GF (2006) Natural history, outcome, and treatment efficacy in children and adults with glutaryl-CoA dehydrogenase deficiency. Pediatr Res 59:840–847

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

Külkens S, Harting I, Sauer S, Zschocke J, Hoffmann GF, Gruber S, Bodamer OA, Kölker 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, 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

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

Lowry OH, Rosebrough 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 I. J Neurosci Res 77:143–147

Melov S, Coskun P, Patel M, Tuinstra R, Cottrell B, Jun AS, Zastawny TH, Dizdaroglu M, Goodman SI, Huang TT, Miziorko H, Epstein CJ, Wallace DC (1999) Mitochondrial disease in superoxide dismutase 2 mutant mice. Proc Natl Acad Sci USA 96:846–851

Mordekar SR, Guthrie P, Bonham JR, Olpin SE, Hargreaves I, Baxter PS (2006) The significance of reduced respiratory chain enzyme activities: Clinical, biochemical and radiological associations. Eur J Paediatr Neurol 10:78–82

Nagy ZS, Esiri MM, LeGris M, Matthews PM (1999) Mitochondrial enzyme expression in the hippocampus in relation to Alzheimer-type pathology. Acta Neuropathol 97:346–354

Porciuncula LO, Dal-Pizzol Jr A, 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 73:93–96

Rafique R, Schapira AH, Cooper JM (2001) Sensitivity of respiratory chain activities to lipid peroxidation: Effect of vitamin E deficiency. Biochem J 357:887–892

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, Gérard B, Rötig A, Saudubray JM, Munnich A (1994) Biochemical and Molecular investigations in respiratory chain deficiencies. Clin Chim Acta 228:35–51

Sauer SW, Okun JG, Fricker G, Mahringer A, Muller I, Crnic LR, Muhlhausen C, Hoffmann GF, Horster F, Goodman SI, Harding CO, Koeller DM, Kolker 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

Sauer SW, Okun JG, Schwab MA, Crnic LR, Hoffmann GF, Goodman SI, Koeller DM, Kölker S (2005) Bioenergetics in glutaryl-coenzyme A dehydrogenase deficiency: a role for glutaryl-coenzyme A. J Biol Chem 280:830–836

Schapira AH, Mann VM, Cooper JM, Dexter D, Daniel SE, Jenner P, Clark JB, Marsden CD (1990) Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. J Neurochem 55:2142–2145

Schuck PF, Leipnitz G, Ribeiro CA, Dalcin KB, Assis DR, Barschak AG, Pulrolnik V, Wannmacher CM, Wyse AT, Wajner M (2002) Inhibition of creatine kinase activity *in vitro* by ethylmalonic acid in cerebral cortex of young rats. Neurochem Res 27:1633–1639

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 DH (2003) Type I glutaric aciduria, part 2: A model of acute striatal necrosis. Am J Med Genet 121:53–70

Strazielle C, Sturchler-Pierrat C, Staufenbiel M, Lalonde R (2003) Regional brain cytochrome oxidase activity in b-amyloid precursor protein transgenic mice with the Swedish mutation. Neuroscience 118:1151–1163

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

Zhang Y, Marcillat O, Giulivi C, Ernster L, Davies KJ (1990) The oxidative inactivation of mitochondrial electron transport chain components and ATPase. J Biol Chem 265:16330–16336

Capítulo III

Evidence for a synergistic action of glutaric and 3-hydroxyglutaric acids disturbing rat brain energy metabolism

Gustavo C. Ferreira, Anelise Tonin, Patrícia F. Schuck, Carolina M. Viegas, Paula C. Ceolato, Alexandra Latini, Marcos L.S. Perry, Angela T.S. Wyse, Carlos S. Dutra-Filho, Clóvis M.D. Wannmacher, Carmen R. Vargas, Moacir Wajner

Artigo publicado no periódico

International Journal of Developmental Neuroscience 25: 391–398, 2007

INTERNATIONAL JOURNAL of DEVELOPMENTAL NEUROSCIENCE

ELSEVIER

Int. J. Devl Neuroscience 25 (2007) 391–398

www.elsevier.com/locate/ijdevneu

Evidence for a synergistic action of glutaric and 3-hydroxyglutaric acids disturbing rat brain energy metabolism

Gustavo C. Ferreira [a], Anelise Tonin [a], Patrícia F. Schuck [a], Carolina M. Viegas [a], Paula C. Ceolato [a], Alexandra Latini [b], Marcos L.S. Perry [a], Angela T.S. Wyse [a], Carlos S. Dutra-Filho [a], Clóvis M.D. Wannmacher [a], Carmen R. Vargas [c,d], Moacir Wajner [a,d,e,*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[b] Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil

[c] Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[d] Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre, RS, Brazil

[e] Universidade Luterana do Brasil, Canoas, RS, Brazil

Received 9 March 2007; received in revised form 2 May 2007; accepted 30 May 2007

Abstract

Glutaric acidemia type I is an inherited metabolic disorder caused by a severe deficiency of the mitochondrial glutaryl-CoA dehydrogenase activity leading to accumulation of predominantly glutaric and 3-hydroxyglutaric acids in the brain tissue of the affected patients. Considering that a toxic role was recently postulated for quinolinic acid in the neuropathology of glutaric acidemia type I, in the present work we investigated whether the combination of quinolinic acid with glutaric or 3-hydroxyglutaric acids or the mixture of glutaric plus 3-hydroxyglutaric acids could alter brain energy metabolism. The parameters evaluated in cerebral cortex from young rats were glucose utilization, lactate formation and $^{14}CO_2$ production from labeled glucose and acetate, as well as the activities of pyruvate dehydrogenase and creatine kinase. We first observed that glutaric (5 mM), 3-hydroxyglutaric (1 mM) and quinolinic acids (0.1 μM) *per se* did not alter these parameters. Similarly, no change of these parameters occurred when combining glutaric with quinolinic acids or 3-hydroxyglutaric with quinolinic acids. In contrast, co-incubation of glutaric plus 3-hydroxyglutaric acids increased glucose utilization, decreased $^{14}CO_2$ generation from glucose, inhibited pyruvate dehydrogenase activity as well as total and mitochondrial creatine kinase activities. The glutaric plus 3-hydroxyglutaric acids-induced inhibitory effects on creatine kinase were prevented by the antioxidants glutathione and catalase plus superoxide dismutase, indicating the participation of reactive oxygen species. Our data indicate a synergic action of glutaric and 3-hydroxyglutaric acids disturbing energy metabolism in cerebral cortex of young rats.
© 2007 ISDN. Published by Elsevier Ltd. All rights reserved.

Keywords: Energy metabolism; Glutaric acid; 3-Hydroxyglutaric acid; Quinolinic acid; Glutaric acidemia type I

1. Introduction

Glutaric acidemia type I (GA I) (GA I; McKusick 23167, OMIM #231670) is an autosomal recessive neurometabolic disorder of the catabolic pathway of lysine, hydroxylysine and tryptophan caused by a deficiency of the mitochondrial flavoenzyme glutaryl-CoA dehydrogenase (GCDH; EC 1.3.99.7) (Goodman and Frerman, 2001). Increased concentrations of glutaric (GA) and 3-hydroxyglutaric (3HG) acids and, to a lesser extent, glutaconic acid are found in the body fluids and brain tissue of GA I affected patients (Goodman et al., 1977; Funk et al., 2005; Kulkens et al., 2005). Clinically, GA I is characterized by macrocephaly and frontotemporal atrophy at birth and by striatal degeneration after episodes of metabolic decompensation precipitated by catabolic events. To date, the exact mechanisms underlying the pathophysiology of GA I are not completely defined. However, various *in vivo* and *in vitro* studies have shown deleterious effects of GA and 3HG, including excitotoxicity (Flott-Rahmel et al., 1997; Lima et al.,

Abbreviations: 3HG, 3-hydroxyglutaric acid; CAT, catalase; CK, creatine kinase; GA, glutaric acid; GA I, glutaric acidemia type I; GSH, reduced glutathione; L-NAME, N^{ω}-nitro-L-arginine methyl ester; PDH, pyruvate dehydrogenase; QA, quinolinic acid; SOD, superoxide dismutase

* Corresponding author at: Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Tel.: +55 51 3308 5571; fax: +55 51 2101 8010.

E-mail address: mwajner@ufrgs.br (M. Wajner).

0736-5748/$30.00 © 2007 ISDN. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijdevneu.2007.05.009

1998; Kölker et al., 1999; Wajner et al., 2004; Rosa et al., 2004), oxidative damage (Kölker et al., 2001a,b; Latini et al., 2002, 2005a; de Oliveira Marques et al., 2003) and disruption of energy metabolism (Ullrich et al., 1999; Silva et al., 2000; Das et al., 2003; Latini et al., 2005b; da C. Ferreira et al., 2005; Ferreira et al., 2005). The fact that a number of GA I patients excrete increased concentrations of lactate, 3-hydroxybutyrate, acetoacetate and dicarboxylic acids reinforces the view that mitochondrial dysfunction plays an important role in the neuropathology of GA I (Gregersen and Brandt, 1979; Floret et al., 1979). However, the literature describes modest effects for GA and 3HGA (Ullrich et al., 1999; Silva et al., 2000; Das et al., 2003; Latini et al., 2005b; da C. Ferreira et al., 2005; Ferreira et al., 2005) when studied individually at millimolar concentrations on brain energy metabolism and to our mind no report described the combined effects of the major organic acids accumulating in this disorder (GA and 3HG) on energy metabolism. Furthermore, it was recently postulated a neurotoxic role for quinolinic acid (QA), a metabolite of the tryptophan kynurenine pathway, in the neurological sequelae of glutaric acidemic patients (Varadkar and Surtees, 2004).

Therefore, in the present work we investigated the combined effects of GA or 3HG with QA, and GA with 3HG on glucose utilization, lactate formation and $^{14}CO_2$ production from labeled glucose and acetate, as well as on the enzyme activities of pyruvate dehydrogenase (PDH) and creatine kinase (CK) in cerebral cortex from young rats. The doses of GA, 3HG and QA utilized were previously found not to influence these parameters of energy production and transfer when added separately to the assays. We also evaluated the influence of the antioxidants glutathione (GSH), catalase (CAT) plus superoxide dismutase (SOD) and the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) on the effects produced by GA plus 3HG on total CK activity.

2. Experimental procedures

2.1. Reagents

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for [U-^{14}C] glucose, [1-^{14}C] acetate and [1-^{14}C] pyruvate, which were purchased from Amersham International plc, UK.

2.2. Animals

A total of 41 thirty-day-old Wistar rats obtained from the Central Animal House of the Department of Biochemistry of the Federal University of Rio Grande do Sul were used. Rats were kept with dams until weaning at 21 days of age. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. The "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

2.3. Cerebral cortex preparation

Animals were sacrificed by decapitation without anesthesia, and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulbs, pons, medulla, cerebellum and striatum were discarded, and the cerebral cortex was peeled away from the subcortical structures, weighed and kept chilled until tissue preparation. Cerebral cortex was cut into two perpendicular directions to produce 400 μM-wide prisms using a McIlwain chopper and used for the glucose utilization and lactate generation assays. For $^{14}CO_2$ production, the cerebral cortex was homogenized (1:10, w/v) in Krebs–Ringer bicarbonate buffer, pH 7.4. For total creatine kinase activity determination, the cerebral cortex was homogenized (1:1000, w/v) in isosmotic saline solution. Mitochondrial and cytosolic fractions were prepared according to Ramirez and Jiménez (2000). Briefly, the homogenates were centrifuged at $800 \times g$ for 10 min at 4 °C and the pellet (P1) discarded. The supernatant was then centrifuged for 30 min at $27,000 \times g$ at 4 °C. The pellet (P2) containing the mitochondria was washed three times with saline solution and used as the mitochondrial fraction for measuring mitochondrial CK activity. The supernatant was further centrifuged for 60 min at $125,000 \times g$ at 4 °C, the microsomal pellet (P3) was discarded and the cytosolic (S) fraction was used for the cytosolic CK enzymatic assay.

The biochemical parameters were determined in the presence of GA, 3HG and QA alone or by combining QA with GA or 3HG or by the simultaneous addition of GA plus 3HG in the medium. Controls did not contain any of these metabolites in the incubation medium. GA, 3HG and QA solutions were prepared on the day of the experiments and had their pH adjusted to 7.4. GA (5 mM), 3HG (1 mM) and QA (0.1–100 μM) were added to the incubation medium at concentrations that *per se* did not alter the parameters evaluated when added separately. In some experiments we also used 6 mM GA in the incubation medium. These concentrations were based on previous reportsn (da C. Ferreira et al., 2005; Latini et al., 2005b; Schuck et al., 2007a,b).

2.4. $^{14}CO_2$ production from glucose and acetate

Homogenates prepared in Krebs–Ringer bicarbonate buffer, pH 7.4, were added to small flasks (11 cm^3) in a volume of 0.45 mL. Flasks were pre-incubated at 35 °C for 15 min in the absence or presence of the metabolites (GA, 5 mM; 3HG, 1 mM; QA, 0.1 μM) in a metabolic shaker (90 oscillations min^{-1}) with 625 μM *n*-dodecyl-β-D-maltoside in order to permeabilize the mitochondrial membranes. After pre-incubation, [U-^{14}C] glucose (0.055 μCi) plus 5 mM of unlabeled glucose or [1-^{14}C] acetate (0.055) μCi plus 1 mM of unlabeled acetate were added to the incubation medium. The total volume of incubation was 525 μL. The flasks were gassed with a O_2/CO_2 (95:5) mixture and sealed with rubber stoppers and Parafilm M. Glass center wells containing a folded 60 mm/4 mm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min incubation at 35 °C in a metabolic shaker (90 oscillations min^{-1}), 0.2 mL of 50% trichloroacetic acid was supplemented to the medium and 0.1 mL of benzethonium hydroxide was added to the center of the wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete $^{14}CO_2$ trapping and then opened. The filter papers were removed and added to vials containing scintillation fluid, and radioactivity was counted. Results were calculated as pmol $^{14}CO_2$ h^{-1} g tissue^{-1}.

2.5. Glucose utilization

Cortical prisms (50 mg) were first pre-incubated at 35 °C for 15 min in the presence of the metabolites (GA, 5 mM; 3HG, 1 mM; QA, 0.01 μM) and then incubated under a O_2/CO_2 (95:5) mixture at 35 °C for 60 min in Krebs–Ringer bicarbonate buffer, pH 7.0 containing 5.0 mM glucose (in a total volume of 0.5 mL) in a metabolic shaker (90 oscillations min^{-1}). Controls did not contain the metabolites in the incubation medium. Glucose was measured in the medium before and after incubation by the glucose oxidase method (Trinder, 1969). Glucose utilization was determined by subtracting the amount after incubation from the total amount measured before incubation (Dutra et al., 1991). Results were calculated as μmol glucose h^{-1} g tissue^{-1}.

2.6. Lactate release

Cortical prisms were initially pre-incubated at 35 °C for 15 min in Krebs–Ringer bicarbonate buffer, pH 7.0, in the presence or absence of the metabolites (GA, 5 mM; 3HG, 1 mM; QA, 0.01 μM), which was followed by the addition of 5 mM glucose. After 60 min incubation at 35 °C in a metabolic shaker (90 oscillations min^{-1}), two volumes of 0.3 N perchloric acid were immediately added to the incubation medium. The excess of perchloric acid was precipitated as

a potassium salt by the addition of one volume of 3 M potassium bicarbonate. After centrifugation for 5 min at 800 × g, lactate was measured in the supernatant by the lactase-peroxidase method (Shimojo et al., 1989). Results were expressed as μmol of lactate h^{-1} g $tissue^{-1}$.

2.7. Measurement of pyruvate dehydrogenase (PDH) activity

Homogenates prepared in Krebs–Ringer bicarbonate buffer, pH 7.4, were added to small flasks (11 cm^3) in a volume of 0.45 mL. Flasks were pre-incubated at 35 °C for 15 min in the absence or presence of the metabolites (GA, 5 mM; 3HG, 1 mM; QA, 0.1 μM) in a metabolic shaker (90 oscillations min^{-1}) with 625 μM *n*-dodecyl-β-D-maltoside in order to permeabilize the mitochondrial membranes. After pre-incubation, [1-^{14}C] pyruvate (0.065 μCi) plus 1.0 mM of unlabeled pyruvate were added to the incubation medium. The total volume of incubation was 525 μL. The flasks were gassed with a O_2/CO_2 (95:5) mixture and sealed with rubber stoppers and Parafilm M. Glass center wells containing a folded 60 mm/4 mm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min incubation at 35 °C in a metabolic shaker (90 oscillations min^{-1}), 0.2 mL of 50% trichloroacetic acid was supplemented to the medium and 0.1 mL of benzethonium hydroxide was added to the center of the wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete $^{14}CO_2$ trapping and then opened. The filter papers were removed and added to vials containing scintillation fluid, and radioactivity was counted (Leighton et al., 1985). Results were calculated as pmol $^{14}CO_2$ h^{-1} g $tissue^{-1}$.

2.8. Spectrophotometric analysis of creatine kinase activity

CK activity was measured in total homogenates, mitochondrial (P2) and cytosolic (S) fractions according to Hughes (1962) with slight modifications (Schuck et al., 2002). Briefly, the reaction mixture consisted of 50 mM Tris buffer, pH 7.5, containing 7.0 mM phosphocreatine, 7.5 mM $MgSO_4$, and 0.5–1.0 μg protein in a final volume of 0.1 mL. We initially carried out a pre-incubation at 37 °C for 15 min with the metabolites (GA, 5 mM; 3HG, 1 mM; QA, 100 μM). The reaction was then started by addition of 4.0 mM ADP and stopped after 10 min by addition of 0.02 mL of 50 mM *p*-hydroxy-mercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL 20% α-naphthol and 0.1 mL 20% diacetyl in a final volume of 1.0 mL and read after 20 min at λ = 540 nm. Some experiments were performed in the presence or absence of catalase (CAT; 50 mU mL^{-1}) plus superoxide dismutase (SOD; 50 mU mL^{-1}), the nitric oxide synthase inhibitor L-NAME (0.5 mM) or GSH (0.5 mM). Results are expressed as μmol of creatine min^{-1} mg $protein^{-1}$ or percentage of controls.

2.9. Protein determination

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

2.10. Statistical analysis

Unless otherwise stated, results are presented as mean ± S.E.M. Assays were performed in duplicate or triplicate and the mean or median was used for the calculations. Data were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc Duncan multiple range test when F was significant or Student t-test for paired samples. Differences between groups were rated significant at $P < 0.05$. Only significant values were given in the text. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

3.1. Inhibition of glucose oxidation by co-incubation of GA with 3HGA in rat cerebral cortex

For these experiments we used 12 animals. Fig. 1 shows that $^{14}CO_2$ production from [U-^{14}C] glucose was significantly inhibited by co-incubation of GA with 3HGA (up to 20%), whereas co-incubation of QA with GA or 3HG did not alter this parameter [$F(3,19) = 3.57$; $P < 0.05$]. Furthermore, the isolated presence of GA (5 mM), 3HG (1 mM) or QA (0.01 μM) in the medium did not alter glucose oxidation (results not shown). It can also be noted in the figure that $^{14}CO_2$ generation from [1-^{14}C] acetate (acetate oxidation) was not changed by these metabolites, indicating that the astrocytic citric acid cycle was not disturbed by these organic acids. These results suggest that GA and 3HGA act synergistically compromising the glycolytic pathway in rat brain.

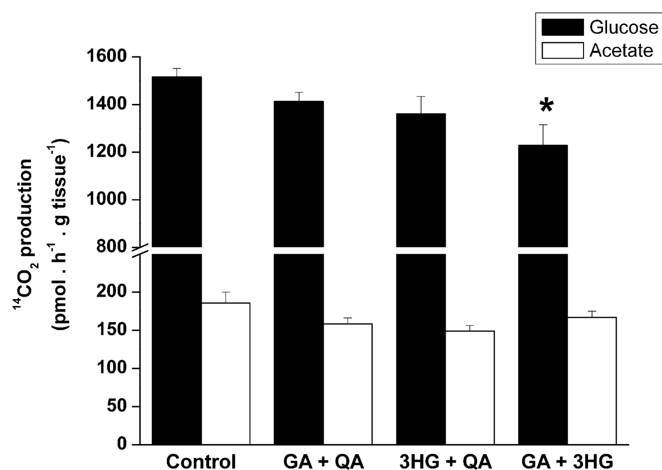

Fig. 1. Effect of glutaric (GA), 3-hydroxyglutaric (3HG) and quinolinic (QA) acids on $^{14}CO_2$ production from [U-^{14}C] glucose (black bars) and [1-^{14}C] acetate (white bars) in rat cortical homogenates. Values are mean ± S.E.M. for six independent experiments (animals) per group expressed as pmol $^{14}CO_2$ h^{-1} g $tissue^{-1}$. $^*P < 0.05$ compared to controls (Duncan multiple range test).

3.2. Glucose utilization is stimulated by co-incubation of GA with 3HGA in rat cerebral cortex

Then we evaluated the effect of GA, 3HGA and QA on glucose utilization and lactate release in cortical prisms. Six animals were used for these experiments. Co-incubation of GA with 3HGA provoked an increase of cortical glucose utilization (around 30%) [$F(3,16) = 3.25$; $P < 0.05$] (Fig. 2A), but did not affect significantly lactate release (Fig. 2B) by prisms from rat cerebral cortex, although there was a tendency of these metabolites to increase this parameter (around 26%). Furthermore, the isolated presence of GA (5 mM), 3HG (1 mM) or QA (0.01 μM) in the medium did not alter glucose oxidation (results not shown).

3.3. Co-incubation of GA with 3HGA leads to an inhibition of pyruvate dehydrogenase activity in rat cerebral cortex

In attempt to explain the decreased $^{14}CO_2$ production from [U-^{14}C] glucose, we evaluated the combined and separated effects of GA plus 3HG on pyruvate dehydrogenase activity in cerebral cortex homogenates. For these experiments we used five animals. Fig. 3 shows that this activity was decreased by GA plus 3HG (up to 24%) [$t(3) = 3.11$; $P < 0.05$], but not when

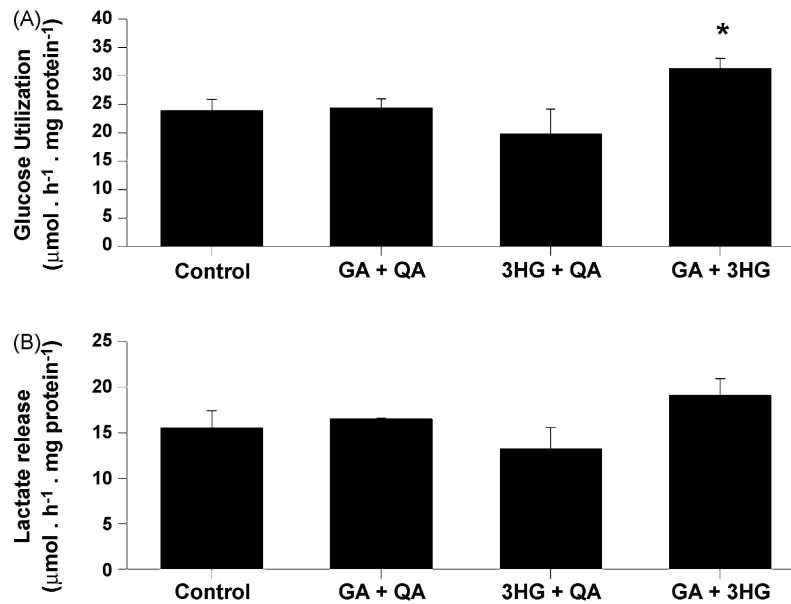

Fig. 2. Effect of glutaric (GA), 3-hydroxyglutaric (3HG) and quinolinic (QA) acids on (A) glucose utilization and (B) lactate release in rat cortical prisms. Values are mean ± S.E.M. for three to six independent experiments (animals) per group expressed as μmol glucose h^{-1} g $tissue^{-1}$ (glucose utilization) or μmol lactate h^{-1} g $tissue^{-1}$ (lactate release), $*P < 0.05$ compared to controls (Duncan multiple range test).

GA (5 mM) or 3HG (1 mM) were added separately to the homogenates (results not shown).

3.4. Inhibition of creatine kinase activity caused by co-incubation of GA with 3HGA in rat cerebral cortex is probably mediated by reactive oxygen species

Finally, we assessed the effect of GA, 3HGA and QA alone or combined on total CK activity in rat cortical homogenates. Five rats were used for these experiments. We observed that co-incubation of GA with 3HGA inhibited this activity (up to 22%) [$F(3,16) = 3.33$; $P < 0.05$] (Fig. 4A). In contrast, this activity was not altered by the presence of GA (5 or 6 mM), 3HG (1 mM) or QA (100 μM), when supplemented separately to the medium and by the combination of QA (100 μM) with GA (5 mM) or 3HG (1 mM) (results not shown). In order to test whether the significant reduction of CK activity was mediated by free radicals, we pre-incubated the cerebral cortex homogenates in the presence of the antioxidants nitric oxide synthase inhibitor L-NAME, catalase (CAT) plus superoxide dismutase (SOD) or reduced glutathione (GSH). Seven animals were used for this experiment. We observed that GSH and CAT plus SOD were able to prevent the inhibitory effect caused by co-incubation with GA and 3HGA [$F(4,30) = 3.81$; $P < 0.05$], suggesting a role for reactive oxygen species in this inhibition (Fig. 4B).

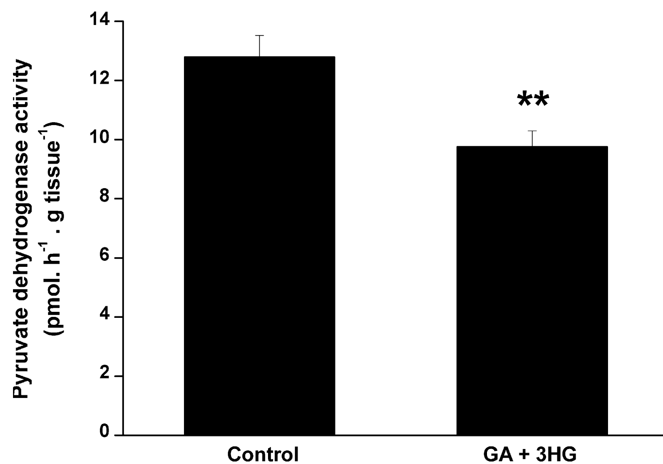

Fig. 3. Combined effect of glutaric (GA) and 3-hydroxyglutaric (3HG) on pyruvate dehydrogenase (PDH) activity in rat cerebral cortex. Values are mean ± S.E.M. for five independent experiments (animals) per group expressed as pmol $^{14}CO_2$ h^{-1} g $tissue^{-1}$. $**P < 0.01$ compared to controls (Student *t*-test for paired samples).

Then, we investigated the effect of a co-incubation of GA with 3HGA on CK activity in mitochondrial (P2) and cytosolic (S) fractions of rat cerebral cortex. For these experiments we used six animals. It can be seen in Fig. 5 that these metabolites significantly inhibited mitochondrial CK activity (up to 35%) [$t(4) = 5.40$; $P < 0.01$], but not the cytosolic CK activity.

4. Discussion

GA I is a cerebral organic aciduria characterized by progressive neurodegeneration with neuroimaging findings of frontotemporal atrophy, delayed myelination with leukoencephalopathy, as well as by acute caudate and putamen degeneration following the characteristic encephalopathic crises when the levels of the accumulated metabolites GA and 3HG dramatically increase to millimolar concentrations (Goodman et al., 1977; Hoffmann and Zschocke, 1999). The neuropathology of GA I is far from understood, although excitotoxicity (Flott-Rahmel et al., 1997; Lima et al., 1998; Ullrich et al., 1999; Porciuncula et al., 2004; de Mello et al., 2001; Frizzo et al., 2004; Rosa et al., 2004), oxidative stress (Kölker et al., 2001a,b; Latini et al., 2002, 2005a; de Oliveira

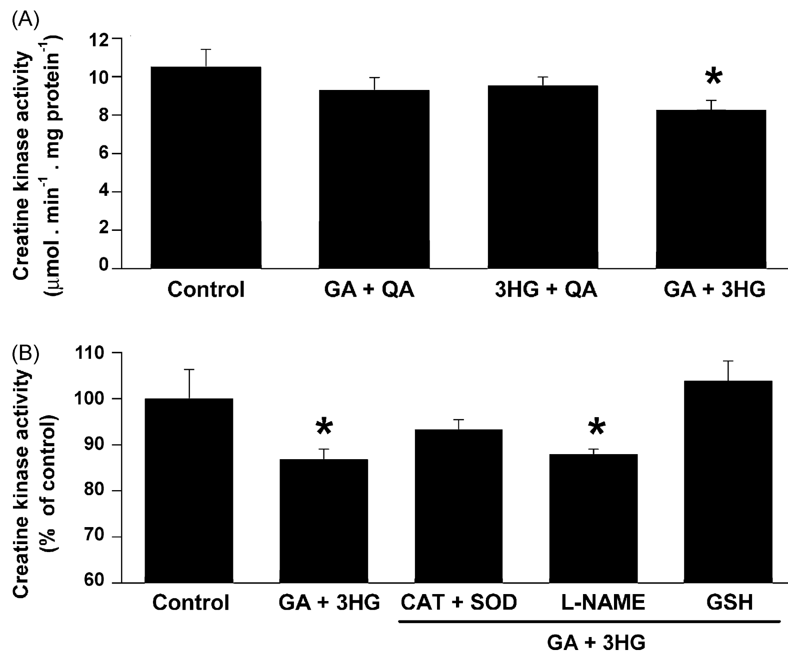

Fig. 4. Effect of glutaric (GA), 3-hydroxyglutaric (3HG) and quinolinic (QA) acids on total creatine kinase (CK) activity in rat cortical homogenates (A). The antioxidants N^{ω}-nitro-L-arginine methyl ester (L-NAME), glutathione (GSH) and catalase (CAT) plus superoxide dismutase (SOD) were used in some assays supplemented by GA and 3HG (B). Values are mean $\pm$ S.E.M. for five to seven independent experiments (animals) per group expressed as nmol creatine min^{-1} mg $protein^{-1}$ or percentage of controls (mean of controls: 15.5 $\pm$ 1.89 nmol creatine min^{-1} mg $protein^{-1}$). $^{*}P < 0.05$ compared to controls (Duncan multiple range test).

Marques et al., 2003; Sauer et al., 2005) and energy disruption (Ullrich et al., 1999; Silva et al., 2000; Das et al., 2003; Sauer et al., 2005; Latini et al., 2005b; da C. Ferreira et al., 2005; Ferreira et al., 2005) have been demonstrated in the last years to be induced by GA and 3HG. However, recent evidence failed to demonstrate an excitotoxic action for 3HG in neuronal cultures (Bjugstad et al., 2001; Freudenberg et al., 2004; Lund et al., 2004). On the other hand, it was postulated a neurotoxic role for quinolinic acid (QA), a metabolite of the tryptophan kynurenine pathway, in the neurological sequelae of glutaric acidemic patients (Varadkar and Surtees, 2004).

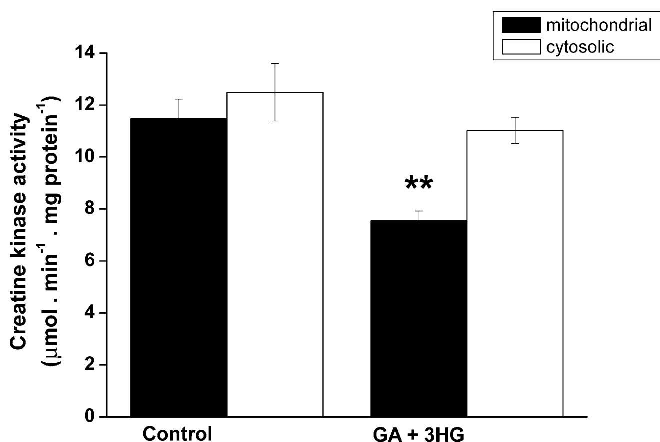

Fig. 5. Combined effect of glutaric (GA) and 3-hydroxyglutaric (3HG) acids on the mitochondrial (black bars) and cytosolic (white bars) creatine kinase activities in rat cerebral cortex. Values are mean $\pm$ S.E.M. for five to six independent experiments (animals) per group expressed as nmol creatine min^{-1} mg $protein^{-1}$. $^{**}P < 0.01$ compared to controls (Student's *t*-test for paired samples).

More recently the importance of mitochondrial dysfunction was emphasized as a possible mechanism for the brain damage in GA I (Strauss and Morton, 2003; Sauer et al., 2005; Bjugstad et al., 2006). This is in line with the fact that some GA I patients excrete increased concentrations of lactate, 3-hydroxybutyrate, acetoacetate and dicarboxylic acids, especially during crises of metabolic decompensation, reflecting a mitochondrial dysfunction (Gregersen and Brandt, 1979; Floret et al., 1979).

Thus, since at present the relevance of bioenergetical impairment and of other pathomechanisms of brain injury are not yet clearly established in GA I, and considering that previous studies did not evaluate the combined effects of GA and 3HG and also of QA on brain energy metabolism, the aim of the present work was to investigate the combined effect of QA with GA or 3HG and GA with 3HG on various parameters of energy metabolism in brain of young rats. We investigated the *in vitro* effect of these metabolites added separately or combined on glucose utilization, lactate generation, glucose and acetate oxidation, as well as on key enzyme activities involved in energy metabolism in brain of developing rats. The control values obtained in the various parameters analyzed in rat cerebral cortex were similar to those found by other investigators (Leighton et al., 1985; Toyomizu et al., 1999; Rasia-Filho et al., 2002; Sgaravatti et al., 2003; Reis de Assis et al., 2004; Schweigert et al., 2004; Rosa et al., 2005; Schuck et al., 2007a,b).

We observed that co-incubation of QA with either GA or 3HG did not alter the parameters of energy metabolism examined, indicating that QA, at the doses utilized (0.01–100 μM) does not apparently compromise energy metabolism. This is in agreement with a recent report showing a mild *in vitro*

effect of QA only at a high concentration on brain energy metabolism (Schuck et al., 2007a,b). Since the concentrations of QA utilized in our experiments are similar or even higher than those achieved in the brain during neuroinflammatory processes, when the kynurenine pathway is activated in microglial cells and/or when invading macrophages infiltrate the brain (Heyes et al., 1992; Schwarcz and Pellicciari, 2002), QA-induced mitochondrial dysfunction as an important pathomechanism of brain damage in GA I is unlikely. We cannot however exclude the possibility that QA associated with GA and 3HG may have a role in the brain damage of GA I inducing oxidative stress (Santamaria and Rios, 1993; Santamaria et al., 2001; Kölker et al., 2001a,b; Latini et al., 2002, 2005a; de Oliveira Marques et al., 2003; Leipnitz et al., 2005) or excitotoxicity (Foster et al., 1983; Schwarcz et al., 1984; Stone, 1993; Flott-Rahmel et al., 1997; Lima et al., 1998; Kölker et al., 1999; Wajner et al., 2004; Rosa et al., 2004).

In contrast, we verified that the combination of GA and 3HG resulted in a synergic effect, compromising various parameters of energy metabolism. GA plus 3HG, but not GA or 3HG alone, caused an inhibition (up to 20%) of $^{14}CO_2$ production from glucose, indicating an impairment of the aerobic glycolytic pathway. In contrast, $^{14}CO_2$ formation from acetate was not changed by GA and 3HG alone or combined. Since acetate oxidation occurs predominantly in astrocytes, it is presumed that these organic acids do not disturb the astrocytic citric acid cycle activity. We also found that the combination of these organic acids significantly inhibited the activity of pyruvate dehydrogenase, an important enzyme that links the glycolytic pathway to the citric acid cycle. Therefore, it may be concluded that the association of GA with 3HG compromises aerobic glycolysis by inhibiting pyruvate dehydrogenase activity. Furthermore, GA plus 3HG significantly increased glucose utilization (up to 30%) by rat cerebral cortex, indicating a stimulation of the transport and/or utilization of this substrate by the brain. Considering that anaerobic glycolysis produces less ATP and needs more substrate (glucose) to compensate the lower energy outcome, it may be assumed that lactate production might be increased by the association of GA plus 3HG. In this context, we observed that the association of these organic acids provoked a moderate (up to 26%) nonsignificant increase of lactate production from glucose. Taken together these observations results on glucose utilization and $^{14}CO_2$ production, it might be presumed that aerobic glycolysis are compromised by GA plus 3HG, whereas anaerobic glycolysis is stimulated.

Finally, we observed that CK activity was significantly inhibited by co-incubating GA with 3HG. We also verified that GSH that acts as a naturally occurring thiol-reducing agent (Meister and Anderson, 1983) and CAT plus SOD, which are excellent trapping agents for superoxide, totally prevented the significant inhibitory role of GA plus 3HG on total CK activity from cerebral cortex, probably indicating that reactive species were involved in this effect. We also demonstrated that the mitochondrial, but not the cytosolic CK activity was inhibited by GA plus 3HG, and this is interesting since critical cysteine residues of the enzyme mitochondrial (P2) fraction are highly vulnerable to free radical attack (Wolosker et al., 1996; Arstall et al., 1998; Stachowiak et al., 1998). These observations are important since this enzyme activity, mainly responsible for ATP transfer within the cell, is crucial for normal brain cell functioning and has been recognized as an important metabolic regulator during health and disease (Wallimann et al., 1998).

The synergic effect between GA and 3HG on energy metabolism is reinforced by the fact that 6 mM GA did not alter the evaluated parameters, as occurred by the combination of 5 mM GA with 1 mM 3HG.

In conclusion, to our knowledge this is the first report showing a synergistic effect of GA and 3HG disrupting cerebral energy metabolism. The fact that GA I patients accumulate both metabolites in their tissues emphasizes the relevance of the present findings. However, at present it is difficult to postulate that energy deficit is mainly responsible for the characteristic cerebral damage of GA I affected patients, as recently postulated (Strauss and Morton, 2003). However, energetic dysfunction may act together with other pathomechanisms making the brain more vulnerable to the effect of the accumulating metabolites in GA I, as recently demonstrated in the genetic knockout GCDH-deficient mouse model (Bjugstad et al., 2006). We cannot however establish whether energy depletion, oxidative stress, excitotoxicity or other mechanisms is mainly responsible for the brain damage in GA I patients. It is likely that these mechanisms act synergistically to cause the devastating on the brain of glutaric acidemic patients.

Acknowledgements

This work was supported by grants from CNPq, PRONEX II, FAPERGS and PROPESQ/UFRGS.

References

Arstall, M.A., Bailey, C., Gross, W.L., Bak, M., Balligand, J.L., Kelly, R.A., 1998. Reversible *S*-nitrosation of creatine kinase by nitric oxide in adult rat ventricular myocytes. J. Mol. Cell. Cardiol. 30, 979–988.

Bjugstad, K.B., Crnic, L.S., Goodman, S.I., Freed, C.R., 2006. Infant mice with glutaric acidaemia type I have increased vulnerability to 3-nitropropionic acid toxicity. J. Inherit. Metab. Dis. 29, 612–619.

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

Das, A.M., 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. Metabol. 78, 108–111.

de Mello, 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.

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

Dutra, J.C., Wajner, M., Wannmacher, C.F., Dutra-Filho, C.S., Wannmacher, C.M.D., 1991. Effects of methylmalonate and propionate on glucose and ketone bodies uptake *in vitro* by brain of developing rats. Biochem. Med. Metab. Biol. 45, 56–64.

da C. Ferreira, G., Viegas, C.M., Schuck, P.F., Latini, A., Dutra-Filho, C.S., Wyse, A.T., Wannmacher, C.M., Vargas, C.R., Wajner, M., 2005. Glutaric

acid moderately compromises energy metabolism in rat brain. Int. J. Dev. Neurosci. 23, 687–693.

Ferreira, G.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. Neurochem. Res. 30, 1123–1131.

Floret, D., Divry, P., Dingeon, N., Monnet, P., 1979. Glutaric aciduria 1. New case. Arch. Francaises de Pediatrie 36, 462–470.

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

Foster, A.C., Collins, J.F., Schwarcz, R., 1983. On the excitotoxic properties of quinolinic acid 2,3-piperidine dicarboxylic acids and structurally related compounds. Neuropharmacology 22, 1331–1342.

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, M.E., Schwarzbold, C., Porciuncula, L.O., Dalcin, K.B., Rosa, R.B., Ribeiro, C.A., 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.

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

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

Goodman, S.I., Frerman, F.E., 2001. Organic acidemias due to defects in lysine oxidation: 2-ketoadipic acidemia and glutaric academia. In: The Metabolic and Molecular Bases of Inherited Disease. 8th ed. McGraw-Hill, New York, pp. 2195–2204.

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

Heyes, M.P., Saito, K., Crowley, J.S., Davis, L.E., Demitrack, M.A., Der, M., Dilling, L.A., Elia, J., Kruesi, M.J., Lackner, A., Larsen, S.A., Lee, K., Leonard, H.L., Markey, S.P., Martin, A., Milstein, S., Mouradian, M.M., Pranzatelli, M.R., Quearry, B.J., Salazar, A., Smith, M., Strauss, S.E., Sunderland, T., Swedo, S.W., Tourtellotte, W.W., 1992. Quinolinic acid and kynurenine pathway metabolism in inflammatory and non-inflammatory neurological disease. Brain 115, 1249–1273.

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.

Hughes, B.P., 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.

Kölker, S., Ahlemeyer, B., Huhne, 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., 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., 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.

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

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., 2005b. 3-Hydroxyglutaric acid moderately impairs energy metabolism in brain of young rats. Neuroscience 135, 111–120.

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

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.

Leighton, B., Budohoski, L., Lozeman, F.J., Challiss, R.A., Newsholme, E.A., 1985. The effect of prostaglandins E1, E2 and F2 alpha and indomethacin on the sensitivity of glycolysis and glycogen synthesis to insulin in stripped soleus muscles of the rat. Biochem. J. 227, 337–340.

Leipnitz, G., Schumacher, C., Scussiato, K., Dalcin, K.B., Wannmacher, C.M., Wyse, A.T., Dutra-Filho, C.S., Wajner, M., Latini, A., 2005. Quinolinic acid reduces the antioxidant defenses in cerebral cortex of young rats. Int. J. Dev. Neurosci. 23, 695–701.

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

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 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. J. Neurosci. Res. 77, 143–147.

Meister, A., Anderson, M.E., 1983. Glutathione. Annu. Rev. Biochem. 52, 711–760.

Porciuncula, L.O., Emanuelli, T., Tavares, R.G., Schwarzbold, C., Frizzo, M.E., Souza, D.O., 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.

Ramirez, O., Jiménez, E., 2000. Opposite transitions of chick brain catalytically active cytosolic creatine kinase isoenzymes during development. Int. J. Dev. Neurosci. 18, 815–823.

Rasia-Filho, A.A., Fin, C.A., Alves, M., Possa, M.A., Roehrig, C., Feoli, A.M., Silva, L., Perry, M.L., 2002. Glucose and lactate utilization by the amygdala of male and female rats. Neurochem. Res. 27, 1583–1588.

Reis de Assis, D., Maria Rde, C., Borba Rosa, R., Schuck, P.F., Ribeiro, C.A., da Costa Ferreira, G., Dutra-Filho, C.S., Terezinha de Souza Wyse, A., Duval Wannmacher, C.M., Santos Perry, M.L., Wajner, M., 2004. Inhibition of energy metabolism in cerebral cortex of young rats by the medium-chain fatty acids accumulating in MCAD deficiency. Brain Res. 1030, 141–151.

Rosa, R.B., Schuck, P.F., de Assis, D.R., Latini, A., Dalcin, K.B., Ribeiro, C.A., da Costa Ferreira, G., Maria, R.C., Leipnitz, G., Perry, M.L., Filho, C.S., Wyse, A.T., Wannmacher, C.M., Wajner, M., 2005. Inhibition of energy metabolism by 2-methylacetoacetate and 2-methyl-3-hydroxybutyrate in cerebral cortex of developing rats. J. Inherit. Metab. Dis. 28, 501–515.

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. Neurochem. Int. 45, 1087–1094.

Santamaria, A., Jimenez-Capdeville, M.E., Camacho, A., Rodriguez-Martinez, E., Flores, A., Galvan-Arzate, S., 2001. *In vivo* hydroxyl radical formation after quinolinic acid infusion into rat corpus striatum. Neuroreport 12, 2693–2696.

Santamaria, A., Rios, C., 1993. MK-801, an *N*-methyl-D-aspartate receptor antagonist, blocks quinolinic acid-induced lipid peroxidation in rat corpus striatum. Neurosci. Lett. 159, 51–54.

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. J. Biol. Chem. 280, 21830–21836.

Schuck, P.F., Tonin, A., da Costa Ferreira, G., Rosa, R.B., Latini, A., Balestro, F., Perry, M.L., Wannmacher, C.M., de Souza Wyse, A.T., Wajner, M., 2007b. *In vitro* effect of quinolinic acid on energy metabolism in brain of young rats. Neurosci. Res. 57, 277–288.

Schuck, P.F., Tonin, A., da Costa Ferreira, G., Viegas, C.M., Latini, A., Duval Wannmacher, C.M., de Souza Wyse, A.T., Dutra-Filho, C.S., Wajner, M.,

2007a. Kynurenines impair energy metabolism in rat cerebral cortex. Cell. Mol. Neurobiol. 27, 147–160.

Schuck, P.F., Leipnitz, G., Ribeiro, C.A., Dalcin, K.B., Assis, D.R., Barschak, A.G., Pulrolnik, V., Wannmacher, C.M., Wyse, A.T., Wajner, M., 2002. Inhibition of creatine kinase activity *in vitro* by ethylmalonic acid in cerebral cortex of young rats. Neurochem. Res. 27, 1633–1639.

Schwarcz, R., Foster, A.C., French, E.D., Whetsell Jr., W.O., Kohler, C., 1984. Excitotoxic models for neurodegenerative disorders. Life Sci. 35, 19–32.

Schwarcz, R., Pellicciari, R., 2002. Manipulation of brain kynurenines: glial targets, neuronal effects, and clinical opportunities. J. Pharmacol. Exp. Therapeut. 303, 1–10.

Schweigert, I.D., Roehrig, C., da Costa, F., Scheibel, F., Gottfried, C.J., Rotta, L.N., Goncalves, C.A., Souza, D.O., Perry, M.L., 2004. High extracellular K+ levels stimulate acetate oxidation in brain slices from well and malnourished rats. Neurochem. Res. 29, 1547–1551.

Sgaravatti, A.M., Rosa, R.B., Schuck, P.F., Ribeiro, C.A., Wannmacher, C.M., Wyse, A.T., Dutra-Filho, C.S., Wajner, M., 2003. Inhibition of brain energy metabolism by the alpha-keto acids accumulating in maple syrup urine disease. Biochim. Biophys. Acta 1639, 232–238.

Shimojo, N., Naka, K., Nakajima, C., Yoshikawa, C., Okuda, K., Okada, K., 1989. Test-strip method for measuring lactate in whole blood. Clin. Chem. 35, 1992–1994.

Silva, C.G., Silva, A.R., Ruschel, C., Helegda, C., Wyse, A.T., Wannmacher, C.M., 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.

Stachowiak, O., Dolder, M., Wallimann, T., Richter, C., 1998. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. J. Biol. Chem. 273, 16694–16699.

Stone, T.W., 1993. Neuropharmacology of quinolinic and kynurenic acids. Pharmacol. Rev. 45, 309–379.

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

Toyomizu, M., Yamahira, S., Shimomura, Y., Akiba, Y., 1999. Metabolic acidosis inhibits pyruvate oxidation in chick liver by decreasing activity of pyruvate dehydrogenase complex. Life Sci. 65, 37–43.

Trinder, P.A., 1969. Determination of blood glucose using on oxidase system with a non-carcinogenic chromogen. J. Clin. Pathol. 22, 158–161.

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.

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

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

Wallimann, T., Dolder, M., Schlattner, U., Eder, M., Hornemann, T., Kraft, T., Stolz, M., 1998. Creatine kinase: an enzyme with a central role in cellular energy metabolism. Magn. Reson. Mater. Phys. Biol. Med. 6, 116–119.

Wolosker, H., Panizzutti, R., Engelender, S., 1996. Inhibition of creatine kinase by *S*-nitrosoglutathione. FEBS Lett. 392, 274–276.

PARTE III

Discussão e Conclusões

III.2. Discussão

A AG I é uma acidúria orgânica cerebral caracterizada por atrofia cortical frontotemporal ao nascimento, hipomielinização com leucoencefalopatia e neurodegeneração progressiva e por necrose aguda do caudado e putamen após crises encefalopáticas, quando as concentrações cerebrais dos metabólitos acumulados, AG e 3HG, estão muito elevados (Goodman et al., 1977; Hoffmann e Zschocke, 1999).

A patogênese do dano cerebral na AG I não está ainda completamente esclarecida embora excitotoxicidade (Flott-Rahmel et al., 1997; Lima et al., 1998; Hoffmann e Zschocke, 1999; Kölker et al., 2000, 2002a; Wajner et al., 2004; Goodman, 2004; Rosa et al., 2004; Magni et al., 2009), estresse oxidativo (Kölker et al., 2001a,b; Latini et al., 2002, 2005a; Sauer et al., 2005), disfunção mitocondrial (Ullrich et al., 1999; Das et al., 2003; Latini et al., 2005b; da C. Ferreira et al., 2005; Ferreira et al., 2005) e rompimento da permeabilidade da barreira hematoencefálica (Strauss e Morton 2003; Zinnanti et al., 2006a; Mühlhausen et al., 2006) sejam considerados importantes mecanismos de neurodegeneração nessa doença.

No que se refere à excitotoxicidade, alguns trabalhos recentes não encontraram efeitos excitotóxicos para o 3HG (Ullrich et al., 1999; Lund et al., 2004; Freudenberg, Lukacs e Ullrich, 2004). Por outro lado, a disfunção energética tem sido considerada nos últimos anos como um importante mecanismo patológico na lesão cerebral que acomete os pacientes afetados por AG I (Strauss e Morton, 2003; Sauer et al., 2005; Bjugstad et al., 2006; Zinnanti et al., 2007). Essa idéia é reforçada pelo fato de que pacientes afetados pela AG I excretam na

urina concentrações aumentadas de lactato, 3-hidroxibutirato, acetoacetato e ácidos dicarboxílicos (Gregersen e Brandt, 1979; Floret et al., 1979). Entretanto, apesar de muitos estudos terem sido realizados no intuito de esclarecer a toxicidade do AG e do 3HG, os mecanismos exatos da neuropatologia da AG I ainda não estão bem estabelecidos e tampouco a relevância dos mesmos nas anormalidades observadas no córtex cerebral e núcleos da base dos pacientes afetados por essa doença.

Modelos animais experimentais de doenças humanas têm sido importantes para investigar os mecanismos que levam aos danos teciduais encontrados em pacientes afetados por diferentes erros inatos do metabolismo, embora não mimetizem vários achados dessas patologias. Nesse sentido, um modelo genético de AG I em camundongos foi desenvolvido (Köeller et al., 2002), apresentando um perfil bioquímico similar ao dos pacientes afetados pela AG I, com excreção urinária de grandes quantidades de AG e 3HG. Entretanto, neste modelo não ocorre lesão estriatal característica da AG I, indicando que um modelo mais representativo desta doença é necessário para melhor entender sua patogênese. Outra desvantagem deste modelo animal genético é que o AG e o 3HG acumulam-se simultaneamente, prejudicando a avaliação da contribuição individual de cada metabólito para a neuropatologia desta doença. Em relação aos testes neuromotores e comportamentais, foi verificado que os animais modificados geneticamente apresentaram um pequeno déficit motor e não se diferenciaram de camundongos normais no que se refere às interações sociais ou ao comportamento no seu habitat (gaiolas) onde foram mantidos durante o seu desenvolvimento (Köeller et al., 2002).

Recentemente, desenvolvemos um modelo agudo químico animal com concentrações altas de AG no cérebro e no músculo esquelético, através da administração subcutânea do AG, o principal metabólito que se acumula na AG I, a ratos. Apesar do fato de que as concentrações cerebrais de AG encontradas em nosso modelo animal foram comparáveis àquelas encontradas em cérebro de pacientes com AG I (Goodman et al., 1975; Kulkens et al., 2005), este modelo não mimetiza o fenótipo bioquímico da doença humana, pois as concentrações plasmáticas de AG foram mais elevadas do que as encontradas em pacientes com AG I. Os animais administrados com AG apresentaram concentrações plasmáticas de AG aproximadamente 10 vezes maiores que no cérebro e duas vezes maiores que em músculo esquelético e coração, indicando que o AG tem uma maior permeabilidade a órgãos periféricos, quando comparado com o cérebro. Neste contexto, foi recentemente sugerido que o transporte de AG através da barreira hemato-encefálica é limitado (Sauer et al., 2006). Observamos que esta administração aguda provocou uma inibição moderada da atividade do complexo I-III da cadeia respiratória em cérebro médio e dos complexos I-III e II-III em músculo esquelético (Ferreira et al., 2005).

Na presente investigação, objetivamos inicialmente estudar se níveis de AG elevados cronicamente nos tecidos de ratos jovens poderiam provocar um prejuízo na performance em tarefas comportamentais, já que não há na literatura nenhum trabalho estudando esse aspecto. O único trabalho disponível na literatura sobre comportamento em modelos animais de AG I estudou apenas o desenvolvimento neuromotor de camundongos geneticamente modificados (*knockout*) no gene da glutaril-CoA desidrogenase como acima mencionado (Köeller et al., 2002), não se

preocupando com a investigação da cognição dos animais. Assim, na primeira etapa desse estudo desenvolvemos um modelo animal experimental crônico de AG I, em que os níveis de AG estivessem cronicamente elevados durante um período de intensa proliferação celular e sinaptogênese (do 5º do 28º dia de vida) em várias estruturas cerebrais envolvidas em processos de aprendizado/memória em ratos (Winick e Noble, 1965; Roisen et al., 1981; Dreyfus et al., 1984; Dutra et al., 1993). Esse tratamento foi baseado em nosso modelo agudo previamente descrito (Ferreira et al., 2005). As concentrações máximas de AG verificadas em vários tecidos dos animais foram aproximadamente 6 mM em plasma, 0,6 mM em cérebro e 4 mM em músculo esquelético. Após a administração crônica, os animais foram deixados em recuperação por 30-45 dias e, após isso, seu desempenho foi avaliado em diversas tarefas comportamentais, tais como labirinto aquático de Morris, labirinto em cruz elevado e campo aberto. Esses resultados estão descritos no Capítulo I deste trabalho.

Observamos inicialmente que a administração crônica de AG não teve efeito sobre o peso corporal dos animais, indicando que este tratamento não provocou malnutrição. Essa observação é importante, pois animais malnutridos podem ter seu desempenho prejudicados em tarefas neurocomportamentais (Smart e Dobbing, 1971; Davis e Squire, 1984). Desse modo, podemos descartar a interferência da desnutrição nas alterações comportamentais observadas neste trabalho. Além disso, a administração crônica de AG não alterou a data de aparecimento dos pelos, abertura dos olhos ou erupção dos dentes incisivos, nem a tarefa do endireitamento em queda-livre, indicando que o desenvolvimento físico e motor dos animais não foi modificado pela administração crônica do AG.

Com relação às tarefas comportamentais, observamos que tanto os animais injetados com solução salina (controles) quanto os animais injetados com AG obtiveram uma melhora na aquisição no labirinto aquático de Morris ao longo dos dias de treinamento, ou seja, diminuiram o tempo de latência para achar a plataforma do primeiro ao quinto dia de treinamento. Entretanto, a latência para achar a platarforma durante os treinamentos no labirinto aquático de Morris foi ligeiramente maior nos animais administrados com AG, quando comparados aos animais controle. Além disso, ratos que receberam a administração crônica de AG ficaram por um período de tempo significativamente menor no quadrante alvo (onde a plataforma foi inicialmente colocada) e um tempo maior no quadrante oposto ao quadrante alvo. Os animais submetidos ao modelo químico com AG também tiveram um menor número de cruzamentos corretos pela posição da plataforma e apresentaram uma maior latência para passar sobre a posição da plataforma no dia do teste, comparando-se aos animais controle. Esses resultados sugerem que a administração crônica de AG logo após o nascimento causou um prejuízo no aprendizado/memória espacial. Como os testes de performance comportamental foram realizados 30 a 45 dias após o tratamento, podemos inferir que esse tratamento causou um dano prolongado ou permanente no cérebro dos animais.

Devemos ressaltar que o déficit na navegação espacial apresentado pelos ratos administrados com o AG não pode ser atribuído a um decréscimo na atividade motora, pois a velocidade de natação no labirinto aquático e o número de cruzamentos no campo aberto foram similares em ambos os grupos, indicando que eles apresentam a mesma atividade locomotora. Adicionalmente, quando

olhamos o padrão das rotas realizadas pelos animais, observamos que os animais administrados com o AG ficaram períodos similares de tempo nos quatro quadrantes da piscina no dia do teste e apresentaram uma rota circular, sugerindo que esses animais utilizaram um padrão circular (busca randômica) como estratégia para achar a plataforma. Entretanto, quando foi avaliada a memória de trabalho uma semana após a sessão de teste, não foi possível diferenciar os animais administrados com AG dos administrados com salina, pois ambos os grupos apresentaram uma diminuição na latência para encontrar a plataforma do primeiro ao último *trial*, dentro de cada dia de treino.

Por outro lado, o comportamento dos ratos na tarefa do labirinto em cruz elevado não foi alterado pela administração do AG, indicando que este tratamento não foi ansiogênico. Similarmente, a administração do AG não alterou o desempenho dos animais na tarefa do campo aberto, já que a diminuição no número de *rearings* e cruzamentos na sessão teste, em relação à sessão treino foi semelhante em ambos os grupos de animais. Esses resultados corroboram os resultados anteriores indicando que a atividade motora dos animais administrados com AG não diferiu dos que receberam solução salina.

Desconhecemos os mecanismos exatos pelos quais o AG prejudica os processos de aprendizado/memória em ratos submetidos ao nosso modelo químico. Entretanto, é possível que as concentrações cerebrais de AG atingidas em nosso modelo animal (0.72 μmol/g = 0.72 mM) possam ter sido suficientes para causar uma disfunção cerebral durante a fase de rápido desenvolvimento e maturação do sistema nervso central em estruturas cerebrais envolvidas com os processos de aprendizado, memória e interação com o ambiente. Convém

salientar que estudos realizados por biópsia e postmortem no cérebro de pacientes com AG I mostraram que as concentrações cerebrais do AG podem chegar a 8 mM (Külkens et al., 2005; Sauer et al., 2006) e, portanto, inferiores às obtidas com esse modelo animal.

Nosso próximo passo foi investigar se concentrações elevadas de AG nos tecidos de ratos jovens por um período prolongado de tempo obtidas por injeções subcutâneas diárias deste ácido orgânico do 5º ao 21º dia de vida, poderiam alterar o metabolismo energético cerebral *in vivo*. Os animais foram sacrificados 12 h após a útlima injeção do AG, sendo então avaliadas as atividades enzimáticas dos complexos da cadeia transportadora de elétrons e da creatina quinase (CK) em cérebro e músculo esquelético de ratos jovens, pelo fato de que pacientes afetados pela AG I apresentam predominantemente sintomas neurológicos associados com hipotonia durante as crises de descompensação metabólica. Esses resultados estão descritos no Capítulo II deste trabalho.

Observamos que a administração crônica de AG inibiu significativamente as atividades dos complexos I-III e II da cadeia transportadora de elétrons em músculo esquelético, sem afetar essas atividades enzimáticas em estruturas cerebrais. Esses resultados diferem dos encontrados em experimentos em que o AG foi administrado agudamente aos animais, onde o tratamento provocou uma inibição das atividades dos complexos I-III da cadeia transportadora de elétrons em cérebro e dos complexos I-III e II-III em músculo esquelético de ratos jovens (Ferreira et al., 2005). Estudos anteriores demonstraram que a administração *in vitro* do AG provocou uma inibição significativa na produção de ATP e nas atividades dos complexos I-III e II-III em córtex cerebral (Silva et al., 2000), bem

como nas atividades das enzimas CK, sucinato desidrogenase e do complexo I-III em cérebro médio de ratos jovens (da Costa Ferreira et al., 2005). Devemos levar em consideração o fato de que as inibições do complexo I-III causado por uma administração *in vivo* aguda de AG ou pela adição *in vitro* deste ácido orgânico (5 mM) aos ensaios com homogeneizados de cérebro de ratos foram possivelmente causadas pelas altas concentrações de AG quando as atividades enzimáticas foram determinadas. No presente estudo, as atividades enzimáticas foram avaliadas 12 h após a última injeção, quando as concentrações de AG eram indetectáveis.

Ullrich e colaboradores (1999) também encontraram uma inibição moderada na atividade do complexo II-III da cadeia transportadora de elétrons por altas doses de AG (4 mM) sem alteração dos outros parâmetros do metabolismo energético em culturas neuronais corticoestriatais de embriões de ratos, enquanto outros investigadores não encontraram nenhuma inibição em atividades isoladas dos complexos da cadeia transportadora de elétrons em partículas submitocondriais de coração bovino e postularam que o AG não interfere diretamente na cadeia transportadora de elétrons (Kölker et al., 2002).

Também devemos considerar que o AG induz a geração de radicais livres (de Oliveira Marques et al., 2003) que podem inativar alguns complexos da cadeia transportadora de elétrons vulneráveis ao ataque oxidativo (Hillered e Ernster, 1983; Zhang et al., 1990; Melov et al., 1999; Rafique et al., 2001) levando assim a uma disfunção energética. Nossos experimentos foram realizados em homogeneizados de tecidos que contém a maquinaria celular completa para gerar radicais livres, enquanto os estudos realizados por Kölker e colaboradores (2002)

foram realizados em membranas submitocondriais purificadas. Assim, esses resultados aparentemente conflitantes podem ser devidos às diferentes abordagens metodológicas, tecidos, preparações teciduais ou concentrações de AG utilizadas nos experimentos que podem não detectar uma ação indireta via produção acelerada de espécies ativas deste ácido orgânico.

Também observamos que a atividade do complexo IV da cadeia transportadora de elétrons estava aumentada em músculo esquelético dos ratos submetidos à administração crônica do AG. Apesar de, nesse momento, não podermos explicar esse achado, atividades aumentadas do complexo IV foram observadas em outras doenças neurodegenerativas, em que a disfunção energética parece desempenhar um importante papel na fisiopatologia dessas doenças (Nagy et al., 1999; Strazielle et al., 2003).

Encontramos ainda que a atividade do ciclo de Krebs, medida pela produção de CO_2 a partir de acetato, nas estruturas cerebrais e no músculo esquelético não foi alterada pela administração crônica do AG, o que pode possivelmente indicar que as inibições da cadeia respiratória provocadas por esse ácido orgânico no músculo esquelético não terem sido fisiologicamente relevantes. Além disso, observamos que a atividade da CK foi marcadamente reduzida em músculo esquelético pela administração crônica de AG, porém não foi alterada no córtex cerebral e no cérebro médio dos animais.

Tomado em seu conjunto, os efeitos inbitórios provocados pelo AG sobre os complexos da cadeia transportadora de elétrons e especialmente sobre a atividade da CK em músculo esquelético sugerem que a produção e a transferência de energia estão prejudicadas nesse tecido após a administração

crônica do AG. A razão pela qual a administração crônica de AG provocou alterações em músculo esquelético sem afetar as estruturas cerebrais pode estar relacionada com as concentrações deste metabólito atingidas nos diferentes tecidos, já que as concentrações cerebrais de AG foram aproximadamente 5 vezes menores que em músculo esquelético (Ferreira et al., 2005). As concentrações cerebrais de AG encontradas em nosso modelo foram similares ou inferiores aquelas previamente descritas em biópsias ou estudos *post mortem* com cérebros de pacientes afetados pela AG I (Goodman et al., 1975; Kölker et al., 2003; Külkens et al., 2005). Assim, não podemos excluir a possibilidade de que níveis cerebrais mais elevados de AG provoquem alterações no metabolismo energético cerebral.

Demonstramos com este trabalho que a administração crônica de AG a ratos provocou um déficit energético em músculo esquelético de ratos jovens que foi refletido por uma marcada inibição da CK e uma moderada inibição da cadeia transportadora de elétrons, nos complexos I-III e II. A relevância fisiopatológica destes achados para a AG I em seres humanos é desconhecida, pois os níveis de AG nos tecidos periféricos dos pacientes ainda não foram descritos. Podemos assim sugerir que, no caso de altas concentrações de AG serem atingidas nos tecidos periféricos de pacientes afetados pela AG I, alterações na bioenergética celular poderiam estar associadas à hipotonia apresentada por estes pacientes, similarmente ao que ocorre em pacientes afetados por doenças mitocondriais primárias (Mordekar et al., 2006).

Considerando que recentemente foi postulado que o ácido quinolínico (AQ), um metabólito da via das quinureninas, poderia estar envolvido na fisiopatologia

da AG I (Varadkar e Surtees, 2004) e que estudos prévios não avaliaram o efeito combinado do AG e do 3HG sobre o metabolismo energético cerebral, também objetivamos investigar o efeito combinado do AQ com o AG ou o 3HG e do AG com o 3HG sobre vários parâmetros do metabolismo energético em cérebro de ratos jovens. Investigamos o efeito *in vitro* destes metabólitos adicionados separadamente ou combinados, sobre a utilização de glicose, a produção de lactato, a oxidação de glicose e acetato, bem como sobre importantes atividades enzimáticas envolvidas no metabolismo energético cerebral de ratos jovens. Esses resultados estão descritos no Capítulo III deste trabalho

Nossos resultados mostraram que a co-incubação de AQ com o AG ou o 3HG não alterou os parâmetros de metabolismo energético examinados, indicando que AQ, nas doses utilizadas, não comprometeu o metabolismo energético. Considerando que as concentrações de AQ utilizadas em nossos experimentos foram similares ou mesmo maiores que as encontradas durante processos neuroinflamatórios em que a via das quinureninas está ativada em células microgliais e/ou macrófagos infiltrados no cérebro (Heyes et al., 1992; Schwarcz and Pellicciari, 2002), o papel da disfunção mitocondrial induzida pelo AQ como um importante mecanismo patológico na AG I é improvável.

Observamos também que tanto o AG como o 3HG não afetaram os parâmetros avaliados quando testados isoladamente. Por outro lado, verificamos que a combinação destes metabólitos resultou em um efeito sinérgico, alterando vários parâmetros do metabolismo energético. A co-incubação do AG com o 3HG provocou uma inibição da produção de CO_2 a partir de glicose sem alterar a produção de CO_2 a partir de acetato, o que indica um prejuízo da via glicolítica. A

combinação destes ácidos orgânicos também inibiu significativamente a atividade da enzima piruvato desidrogenase, uma importante enzima que une a via glicolítica ao ciclo de Krebs. Assim, podemos concluir que a associação do AG com o 3HG causa um prejuízo à glicólise aeróbica. Além disso, a adição simultanea do AG com o 3HG aumentou significativamente a utilização de glicose em córtex cerebral de ratos, indicando uma estimulação do transporte e/ou utilização deste substrato (glicose) pelo cérebro. Considerando que a glicólise anaeróbica produz menos ATP e necessita, assim, mais substrato para compensar a menor geração de energia, podemos presumir que a produção de lactato pode estar aumentada pela associação do AG com o 3HG. Neste sentido, observamos que a combinação destes ácidos orgânicos provocou um moderado aumento na produção de lactato a partir de glicose, apesar de não ser significativo.

Finalmente, observamos que a atividade da CK estava significativamente inibida pela co-incubação do AG com o 3HG, mas não pela adição isolada desses compostos. Também observamos que GSH que atua com um agente fisiológico redutor de tióis e a combinação das enzimas CAT mais SOD que são excelentes agentes sequestradores de peróxido de hidrogênio, preveniram totalmente a inibição do AG mais 3HG sobre a ativdade da enzima CK, indicando que a formação de espécies reativas estaria envolvida neste efeito. Também demonstramos que a fração mitocondrial desta enzima, mas não a citosólica, foi inibida pela combinação de AG mais 3HG. Esse achado é interessante, pois os resíduos críticos de cisteína da fração mitocondrial (P2) desta enzima são altamente vulneráveis ao ataque de radicais livres (Wolosker et al., 1996; Arstall et

al., 1998; Stachowiak et al., 1998). Tendo em vista que essa atividade enzimática é fundamental para o funcionamento normal do cérebro (Wallimann et al., 1998), é possível que uma inibição da CK, como a encontrada no presente trabalho, seja importante na fisiopatologia do dano cerebral presente na AG I. Em resumo, demonstramos que o AG e o 3HG atuam sinergisticamente *in vitro* prejudicando o metabolismo energético cerebral. O fato de que os pacientes afetados pela AG I acumulam ambos os metabólitos em seus tecidos enfatiza a relevância destes achados.

Concluindo, demonstramos neste trabalho que a administração crônica de AG provoca alterações comportamentais que comprometem o aprendizado/memória, além de prejudicar o metabolismo energético em ratos. Mostramos também que a combinação do AG com o 3HG leva a alterações *in vitro* de vários parâmetros do metabolismo energético não observadas com a adição isolada desses metabólitos ao meio de incubação, o que indica um efeito sinérgico entre esses metabólitos. Entretanto, com base nos presentes resultados de inibição moderada do metabolismo energético, é difícil postularmos que o déficit energético é o principal mecanismo responsável pelo característico dano cerebral apresentado por pacientes afetados pela AG I, como foi recentemente sugerido (Strauss e Morton, 2003). Acreditamos, porém, que a disfunção energética possa atuar conjuntamente com outros mecanismos patológicos, como o estresse oxidativo, excitotoxicidade, entre outros mecanismos, tornando o cérebro mais vulnerável ao efeito dos metabólitos acumulados na AG I e causando os pronunciados achados patológicos encontrados no cérebro dos pacientes afetados por esta doença. Finalmente, tendo em vista que deficiência da produção e/ou

transferência de energia leva a déficit importante da cognição (Saydoff et al., 1994; Martínez et al., 2000) e que substratos energéticos como creatina e sucinato foram capazes de prevenir prejuízos à memória/aprendizado em um modelo animal de acidemia metilmalônica (Vasques et al., 2006), presume-se que o tratamento adjuvante com esses substratos pode representar potencialmente uma nova estratégia terapêutica para os pacientes afetados por AG I.

III.2. CONCLUSÕES

III.2.1. Efeitos *in vivo* da administração crônica de AG a ratos jovens sobre tarefas comportamentais

- Estabelecemos um modelo químico experimental de AG I em ratos através de injeções subcutâneas de AG (do 5º ao 28º dia de vida).
- A administração crônica subcutânea de AG não alterou o peso e o desenvolvimento físico e motor (tempo de aparecimento dos pelos, abertura de olhos e erupção dos incisivos superiores, bem como o endireitamento em queda livre) dos animais.
- Os ratos administrados cronicamente com AG apresentaram déficit de memória espacial, enquanto a memória de trabalho foi preservada na tarefa do labirinto aquático de Morris.
- Os ratos administrados com AG não alteraram sua performance (número de entradas, tempo de permanência nos braços abertos e número de entradas nos braços fechados) na tarefa do labirinto em cruz elevado.
- Os ratos administrados com AG não apresentaram alterações comportamentais (latência para deixar o primeiro quadrante, número de cruzamentos, bolos fecais ou *rearings)* na tarefa do campo aberto.

III.2.2. Efeitos *in vivo* da administração crônica de AG sobre parâmetros de metabolismo energético

- A administração crônica subcutânea de AG ocasionou uma inibição da atividade dos complexos I-III e II e um aumento da atividade do complexo IV da cadeia transportadora de elétrons em músculo esquelético, sem alterar as atividades dos complexos I-IV da cadeia respiratória em córtex cerebral e cérebro médio de ratos jovens.

- A administração crônica subcutânea de AG ocasionou uma inibição da atividade da enzima creatina quinase em músculo esquelético, mas não no córtex cerebral e cérebro médio de ratos jovens.

- A administração crônica subcutânea de AG não alterou a produção de CO_2 a partir de acetato em músculo esquelético, córtex cerebral e cérebro médio de ratos.

III.2.3. Efeitos *in vitro* da co-incubação de AQ, AG e 3HG sobre parâmetros de metabolismo energético

- A incubação isolada de AQ, AG e 3HG ou a co-incubação de AQ com AG ou 3HG não afetou a utilização de glicose, liberação de lactato, produção de CO_2 a partir de [U-^{14}C]glicose ou [1-^{14}C]acetato e a atividade da enzima creatina quinase em córtex cerebral de ratos jovens.

- A co-incubação de AG com 3HG provocou um aumento na captação de glicose e uma inibição da produção de CO_2 a partir de glicose, da atividade da enzima piruvato desidrogenase e da fração mitocondrial da enzima creatina quinase em córtex cerebral de ratos jovens, indicando um efeito sinérgico dos AG e 3HG.

- A inibição da enzima creatina quinase causada pela co-incubação de AG com 3HG foi prevenida pela adição de glutationa reduzida e da combinação das enzimas catalase e superóxido dismutase, sugerindo o envolvimento de espécies reativas no efeito causado pelos ácidos.

II.3. PERSPECTIVAS

- Avaliar o efeito da administração intracerebral do AG e do 3HG sobre parâmetros de metabolismo energético em cérebro de ratos jovens.
- Avaliar o efeito da neuroinflamação induzida agudamente ou cronicamente por lipopolissacarídeo em ratos administrados agudamente (intracerebral) e cronicamente (subcutânea) com AG sobre parâmetros de metabolismo energético em cérebro de ratos jovens.
- Avaliar o efeito de agentes neuroprotetores sobre os efeitos *in vivo* causados pelos AG e 3HG sobre o metabolismo energético cerebral.
- Determinar o efeito da administração intracerebral dos ácidos glutárico e 3-hidroxiglutárico sobre as concentrações cerebrais de ATP, ADP, fosfocreatina e creatina em várias estruturas cerebrais.

III.4. REFERÊNCIAS BIBLIOGRÁFICAS

Abeles RH, Frey PA, Jenks WP. Biochemistry. London: Jones and Bartlett, p. 603-632, 1992.

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

Arstall MA, Bailey C, Gross WL, Bak M, Balligand JL, Kelly RA. Reversible S-nitrosation of creatine kinase by nitric oxide in adult rat ventricular myocytes. J Mol Cell Cardiol 30:979–9881998.

Arteni NS, Salgueiro J, Torres I, Achaval M, Netto CA. Neonatal cerebral hypoxia–ischemia causes lateralized memory impairments in the adult rat. Brain Res 973:171–178, 2003.

Baric I, Furnic K, Hoffmann GF. 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 SI, Leonard JV, Muller E, Morton DH, Superti-Furga A, Hoffmann GF. Diagnosis and management of glutaric aciduria type I. J Inherit Metab Dis 21:326-340, 1998.

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

Biery BJ, Stein DE, Morton DH, Goodman SI. 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 KB, Zawada WM, Goodman S, Free CR. IGF-1 and bFGF reduce glutaric acid and 3-hydroxyglutaric acid toxicity in striatal cultures. J Inherit Metab Dis 24: 631-647, 2001.

Brismar J, Ozand PT. 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 AP, Zara G, Hoffmann GF, Zschocke J, Burlina AB. 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 IT, Garavaglia B, Rimoldi M, Rivera I, Uziel G, Cabral A, Coll MJ, 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 RA, Bain MD, Zschocke J. Riboflavin-responsive glutaryl CoA dehydrogenase deficiency. Mol Genet Metab 88: 29-37, 2006.

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

Chalmers RA, Purkiss P, Watts RW, Lawson AM. Screening for organic acidurias and amino acidopathies in newborns and children. J Inherit Metab Dis 3:27-43, 1980.

Chow CW, Haan EA, Goodman SI, Anderson RM, Evans WA, Kleinschimidt-DeMasters BK, Wise G, McGill JJ, Danks DM. Neuropathology in glutaric acidaemia type I. Acta Neuropathol (Berl) 76: 590-594, 1988.

Clark JB, Bates TE, Cullingford T. et al. Development of enzymes of energy metabolism in the neonatal mammalian brain. Dev Neurosci-Basel 17:174-180, 1993.

Cremer JE, Braun LD, Oldendorf WB. Changes during development in transport process of the blood-brain barrier. Biochim Biophys Acta 448:633-637, 1976.

Crone C. Facilited transfer of glucose from blood into brain tissue. J Physiol (Lond) 181:103-106, 1965.

Cruz-Morales SE, Santos NR, Brandão ML. One-trial tolerance to midazolam is due to enhancement of fear and reduction of anxiolytic-sensitive behaviors in the elevated plus-maze retest in the rat. Pharmacol Biochem Behav 72:973-8, 2002.

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

Das AM, 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.

Davis PH, Squire LR. Protein synthesis and memory: a review. Psychol Bull 96:518–559, 1984.

de Mello CF, Kölker S, Ahlemeyer B, de Souza FR, Fighera MR, Mayatepek E, Krieglstein J, Hoffmann GF, 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 ME, Pederzolli CD, Sgaravatti AM, Durigon K, Testa CG, Wannmacher CMD, de Souza Wyse AT, Wajner M, Dutra-Filho CS. Glutaric acid induces oxidative stress in brain of young rats. Brain Res 964:153-158, 2003.

Di Donato S. Disorders related to mitochondrial membranes: pathology of the respiratory chain and neurodegeneration. J Inherit Metab Dis 23:247-263, 2000.

Dreyfus H, Ferret B, Harth S, Giorgio A, Durand M, Freysy L, Massarelli R. Metabolism and function of gangliosides in developing neurons. J Neurosci Res 12:311-312, 1984.

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

Dutra JC,Wajner M,Wannmacher CMD, Dutra-Filho CS, Rache E. Inhibition of succinate dehydrogenase and beta-hydroxybutyrate dehydrogenase activities by methylmalonate in brain and liver of developing rats. J Inherited Metab Dis 16:147–153, 1993.

Erecinska M, Silver IA. Ions and energy in mammalian brain. Prog Neurobiol 43:37-71, 1994.

Ferreira GC, Viegas CM, Schuck PF, Tonin A, Ribeiro CAJ, Coelho DM, Dalla-Costa T, Latini A, Wyse ATS, Wannmacher CMD, Vargas CR, Wajner M. Glutaric acid administration impairs energy metabolism in midbrain and skeletal muscle of young rats. Neurochem Res 30:1123-1131, 2005.

Floret D, Divry P, Dingeon N, Monnet P. Glutaric aciduria. 1 new case. Arch Fr Pediatr 36:462-470, 1979.

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.

Forstner R, Hoffmann GF, Gassner I, Heideman P, De Klerk JB, Lawrenz-Wolf B, Doringer E, Weiss-Wichert P, Troger J, Colombo JP, 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.

Funk CB, Prasad AN, Frosk P, Sauer S, Kölker S, Greenberg CR, Del Bigio MR. Neuropathological, biochemical and molecular findings in a glutaric acidemia type 1 cohort. Brain 128:711-722, 2005.

Goldman-Rakic PS. The 'psychic' neuron of the cerebral cortex. Ann N Y Acad Sci 868:13–26, 1999.

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

Goodman SI, Frerman FE. 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. 8[a] edition. New York, McGraw-Hill. 2195-2204, 2001.

Goodman SI, Markey SP, Moe PG, Miles BS, Teng CC. Glutaric aciduria: a 'new' disorder of amino acid metabolism. Biochem Med 12:12-21, 1975.

Goodman SI, Norenberg MD, Shikes RH, Breslich DJ, Moe PG. Glutaric aciduria: biochemical and morphologic considerations. J Pediatr 90:746-750, 1977.

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

Greenberg CR, Reimer D, Singal R, Triggs-Raine B, Chudley AE, Dilling LA, Philipps S, Haworth JC, Seargeant LE, Goodman SI. 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.

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

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.

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

Hillered L, Ernster L. Respiratory activity of isolated rat brain mitochondria following *in vitro* exposure to oxygen radicals. J Cereb Blood Flow Metab 3:207-214, 1983.

Ho YJ, Eichendorff J, Schwarting RK. Individual response profiles of male Wistar rats in animal models for anxiety and depression. Behav Brain Res 136:1-12, 2002.

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

Hoffmann GF, Athanassopoulos S, Burlina AB, Duran M, de Klerck JBC, Lehnert W, Leonard JV, Monavari AA, Müller E, Muntau AC, Naughten ER, 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 GF, Bohles HJ, Burlina A, Duran M, Herwig J, Lehnert W, Leonard JV, Muntau A, Plecko-Starting FK, 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 GF, Seppel CK, Holmes B, Mitchell L, Christen HJ, Hanefeld F, Rating D, Nyhan WL. Quantitative organic acid analysis in cerebrospinal fluid and plasma: reference values in a pediatric population. J Chromatogr 617:1-10, 1993.

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

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

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

Izquierdo I. Aprendizaje y memoria. In: Fisiologia Humana, de B. Housay, Tomo 4- Neurofisiologia. Cingolani, HE e Houssay, AB. (eds), El Ateneo, Buenos Aires, pp. 311-319, 1989.

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

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. 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, 2003.

Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF. 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 GF. 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, Garbade SF, Greenberg CR, Leonard JV, Saudubray JM, Ribes A, Kalkanoglu HS, Lund AM, Merinero B, Wajner M, Troncoso M, Williams M, Walter JH, Campistol J, Marti-Herrero M, Caswill M, Burlina AB, Lagler F, Maier EM,

Schwahn B, Tokatli A, Dursun A, Coskun T, Chalmers RA, Koeller DM, Zschocke J, Christensen E, Burgard P, Hoffmann GF. Natural history, outcome, and treatment efficacy in children and adults with glutaryl-CoA dehydrogenase deficiency. Pediatr Res 59: 840-847, 2006a.

Kölker S, Koeller DM, Okun JG, Hoffmann GF. Pathomechanisms of neurodegeneration in glutaryl-CoA dehydrogenase deficiency. Ann Neurol 55:7-12, 2004a.

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

Kölker S, Kohr G, Ahlemeyer B, Okun JG, Pawlak V, Horster F, Mayatepek E, Krieglstein J, Hoffmann GF. Ca^{2+} 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 JG, Ahlemeyer B, Wyse AT, Horster F, Wajner M, Kohlmuller D, Mayatepek E, Krieglstein J, Hoffmann GF. 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 SW, Okun JG, Hoffmann GF, Koeller DM. Lysine intake and neurotoxicity in glutaric aciduria type I: towards a rationale for therapy? Brain 129: e54, 2006b.

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

Latini A, Ferreira GC, Scussiato K, Schuck PF, Solano AF, Dutra-Filho CS, Vargas CR, 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 RB, Scussiato K, Leipnitz G, de Assis DR, Ferreira GC, Funchal C, Jacques-Silva MC, 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 RB, 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 CS, Wajner M. Promotion of oxidative stress by 3-hydroxyglutaric acid in rat striatum. J Inherit Metab Dis 28:57-67, 2005b.

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

Leibel RL, Shih VE, Goodman S, Bauman ML, McCabe ER, Zwerdling RG, Bergman I, Costello C. Glutaric acidemia: a metabolic disorder causing progressive choreoathetosis. Neurology 30:1163-1168, 1980.

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

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

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

Magni DV, Furian AF, Oliveira MS, Souza MA, Lunardi F, Ferreira J, Mello CF, Royes LF, Fighera MR. Kinetic characterization of l-[(3)H]glutamate uptake inhibition and increase oxidative damage induced by glutaric acid in striatal synaptosomes of rats. Int J Dev Neurosci 27:65-72, 2009.

Marks DB, Marks AD, Smith C. M. Basic Medical Biochemisty, A Clinical Approach. Lippincott Willians & Wilkins, 1996.

Martínez M, Hernández AI, Martínez N. N-Acetylcysteine delays age-associated memory impairment in mice: role in synaptic mitochondria. Brain Res. 2000 855:100-106.

Mayatepek E, Hoffmann GF, Baumgartner R, Schulze A, Jakobs C, Trefz FK, Bremer HJ. Atypical vitamin B12-uresponsive methylmalonic aciduria in sibship with severe progressive encephalomyopathy: a new genetic disease? Eur J Pediatr 155:398-403, 1996.

McDonald JW, Silverstein FS, Johnston MV. Neurotoxicity of N-methyl-D-aspartate is markedly enhanced in developing rat central nervous system. Brain Res 459:200-203, 1988.

Melov S, Coskun P, Patel M, Tuinstra R, Cottrell B, Jun AS, Zastawny TH, Dizdaroglu M, Goodman SI, Huang TT, Miziorko H, Epstein CJ, Wallace DC. Mitochondrial disease in superoxide dismutase 2 mutant mice. Proc Natl Acad Sci US A 96:846-851, 1999.

Merinero B, Perez-Cerda C, Font LM, Garcia MJ, 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 AL, Kiney CA, Corddry DH, Staton DM. Interactions between glucose and ketone body use by developing brain. Brain Res 256:443-450, 1982.

Mordekar SR, Guthrie P, Bonham JR, Olpin SE, Hargreaves I, Baxter PS. The significance of reduced respiratory chain enzyme activities: Clinical, biochemical and radiological associations. Eur J Paediatr Neurol 10:78–82, 2006.

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.

Nagy ZS, Esiri MM, LeGris M, Matthews PM. Mitochondrial enzyme expression in the hippocampus in relation to Alzheimer-type pathology. Acta Neuropathol 97:346–354, 1999.

Nelson DL, Cox MM. Lehninger, Principles of Biochemistry. 3a ed. New York: Worth Publishers, 2000.

Nelson DL, Cox MM. Lehninger, Principles of Biochemistry. 4a ed. New York: Worth Publishers, 2004.

Olton DS, Markowska AL. Memory and hipocampal function as targets for neurotoxic substances. Neurotoxicology. 15:439-444, 1994.

Porciúncula LO, Dal-Pizzol JrA, Coitinho AS, Emanuelli T, Souza DO, 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 LO, Emanuelli T, Tavares RG, Schwarzbold C, Frizzo ME, Souza DO, 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.

Rafique R, Schapira AH, Cooper JM. Sensitivity of respiratory chain activities to lipid peroxidation: effect of vitamin E deficiency. Biochem J 357(Pt 3):887-892, 2001.

Rashed M, Ozand PT, al Aqeel A, Gascon GG. Experience of King Faisal Specialist Hospital and Research Center with Saudi organic acid disorders. Brain Dev 16:1-6, 1994.

Roisen FJ, Bartfeld H, Nagele R, Yorke G. Ganglioside stimulation of axonal sprouting *in vivo*. Science. 214:577-578, 1981.

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

Saudubray JM, Sedel F, Walter JH. Clinical approach to treatable inborn metabolic diseases: an introduction. J Inherit Metab Dis 29:261-274, 2006.

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. 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.

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

Save E, Poucet B. Involvement of the hippocampus and associative parietal cortex in the use of proximal and distal landmarks for navigation. Behav. Brain Res 109: 195-206, 2000.

Saydoff JA, Liu LS, Garcia RA, Hu Z, Li D, von Borstel RW. Oral uridine pro-drug PN401 decreases neurodegeneration, behavioral impairment, weight loss and mortality in the 3-nitropropionic acid mitochondrial toxin model of Huntington's disease. Brain Res 994:44-54, 2003.

Schwarcz R, Pellicciari R. Manipulation of brain kynurenines: glial targets, neuronal effects, and clinical opportunities. J Pharmacol Exp Therapeut 303:1–10, 2002.

Scriver CR, Beaudet AL, Sly WS, Valle D. (Eds). The Metabolic and Molecular Bases of Inherited Disease. 8ª edition. New York, McGraw-Hill. 3-45, 2001.

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

Smart JL, Dobbing J. Vulnerability of developing brain. II. Effect of early nutritional deprivation on reflex ontogeny and development of behavior in the rat. Brain Res 28:85–95, 1971.

Stachowiak O, Dolder M, Wallimann T, Richter C. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. J Biol Chem 273:16694–16699, 1998.

Stokke O, Goodman SI, Moe PG. 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 KA, Morton DH. 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 KA, Puffenberger EG, Robinson DL, 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.

Strazielle C, Sturchler-Pierrat C, Staufenbiel M, Lalonde R. Regional brain cytochrome oxidase activity in b-amyloid precursor protein transgenic mice with the Swedish mutation. Neuroscience 118:1151–1163, 2003.

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.

Vasques V, Brinco F, Viegas CM, Wajner M. Creatine prevents behavioral alterations caused by methylmalonic acid administration into the hippocampus of rats in the open field task. J Neurol Sci. 244:23-29, 2006.

Vianna MR, Alonso M, Viola H, Quevedo J, de Paris F, Furman M, de Stein ML, Medina JH, Izquierdo I. Role of hippocampal signaling pathways in long-term memory formation of a nonassociative learning task in the rat. Learn Mem 7:333-340, 2000.

Vicario C, Arizmendi C, Malloch G, Clark JB, Medina JM. Lactate utilization by isolated cells from early neonatal rat brain. J Neurochem 57:1700-1707, 1991.

Voet D, Voet JG. Biochemistry. New York: John Wiley & Sons, 1990.

Wajner M, Kölker S, Souza DO, Hoffmann GF, de Mello CF. Modulation of glutamatergic and GABAergic neurotransmission in glutary-CoA dehydrogenase deficiency. J Inherit Metab Dis 27:825-828, 2004.

Wallimann T, Dolder M, Schlattner U, Eder M, Hornemann T, Kraft T, Stolz M. Creatine kinase: an enzyme with a central role in cellular energy metabolism. Magn Reson Mater Phys Biol Med 6:116–119, 1998.

Winick M, Noble A. Quantitative changes in DNA, RNA and protein during prenatal and postnatal growth in rat. Dev Biol 12:451-66, 1965.

Wolosker H, Panizzutti R, Engelender S. Inhibition of creatine kinase by S-nitrosoglutathione. FEBS Lett 392:274–276, 1996.

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.

Zhang Y, Marcillat O, Giulivi C, Ernster L, Davies KJ. The oxidative inactivation of mitochondrial electron transport chain components and ATPase. J Biol Chem 265:16330-16336, 1990.

Ziadeh R, Hoffman EP, Finegold DN, Hoop RC, Brackett JC, Strauss AW, 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 WJ, Lazovic J, Housman C, Lanoue K, O'callaghan JP, Simpson I, Woontner M, Goodman SI, Connor JR, Jacobs RE, Cheng KC. Mechanism of age-dependent susceptibility and novel treatment strategy in glutaric acidemia type I. J Clin Invest 117: 3258-3270, 2007.

Zinnanti WJ, Lazovic J, Wolpert EB, Antonetti DA, Smith MB, Connor JR, Woontner M, Goodman SI, Cheng KC. A diet-induced mouse model for glutaric aciduria type I. Brain 129:899-910, 2006a.

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

// Livros Grátis

(http://www.livrosgratis.com.br)

Milhares de Livros para Download:

Baixar livros de Administração
Baixar livros de Agronomia
Baixar livros de Arquitetura
Baixar livros de Artes
Baixar livros de Astronomia
Baixar livros de Biologia Geral
Baixar livros de Ciência da Computação
Baixar livros de Ciência da Informação
Baixar livros de Ciência Política
Baixar livros de Ciências da Saúde
Baixar livros de Comunicação
Baixar livros do Conselho Nacional de Educação - CNE
Baixar livros de Defesa civil
Baixar livros de Direito
Baixar livros de Direitos humanos
Baixar livros de Economia
Baixar livros de Economia Doméstica
Baixar livros de Educação
Baixar livros de Educação - Trânsito
Baixar livros de Educação Física
Baixar livros de Engenharia Aeroespacial
Baixar livros de Farmácia
Baixar livros de Filosofia
Baixar livros de Física
Baixar livros de Geociências
Baixar livros de Geografia
Baixar livros de História
Baixar livros de Línguas

Baixar livros de Literatura
Baixar livros de Literatura de Cordel
Baixar livros de Literatura Infantil
Baixar livros de Matemática
Baixar livros de Medicina
Baixar livros de Medicina Veterinária
Baixar livros de Meio Ambiente
Baixar livros de Meteorologia
Baixar Monografias e TCC
Baixar livros Multidisciplinar
Baixar livros de Música
Baixar livros de Psicologia
Baixar livros de Química
Baixar livros de Saúde Coletiva
Baixar livros de Serviço Social
Baixar livros de Sociologia
Baixar livros de Teologia
Baixar livros de Trabalho
Baixar livros de Turismo