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AVALIAÇÃO DA HOMEOSTASE ENERGÉTICA EM VÁRIOS TECIDOS E HISTOPATOLOGIA CEREBRAL EM CAMUNDONGOS NOCAUTE PARA A ENZIMA GLUTARIL-COA DESIDROGENASE

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Aos meus pais,

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“A morte não é nada, mas viver vencido

e sem glória é morrer todos os dias”

(Napoleão Bonaparte)

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RESUMO

Estudamos a homeostase energética no cérebro (córtex cerebral, estriado e hipocampo) e tecidos periféricos (coração e músculo esquelético) de camundongos selvagens (WT) e nocaute para a enzima glutaril-CoA desidrogenase (*Gcdh-/-*), modelo animal genético para estudo da acidemia glutárica tipo I (AG I), com 15 e 30 dias de vida. Esses animais também foram submetidos a uma sobrecarga de lisina através de uma injeção intraperitoneal (8 µmol/g) desse aminoácido ou de uma dieta rica em lisina (4,7 %) por 60 horas. Os parâmetros da homeostase energética analisados foram as atividades dos complexos I-III, II, II-III e IV da cadeia respiratória, das enzimas do ciclo do ácido cítrico (CAC) citrato sintase (CS), aconitase, isocitrato desidrogenase (IDH), α-cetoglutarato desidrogenase, sucinato desidrogenase e malato desidrogenase, da creatina quinase (CK) e da Na^+, K^+ - ATPase, bem como a liberação de lactato, os parâmetros respiratórios mitocondriais estados 3 e 4, razão de controle respiratório e o estado desacoplado, além do potencial de membrana mitocondrial na presença ou ausência de Ca^{2+}. Estudos histológicos também foram conduzidos no córtex cerebral e estriado dos camundongos WT e *Gcdh-/-* de 30, 60 e 90 dias de vida submetidos por um pequeno (60 horas) ou longo (30 dias) período com dieta com alta concentração de lisina (4,7 %). Verificamos leves alterações nas atividades dos complexos da cadeia respiratória no cérebro, coração e músculo esquelético dos animais *Gcdh-/-* quando comparados aos WT com 15 e 30 dias de vida. Além disso, demonstramos uma diminuição significativa das atividades da CS e IDH em preparações mitocondriais de estriado de camundongos *Gcdh-/-* submetidos a uma sobrecarga de lisina associada a um pequeno aumento na liberação de lactato. No entanto, não encontramos alterações nos parâmetros respiratórios e no potencial de membrana em mitocôndrias de estriado dos camundongos *Gcdh-/-*quando comparados aos WT. Por outro lado, as atividades da Na^+, K^+-ATPase (cérebro) e CK (cérebro e músculo esquelético) foram significativamente menores em camundongos *Gcdh-/-* com 15 dias de vida quando submetidos a uma injeção intraperitoneal de lisina. Além disso, encontramos uma redução na atividade da Na^+, K^+-ATPase associada com uma diminuição da sua expressão em córtex cerebral, mas não em estriado e hipocampo, de camundongos *Gcdh-/-* com 30 dias de vida submetidos ou não a uma dieta rica em lisina. Finalmente, a análise histológica revelou a presença de vacúolos no córtex cerebral dos camundongos *Gcdh-/-* com 60 e 90 dias de vida, bem como no estriado dos animais *Gcdh-/-* com 90 dias de vida que foram alimentados com uma dieta rica em lisina por 30 dias. Concluindo, presumimos que uma redução das atividades da Na^+, K^+-ATPase e CK possa contribuir para o dano neurológico encontrado nos camundongos *Gcdh-/-* e possivelmente nos pacientes com AG I.

ABSTRACT

We studied energy homeostasis in the brain (cerebral cortex, striatum and hippocampus) and peripheral tissues (heart and skeletal muscle) from 15 and 30-day-old wild type (WT) and glutaryl-CoA dehydrogenase deficient (*Gcdh-/-*) mice, which is a genetic animal model to study glutaric academia type I (GA I). These animals were also submitted to lysine overload through an intraperitoneal injection (8 µmol/g) of this amino acid or supplementing the mice with a high lysine (4.7 %) diet for 60 hours. The energy homeostasis parameters evaluated were the activities of the respiratory chain complexes I-III, II, II-III and IV, of the citric acid cycle (CAC) enzymes citrate synthase (CS), aconitase, isocitrate dehydrogenase (IDH), α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase, creatine kinase (CK) and Na^+, K^+ - ATPase, as well as the lactate release, the mitochondrial respiratory parameters states 3 and 4, respiratory control ratio and uncoupled state, besides the mitochondrial membrane potential in the presence or absence of Ca^{2+}. Histological studies were also conducted in the cerebral cortex and striatum from 30, 60 and 90-day-old WT and *Gcdh-/-* mice submitted for a short (60 hours) or long (30 days) period to a high lysine (4.7 %) diet. We verified mild alterations in the respiratory chain activity in the brain, heart and skeletal muscle from *Gcdh-/-* animals when compared to 15 and 30-day-old WT mice. Furthermore, we demonstrated a reduction in the activities of CS and IDH in striatum mitochondrial preparations from *Gcdh-/-* mice submitted to a lysine overload associated with a mild increase of lactate release. However, we did not find alterations in the respiratory parameters and membrane potential in striatum mitochondria from *Gcdh-/-* mice when compared to WT. On the other hand, the activities of Na^+, K^+-ATPase (brain) and CK (brain and skeletal muscle) were significantly reduced in 15-day-old *Gcdh-/-* mice when received an intraperitoneal injection of lysine. Moreover, a reduction in the Na^+, K^+-ATPase activity associated with a diminution of its expression was observed in the cerebral cortex, but not in striatum and hippocampus, from 30-day-old *Gcdh-/-* mice submitted or not to a high lysine diet. Finally, the histological analyses revealed the presence of vacuoles in the cerebral cortex from 60 and 90-day-old *Gcdh-/-* mice, as well as in the striatum from 90-day-old *Gcdh-/-* animals that were fed a high Lys chow for 30 days. In conclusion, we presume that a reduction in the activities of Na^+, K^+-ATPase and CK may contribute to the brain damage found in *Gcdh-/-* mice and possibly in GA I patients.

LISTA DE ABREVIATURAS

AG I – acidemia glutárica tipo I;

AG – ácido glutárico;

ACO – aconitase;

ADP - adenosina-5’-difosfato;

ATP - adenosina-5’-trifosfato;

α-CGDH - α-cetoglutarato desidrogenase;

CAC – ciclo do ácido cítrico;

CS – citrato sintase;

CK – creatina quinase;

FAD - flavina adenina dinucleotídeo;

$FADH_2$ - flavina adenina dinucleotídeo reduzido;

GABA - ácido gama-aminobutírico;

GCDH – glutaril-CoA desidrogenase;

Gcdh-/- - camundongos nocautes para glutaril-CoA desidrogenase;

HE – hematoxilina-eosina;

3HG – ácido 3-hidróxi-glutárico;

IDH – isocitrato desidrogenase;

MDH – malato desidrogenase;

NAD^+ - adenina dinucleotídeo;

NADH - nicotinamida adenina dinucleotídeo reduzido;

NADPH – nicotinamida adenina dinucleotídeo fosfato reduzido;

NMDA – N-metil-D-aspartato;

RCR – razão de controle respiratório;

SDH – sucinato desidrogenase;

SNC – sistema nervoso central.

I.1. INTRODUÇÃO

I.1.1. Erros inatos do metabolismo

Erros inatos do metabolismo são distúrbios hereditários, majoritariamente de herança autossômica recessiva, cuja característica bioquímica principal é a deficiência ou ausência da atividade de uma enzima específica de uma rota metabólica. Além das enzimas, outras proteínas com função alterada como proteínas de transporte e proteínas estruturais, imunoglobulinas, hormônios, entre outras, podem estar afetadas nos erros inatos do metabolismo. O resultado da deficiência de uma atividade enzimática leva a um bloqueio da rota metabólica levando ao acúmulo de substâncias tóxicas nos tecidos e líquidos corporais ou à falta de substâncias essenciais, muitas vezes, acarretando prejuízo no desenvolvimento mental e/ou físico dos indivíduos afetados (Scriver, 2001). Além disso, rotas alternativas também originam outras substâncias tóxicas (Bickel, 1987). Até o momento, foram descritos mais de 600 erros inatos do metabolismo, a maioria deles envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (Scriver, 2001). Embora individualmente raras, essas doenças afetam aproximadamente 1 a cada 500-1000 recém nascidos vivos (Baric et al., 2001).

I.1.2. Acidemias orgânicas

As acidemias ou acidúrias orgânicas constituem um grupo de erros inatos do metabolismo bioquimicamente caracterizados pelo acúmulo de um ou mais ácidos orgânicos (carboxílicos) 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; Ozand e Gascon, 1991). A frequência destas doenças na população em geral é pouco conhecida, o que pode ser creditado à falta de laboratórios especializados para o seu diagnóstico e ao desconhecimento médico sobre essas enfermidades. Na Holanda, país considerado referência para o diagnóstico de erros inatos do metabolismo, a incidência de acidemias orgânicas é estimada em 1: 2.200 recém-nascidos, enquanto que na Alemanha, Israel e Inglaterra é de aproximadamente 1: 6.000 - 1: 9.000 recém-nascidos (Hoffmann et al., 2004). Na Arábia Saudita, onde a taxa de consanguinidade é elevada, a frequência é de 1: 740 nascidos vivos (Rashed et al., 1994).

Clinicamente, os pacientes afetados por acidemias orgânicas apresentam predominantemente disfunção neurológica em suas mais diversas formas de expressão, incluindo regressão neurológica, convulsões, coma, ataxia, hipotonia, hipertonia, irritabilidade, tremores, movimentos coreatetóticos, tetraparesia espástica, atraso no desenvolvimento psicomotor, retardo mental e outras manifestações. As mais frequentes alterações laboratoriais são cetose, cetonúria, neutropenia, trombocitopenia, acidose metabólica, baixos níveis de bicarbonato, hiperglicinemia, hiperamonemia, hipo / hiperglicemia, acidose lática, aumento dos níveis séricos de ácidos graxos livres, dentre outras (Scriver, 2001). Com o uso da tomografia computadorizada, alterações de substância branca (hipomielização e / ou desmielização), atrofia cerebral generalizada ou dos gânglios da base (necrose ou calcificação), macrocefalia, atrofia frontotemporal e atrofia cerebelar são

encontradas na maioria dos pacientes afetados por essas doenças (Mayatepek et al., 1996).

I.1.3. Acidemia glutárica tipo I (AG I)

A acidemia glutárica tipo I (AG I, OMIM # 231670) é uma acidemia orgânica que foi inicialmente descrita em 1975 por Goodman e colaboradores (Goodman et al., 1975), sendo causada pela deficiência na atividade da enzima mitocondrial glutaril-CoA desidrogenase (GCDH, EC 1.3.99.7) (Goodman e Frerman, 2001). A GCDH catalisa a descarboxilação oxidativa da glutaril-CoA formando crotonil-CoA e CO_2, transferindo os elétrons para a cadeia respiratória via a proteína flavoproteína transferidora de elétrons. Essa reação possui duas diferentes etapas: a desidrogenação de glutaril-CoA a glutaconil-CoA e a descarboxilação de glutaconil-CoA a crotonil-CoA (Hartel et al., 1993). O gene da GCDH localiza-se no cromossomo 19p 13.2 e codifica um polipeptídeo de 438 aminoácidos que sofre uma clivagem na porção *N*-terminal na qual são retirados 44 aminoácidos, formando a proteína madura dentro da matriz mitocondrial (Goodman et al., 1998). A maioria das mutações conhecidas está relacionada com simples mudanças de bases, como no caso da mais frequente mutação em caucasianos (R402W) (Goodman et al., 1998; Zschocke et al., 2000). Existe uma grande heterogeneidade de mutações na deficiência da GCDH; no entanto, dentro de comunidades específicas, o padrão de mutações é mais homogêneo (Busquets et al., 2000). Apesar do conhecimento de diferentes mutações, não há correlação entre o genótipo e a atividade enzimática, bem como o fenótipo bioquímico, clínico e o prognóstico dos pacientes (Goodman et al., 1998; Hoffmann e Zschocke, 1999;

Kolker et al., 2006). Com o bloqueio da atividade enzimática, formam-se rotas metabólicas alternativas que culminam na presença de concentrações elevadas dos ácidos glutárico (AG), 3-hidroxiglutárico (3HG) e, algumas vezes, glutacônico nos tecidos e líquidos biológicos (plasma, urina e líquor) dos indivíduos afetados (Goodman et al., 1977; Goodman e Frerman, 2001) (Figura 1).

As concentrações plasmáticas destes ácidos variam entre 5 e 400 µmol/L (Hoffmann et al., 1991; Merinero et al., 1995), mas as cerebrais podem atingir 500–5000 µmol/L para o AG e 40–200 µmol/L para o 3HG (Funk et al., 2005; Sauer et al., 2006). Tais diferenças podem ser explicadas pelo fato de que o AG e o 3HG serem 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 (SNC), o que constitui num fator de risco na neurodegeneração característica dos pacientes afetados (Hoffmann et al., 1993; Kolker et al., 2006; Sauer et al., 2006).

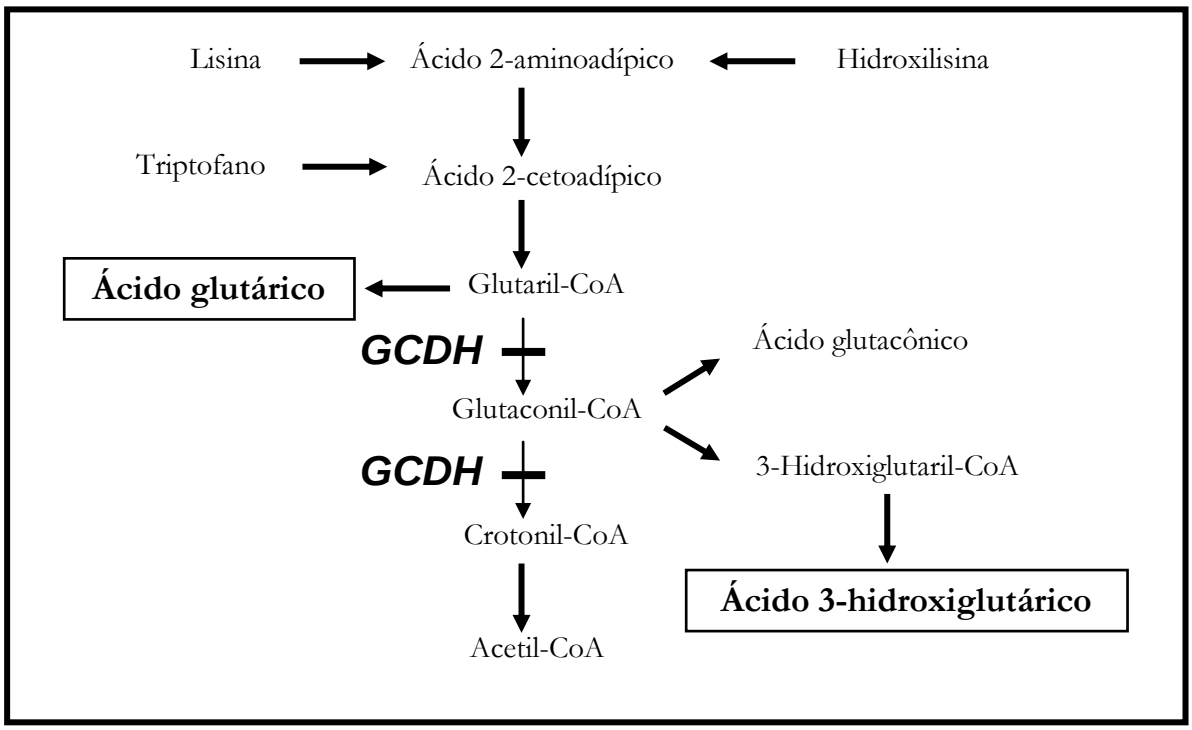

Figura 1. Deficiência da enzima glutaril-CoA desidrogenase (GCDH) e acúmulo dos ácidos glutárico (AG) e 3-hidroxiglutárico (3HG) (Adaptado de Goodman e Frerman, 2001).

A prevalência da doença é estimada em 1: 30.000 - 1:100.000 nascidos vivos, podendo atingir uma prevalência maior (até 1: 300 nascidos vivos) em algumas comunidades fechadas, como os Amish e índios canadenses (Goodman et al., 1977; Goodman e Frerman, 2001; Lindner et al., 2004; Morton et al., 1991).

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, as

quais são caracterizadas por convulsões e coma e associadas à destruição aguda dos núcleos da base caudato e putamen (Hoffmann et al., 1996). Após as crises que ocorrem entre os 6 e os 36 meses de idade surgem sintomas relacionados à destruição estriatal, como distonia e discinesia, hipotonia, convulsões, rigidez muscular e espasticidade (Hoffmann e Zschocke, 1999; Kolker et al., 2004; Neumaier-Probst et al., 2004; Strauss et al., 2003). Tal fato sugere uma "janela de vulnerabilidade" para o aparecimento dos sintomas, provavelmente relacionada ao período de desenvolvimento cerebral. Ataxia, irritabilidade, retardo mental e demência também estão entre os achados clínicos da AG I (Kulkens 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 determinante para um melhor prognóstico para os pacientes afetados. Usualmente, o marcador bioquímico da AG I é a presença de quantidades elevadas de AG e 3HG nos líquidos biológicos (principalmente urina) dos pacientes (Funk et al., 2005; Goodman et al., 1977; Kolker et al., 2006). O diagnóstico é geralmente realizado através da detecção desses compostos e seus ésteres de glicina e carnitina na urina por cromatografia gasosa acoplada à espectrometria de massa (Hoffmann, 1994; Kolker et al., 2006). O perfil de acilcarnitinas e a diminuição de carnitinas livres nos líquidos biológicos determinados por espectrometria de massa em Tandem podem ser usados como métodos auxiliares no diagnóstico (Ziadeh et al., 1995). A análise mutacional não é muito utilizada para fins de diagnóstico devido ao grande número de mutações conhecidas, apresentando maior valor em

estudos de comunidades onde a consanguinidade é elevada e para fins de pesquisa (Busquets et al., 2000; Kolker et al., 2006).

Alguns pacientes apresentam excreção pouco elevada, intermitente, ausente ou normal de AG (Baric et al., 1998; Hoffmann et al., 1996; Merinero et al., 1995) e nesses casos a determinação da atividade da GCDH em fibroblastos ou leucócitos deve ser realizada sempre que houver fortes suspeitas clínicas e neurorradiológicas da doença (Goodman e Frerman, 2001).

O diagnóstico neonatal através dos testes de triagem neonatal tem sido realizado em alguns países no intuito de diagnosticar precocemente essa doença e prevenir as crises encefalopáticas com todas suas consequências funestas por um tratamento precoce (Kolker et al., 2006; Lindner et al., 2004).

I.1.3.3. Achados neuropatológicos

Os achados neuropatológicos da deficiência da GCDH incluem atrofia frontotemporal cortical ao nascimento, formação espongiforme e diminuição de substância branca (leucoencefalopatia) progressiva, além de 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 6 e os 36 meses de idade (Amir et al., 1987; Brismar e Ozand, 1995; Chow et al., 1988; Harting et al., 2009; Hoffmann e Zschocke, 1999; Neumaier-Probst et al., 2004; Strauss et al., 2003). Imagem por ressonância magnética geralmente mostra alterações espongiformes progressivas na substância branca (leucoencefalopatia) com hipoplasia cortical e vacuolização, hemorragia subdural e degeneração dos gânglios da base (Bodamer et al., 2004;

Goodman et al., 1977; Harting et al., 2009; Hoffmann e Zschocke, 1999; Neumaier-Probst et al., 2004; Nunes et al., 2013; Perez-Duenas et al., 2009; Strauss e Morton, 2003).

I.1.3.4. Tratamento

Restrição dietética de proteína é essencial para o bom prognóstico dos indivíduos afetados, evitando as crises agudas com destruição do estriado em até dois terços dos casos (Goodman e Frerman, 2001; Kolker et al., 2006). Além disso, suplementação com dieta hipercalórica especialmente durante as crises, e com L-carnitina e riboflavina em alguns casos, têm mostrado resultados positivos na diminuição das crises encefalopáticas e da lesão progressiva do SNC dos pacientes (Chalmers et al., 2006; Hoffmann et al., 1996; Kulkens et al., 2005).

Diversos fármacos foram testados na terapia da AG I, sendo que anticolinérgicos e toxina botulínica (Burlina et al., 2004), anticonvulsivantes (Hoffmann et al., 1996; Yamaguchi et al., 1987) e antioxidantes (Hoffmann e Zschocke, 1999), não mostraram resultados satisfatórios. Posteriormente, baseados em estudos prévios em um modelo animal de AG I (Zinnanti et al., 2006), alguns autores propuseram a utilização da suplementação com glicose e homoarginina para reduzir o acúmulo cerebral dos metabólitos tóxicos gerados pela deficiência da GCDH (Sauer et al., 2011; Zinnanti et al., 2007; Zinnanti e Lazovic, 2010). Desde então, a suplementação dietética com arginina, que é capaz de competir com a lisina pelo system Y de transporte da barreira hematoencefálica, tem sido utilizada no tratamento da AG I para diminuir a entrada

de lisina com posterior formação dos AG e 3HG no cérebro dos pacientes, demonstrando resultados benéficos (Kolker et al., 2012; Strauss et al., 2011).

I.1.3.5. Modelos animais de acidemia glutárica tipo I (AG I)

O desenvolvimento de modelos animais que mimetizem as características metabólicas e neuropatológicas apresentadas pelos pacientes com AG I também se constitui num desafio. Um modelo químico em ratos foi proposto por Ferreira e colaboradores (Ferreira et al., 2005a) através da administração subcutânea de AG diariamente do 7^{o} ao 22^{o} dia de vida, onde os animais apresentavam altas concentrações desse ácido orgânico no cérebro. Além disso, Strauss e Morton (Strauss e Morton, 2003) propuseram um modelo de degeneração estriatal aguda com o uso de ácido 3-nitropropiônico, um inibidor clássico do complexo II da cadeia respiratória utilizado em modelos de doença de Huntington, que apresenta características neurorradiológicas idênticas às observadas em pacientes com AG I.

Em 2002, Koeller e colaboradores desenvolveram um modelo nocaute para o gene da GCDH em camundongos (*Gcdh-/-*) (Koeller et al., 2002). 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 as alterações neurológicas e particularmente a degeneração estriatal característica dos pacientes afetados. Um aperfeiçoamento deste modelo foi proposto por Zinnanti e colaboradores com a administração via oral de uma sobrecarga de lisina aos animais (Zinnanti et al., 2006). Neste particular, foi verificado que as concentrações de AG no cérebro dos camundongos *Gcdh-/-* aumentaram significativamente e que os mesmos apresentaram lesão estriatal

semelhante aos pacientes afetados pela AG I, além de provocar a perda de seletividade da barreira hematoencefálica.

I.1.3.6. Fisiopatologia

Nos últimos anos, distintos mecanismos foram propostos para explicar a fisiopatogenia do dano cerebral da AG I. O fato de alguns pacientes excretarem altas concentrações de lactato, 3-hidroxibutirato, acetoacetato e ácidos dicarboxílicos, sugere que uma disfunção mitocondrial exerce um importante papel na neuropatologia dos pacientes acometidos pela AG I (Floret et al., 1979; Gregersen e Brandt, 1979). Neste sentido, foi demonstrado que o AG e 3HG *in vitro* alteram de maneira moderada a atividade de alguns complexos da cadeia respiratória, os níveis de fosfocreatina, a produção de CO_2, a atividade da creatina quinase (CK) e os níveis de ATP em cérebro e culturas de neurônios de ratos (Das et al., 2003; Ferreira et al., 2005b; Ferreira et al., 2007a; Kolker et al., 2002a; Kolker et al., 2002b; Latini et al., 2005a; Silva et al., 2000; Ullrich et al., 1999). Além disso, estudos *in vivo* em músculo esquelético e cérebro de ratos tratados cronicamente com AG mostraram inibições moderadas nas atividades dos complexos I-III, II, II-III e da enzima CK (Ferreira et al., 2005a; Ferreira et al., 2007b). Por outro lado, uma inibição da enzima Na^+, K^+ - ATPase pelo AG foi relatada em córtex cerebral de ratos *in vitro* (Kolker et al., 2002b), assim como em cérebro de ratos tratados cronicamente ou através de uma injeção intraestriatal desse ácido orgânico (Fighera et al., 2006; Rodrigues et al., 2013). Sauer e colaboradores também descreveram que o glutaril-CoA, diferentemente do AG e 3HG, foi capaz de inibir de maneira não-competitiva a enzima α-cetoglutarato

desidrogenase (α-CGDH) purificada (Sauer et al., 2005). Finalmente, a presença de disfunção mitocondrial foi detectada em cultura de astrócitos de ratos tratados com AG ou 3HG (Olivera et al., 2008).

Vários outros estudos *in vivo* e *in vitro* demonstraram efeitos deletérios de AG e 3HG, incluindo excitotoxicidade e estresse oxidativo. Neste contexto, estudos *in vitro* mostraram que tanto o AG (de Oliveira Marques et al., 2003) como o 3HG (Latini et al., 2002; Latini et al., 2005b) aumentam a lipoperoxidação e diminuiem as defesas antioxidantes e os níveis de glutationa reduzida em cérebro de ratos. A produção de espécies reativas de oxigênio na presença de 3HG também foi evidenciada em culturas de neurônios de telencéfalos de embriões de pinto (Kolker et al., 2001). Além disso, foi demonstrada que a administração aguda e crônica de AG aumenta a lipoperoxidação e diminuíram as defesas antioxidantes em diferentes estruturas cerebrais, fígado e eritrócitos de ratos (Latini et al., 2007).

A excitotoxicidade também é um mecanismo muito relacionado com a fisiopatogenia da AG I. Inicialmente, foi demonstrado um comprometimento da neurotransmissão GABAérgica causada pelo metabólitos acumulados na AG I (Leibel et al., 1980; Stokke et al., 1976; Wajner et al., 2004). Além disso, vários trabalhos sugerem que a neurotoxicidade da AG I possa ocorrer devido à interação dos AG e 3HG com receptores e transportadores glutamatérgicos em culturas de células e cérebro de ratos (Bjugstad et al., 2001; de Mello et al., 2001; Flott-Rahmel et al., 1997; Kolker et al., 1999, 2000; Kolker et al., 2002a; Kolker et al., 2002b; Porciuncula et al., 2000; Porciuncula et al., 2004; Rosa et al., 2004).

Outros trabalhos sugerem que uma disfunção endotelial com perda da integridade da barreira hematoencefálica esteja envolvida na lesão neurológica característica dos pacientes com AG I e outras acidemias orgânicas (Muhlhausen et al., 2006; Strauss e Morton, 2003; Zinnanti et al., 2006). Neste contexto, foi demonstrado recentemente um aumento na permeabilidade da barreira hematoencefálica em cérebro de camundongos *Gcdh-/-* (Zinnanti et al., 2014). Além disso, metabólitos da via das quinureninas, uma das rotas de catabolismo do triptofano, associados com outras substâncias acumuladas na AG I, podem também estar envolvidos na neurodegeneração dessa doença (Heyes, 1987; Lehnert e Sass, 2005; Varadkar e Surtees, 2004).

Enfatize-se que todos os resultados citados acima foram obtidos através de estudos *in vitro* e *in vivo* em ratos selvagens ou em culturas de células obtidas de animais com atividade normal da GCDH.

Estudos recentes com animais *Gcdh-/-* demonstraram que o transporte de sucinato dos astrócitos para os neurônios, via o transportador de dicarboxilatos, foi inibido pelos AG e 3HG em culturas primárias de astrócitos e neurônios de camundongos *Gcdh-/-*, possivelmente comprometendo a reposição de intermediários do ciclo do ácido cítrico (CAC) para os neurônios e, consequentemente, prejudicando principalmente a produção dos neurotransmissores glutamato e GABA, assim como de ATP (Lamp et al., 2011). Neste contexto, Zinnanti e colaboradores encontraram uma diminuição nos níveis de ATP, fosfocreatina, α-cetoglutarato, CoA, glutamato e GABA, bem como um aumento de acetil-CoA, em cérebro de camundongos *Gcdh-/* (Zinnanti et al., 2007). Além disso, foi recentemente verificado a indução de estresse oxidativo em

cérebro de camundongos *Gcdh-/-* submetidos a uma sobrecarga de lisina (Seminotti et al., 2012; Seminotti et al., 2013).

No entanto, apesar da intensa investigação, as causas da susceptibilidade frontotemporal cortical durante a gestação e da janela de vulnerabilidade estriatal durante os primeiros anos de vida, assim como o papel da disfunção mitocondrial, permanecem obscuras na patogênese da AG I.

I.1.4. Metabolismo energético cerebral

O cérebro é um dos órgãos mais ativos metabolicamente, mas possui reservas energéticas extremamente pequenas em relação a sua alta taxa metabólica (Attwell e Laughlin, 2001; Dickinson, 1996).

A glicose é o principal composto energético do cérebro (Erecinska et al., 2004). Em condições normais, o metabolismo energético nos tecidos neurais é mantido, quase que exclusivamente, pelo metabolismo oxidativo da glicose (Mergenthaler et al., 2013). A oxidação da glicose no cérebro ocorre mais rapidamente do que em outros órgãos como fígado, coração ou rins. Em contraste com outros tecidos, o cérebro não necessita de insulina para captar e oxidar a glicose. Entretanto, durante o estado de jejum, os corpos cetônicos podem substituir mais de 50% das necessidades energéticas cerebrais (Dickinson, 1996).

A oxidação da glicose através da via glicolítica forma piruvato, que é convertido a CO_2 e H_2O no CAC e na cadeia transportadora de elétrons. O acoplamento entre a cadeia transportadora de elétrons e a fosforilação oxidativa gera grande parte do ATP necessário ao cérebro (Erecinska et al., 2004).

I.1.5. Ciclo do ácido cítrico (CAC), fosforilação oxidativa, cadeia transportadora de elétrons e parâmetros respiratórios

O CAC é a via comum de oxidação dos glicídeos, aminoácidos e ácidos graxos utilizando enzimas como citrato sintase (CS), aconitase (ACO), isocitrato desidrogenase (IDH), α-CGDH, sucinato desidrogenase (SDH) e malato desidrogenase (MDH). Enfatizamos que a IDH3 é a isoforma mitocondrial e dependente de NAD em camundongos, possuindo três diferentes subunidades onde a α é a catalítica (Kim et al., 1999). 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 o processo mitocondrial 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, 2008). As mitocôndrias são corpúsculos envoltos 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 (Nelson e Cox, 2008). 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. Essa transferência de elétrons é impulsionada por um crescente potencial redox

existente entre os equivalentes reduzidos (NADH e o $FADH_2$), os complexos enzimáticos da cadeia transportadora de elétrons e o O_2, que é o aceptor final dessa cadeia de reações de oxidação.

A cadeia respiratória é composta por vários complexos enzimáticos e uma coenzima lipossolúvel, a coenzima Q ou ubiquinona. O complexo I conhecido como NADH desidrogenase ou NADH: ubiquinona oxidorredutase transfere os elétrons do NADH para a ubiquinona. O complexo II reduz a ubiquinona com elétrons do $FADH_2$ provenientes da oxidação do sucinato a fumarato no CAC. O complexo III citocromo bc_1 ou ubiquinona-citocromo *c* oxidoredutase catalisa a redução do citocromo *c* a partir da ubiquinona reduzida. Na parte final da cadeia de transporte de elétrons, o complexo IV (citocromo *c* oxidase) catalisa a transferência de elétrons de moléculas reduzidas de citocromo *c* para O_2, formando H_2O.

O fluxo de elétrons através dos complexos da cadeia respiratória é acompanhado pelo bombeamento de prótons da matriz mitocondrial para o espaço intermembrana, através dos complexos I, III e IV, gerando um potencial de membrana. Assim, cria-se um gradiente eletroquímico transmembrana que pode ser utilizado por um quinto complexo proteico, 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 potencial de membrana é dissipado pelo fluxo de prótons a favor do gradiente eletroquímico, a energia liberada é utilizada pela ATP sintase que atua como uma bomba de prótons ATP-dependente (Nelson e Cox, 2008).

Desse modo, a respiração mitocondrial pode ser estimada através da medida do consumo de O_2. Apesar do fato de que essa medida determina diretamente apenas a velocidade de uma única reação (transferência final de elétrons para O_2), muitas informações sobre outros processos mitocondriais podem ser obtidos simplesmente pela adaptação das condições de incubação. Vários passos podem ser investigados, incluindo o transporte de substratos através da membrana mitocondrial, a atividade das desidrogenases, a atividade dos complexos da cadeia respiratória, o transporte de nucleotídeos de adenina pela membrana mitocondrial, a atividade da ATP sintase e a permeabilidade da membrana mitocondrial a prótons (Nicholls e Ferguson, 2001). Experimentalmente, pode-se dividir a respiração mitocondrial em 5 estágios, conforme ilustra a figura 2. No entanto, apenas os parâmetros estados 3 e 4 são comumente utilizados. O estado 3 representa o consumo de oxigênio quando as mitocôndrias, em um meio contendo substrato oxidável, são expostas a ADP, estimulando o consumo de O_2 e produzindo ATP (estado fosforilante). O estado 4 reflete o consumo de O_2 após as mitocôndrias já terem depletado todo o ADP disponível ou podendo ser estimulado por oligomicina A (inibidor da ATP sintase), reduzindo a taxa da respiração (estado não-fosforilante) (Nicholls e Ferguson, 2001). Para que a ATP sintase esteja ativa, são necessários dois fatores: disponibilidade de ADP e potencial de membrana suficientemente alto (Nelson e Cox, 2008). Neste contexto, o acoplamento da respiração mitocondrial é definido como a capacidade da mitocôndria gerar energia (ATP) quando exposta ao ADP, ou seja, unir (acoplar) os processos de oxidação e de fosforilação, o que pode ser avaliado experimentalmente pela medida da razão de controle respiratório (RCR; razão do

estado 3 / estado 4). A dissipação do gradiente eletroquímico de prótons no espaço mitocondrial intermembrana, determinado por dano ou aumento da permeabilidade da membrana mitocondrial interna, desacopla o transporte de elétrons (oxidação) da síntese de ATP (fosforilação), resultando em um aumento do consumo de oxigênio no estado 4 (atividade respiratória aumentada) com reduzida formação de ATP (Nicholls e Ferguson, 2001). Além disso, pode-se avaliar exclusivamente a parte oxidativa e, portanto, excluindo as etapas de fosforilação, no estado desacoplado da respiração mitocondrial, adicionando-se um desacoplador (dinitrofenol, CCCP ou FCCP) ao meio de incubação (Nicholls e Ferguson, 2001).

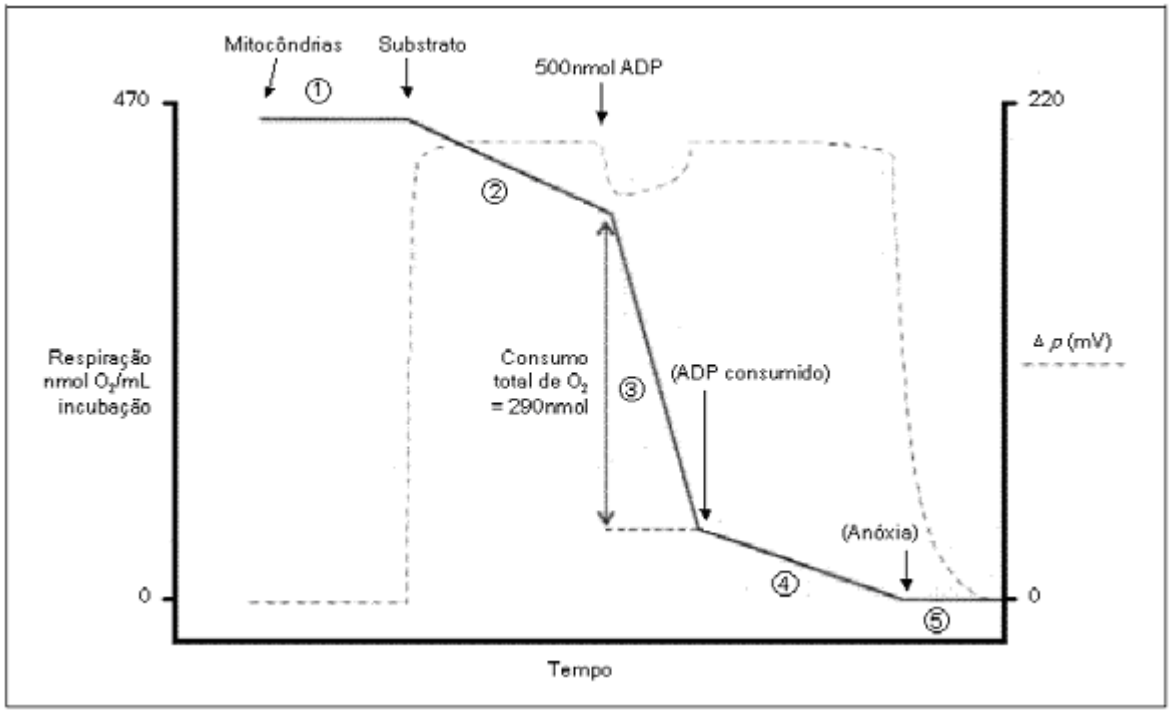

Figura 2. Estados da respiração mitocondrial. (Adaptado de Nicholls e Ferguson, 2001).

Além da regeneração do ATP, que é a sua principal função, a mitocôndria desempenha outras funções importantes. Esta organela é a principal fonte de espécies reativas de oxigênio e de defesas antioxidantes nas células (Cadenas e Davies, 2000), gerando ânions superóxido ($O^{2\bullet-}$) no espaço intermembrana pelo vazamento de elétrons que se combinam com o oxigênio molecular, principalmente no complexo III, em um processo que é dependente de potencial de membrana na matriz (Han et al., 2001). Além disso, a mitocôndria participa ativamente da homeostase celular de Ca^{2+} (Nicholls e Akerman, 1982) e está envolvida em diversos processos que levam à morte celular por apoptose, incluindo liberação de citocromo c (Liu et al., 1996). É possível medir experimentalmente, além da respiração mitocondrial, o potencial de membrana, o inchamento, a produção de peróxido de hidrogênio, a capacidade de retenção de Ca^{2+} e o conteúdo de NAD(P)H mitocondrial (Maciel et al., 2004; Saito e Castilho, 2010).

I.1.6. Creatina quinase (CK)

A CK é a enzima responsável pelo processo reversível de fosforilação da creatina formando fosfocreatina, e desfosforilação da fosfocreatina formando creatina.

Tecidos que possuem uma alta demanda de energia, como músculo esquelético, cardíaco e cérebro, apresentam uma maior concentração de CK, porque esta enzima regenera o ATP, que é muito consumido nesses tecidos (Wyss et al., 1992). Atualmente são conhecidas cinco isoformas da CK. Três são encontradas no citosol e duas na mitocôndria (CKmi). As isoformas citosólicas

formam dímeros, chamados MM-CK, MB-CK e BB-CK, compostos por dois tipos de subunidades (monômeros): o monômero M (tipo muscular) e o monômero B (tipo cerebral). A isoforma MM-CK é encontrada predominantemente em músculo esquelético adulto e no músculo cardíaco, a BB-CK está presente principalmente em tecidos neurais, e a MB-CK é somente encontrada em coração (Boehm et al., 1996; Wyss et al., 1992).

A atividade da CK e as concentrações de creatina são importantes para o tamponamento energético e para a transferência de energia dos sítios de produção de ATP para os sítios de consumo que utilizam as ATPases, evitando assim grandes variações nos níveis celulares de ATP e ADP do metabolismo celular (O'Gorman et al., 1996; Wyss et al., 1992). Alterações na atividade da CK são associadas com vários estados patológicos. A diminuição da atividade da CK no coração está relacionada à cardiomiopatias e falência cardíaca, além de pacientes com miopatias mitocondriais apresentarem mitocôndrias inchadas devido à inibição da CKmi (O'Gorman et al., 1996).

I.1.7. Na^+, K^+-ATPase

A enzima Na^+, K^+-ATPase é uma proteína transmembrana constituída principalmente por dois tipos de subunidades: a subunidade α de 110kDa, que contém os sítios catalíticos e de ligação de íons, e a subunidade β, que é uma glicoproteína de 55kDa essencial para a atividade da enzima, mas de função não totalmente esclarecida, formando uma estrutura dimérica ($\alpha\beta$)2 (Morth et al., 2007). A subunidade catalítica da Na^+, K^+-ATPase se apresenta com 3 isoformas

em mamíferos, sendo que a α1 está expressa em todo o animal, a α2 em cérebro (principalmente em astrócitos), músculo esquelético, coração e adipócitos, enquanto que a α3 principalmente em neurônios (Blanco e Mercer, 1998; Chen et al., 2013; Kawakami e Ikeda, 2006; Zhang et al., 2013).

A função dessa enzima é translocar os cátions Na^+ e K^+ através da membrana plasmática contra seus respectivos gradientes de concentração, utilizando a energia fornecida pela hidrólise de ATP. A enzima transporta simultaneamente 3 íons Na^+ para fora e 2 íons K^+ para dentro da célula. A saída de Na^+ capacita as células animais a controlar osmoticamente seu conteúdo hídrico (Aperia, 2007; Jorgensen et al., 2003). Visto que três cargas positivas são transportadas para o meio extracelular e somente duas são transportadas para o meio intracelular, o fluxo de íons Na^+ e K^+ produz um gradiente eletroquímico através da membrana celular (Kaplan, 2002). Esse gradiente é usado como fonte de energia para a despolarização e repolarização do potencial de membrana, para a manutenção e regulação do volume celular, para transporte ativo secundário dependente de íons Na^+, transporte de glicose, de aminoácidos, de neurotransmissores e outros íons (Geering, 1990). Alteração nos mecanismos que mantêm o equilíbrio entre a taxa de Na^+ e K^+ intra e extracelular pode causar graves consequências para as células do SNC (Erecinska et al., 2004), tendo sido associadas a estados de neurodegeneração como nas doenças de Alzheimer, Parkinson e esclerose lateral amiotrófica (Bagh et al., 2008; Dickey et al., 2005; Ellis et al., 2003; Vignini et al., 2007).

I.1.8. Metabolismo energético e doenças neurodegenerativas

Numerosas hipóteses têm sido propostas para explicar a fisiopatologia de doenças neurodegenerativas mais comuns, como as doenças de Alzheimer e Parkinson sem, no entanto, obter até o momento uma explicação satisfatória para o dano cerebral dessas doenças. Entretanto, acredita-se que alterações do metabolismo energético, indução de estresse oxidativo e neurotoxicidade mediada por receptores glutamatérgicos do tipo NMDA, ou, possivelmente, um somatório desses fatores possam estar envolvidos na neurodegeneração (Camins et al., 2008; Nakamura e Lipton, 2011; Rose e Henneberry, 1994). Uma das hipóteses é de que alterações na cadeia transportadora de elétrons seria o evento etiológico primário na maioria dessas doenças (Chaturvedi e Flint Beal, 2013; Parker et al., 1990; Swerdlow et al., 1998).

O cérebro é altamente dependente de energia para seu funcionamento normal e a mitocôndria é a organela intracelular que mantém os suprimentos de energia para o cérebro. Uma alteração funcional nessa organela pode levar, portanto, a consequências patológicas aos neurônios e astrócitos (Beal, 1995) (Bowling e Beal, 1995; Davis et al., 1995). Assim, numerosas evidências relacionam doenças neurodegenerativas a um comprometimento do metabolismo energético. Estudos anteriores demonstraram uma diminuição na atividade do complexo I da cadeia respiratória em cérebros *postmortem* de pacientes portadores de doença de Parkinson (Gautier et al., 2013; Janetzky et al., 1994; Parker et al., 2008). Também há relatos de defeitos nas atividades dos complexos

II e III da cadeia respiratória e da enzima α-CGDH nessa doença (Mizuno et al., 1990; Shen et al., 2000).

No que diz respeito a mais comum dentre as doenças neurodegenerativas, a doença de Alzheimer, já foi relatado uma redução na atividade do complexo IV da cadeia respiratória (Bobba et al., 2013; Maurer et al., 2000). Estudos em cérebros postmortem de pacientes portadores dessa doença demonstraram uma diminuição nas atividades do complexo enzimático da piruvato desidrogenase e da α-CGDH (Gibson et al., 1988; Gibson et al., 2012; Mastrogiacomo et al., 1993; Perry et al., 1980).

I.2. OBJETIVOS

I.2.1. Objetivo geral

O objetivo do presente trabalho foi o de investigar importantes parâmetros da homeostase energética celular em tecidos cerebrais (córtex cerebral, estriado e hipocampo) e periféricos (coração e músculo esquelético), bem como alterações histológicas no cérebro de camundongos *Gcdh-/-* e selvagens (WT) submetidos a uma dieta normal ou a uma sobrecarga de lisina (injeção intraperitoneal ou dieta rica em lisina), visando a uma melhor compreensão da fisiopatologia do dano tecidual na AG I.

I.2.2. Objetivos específicos

1. Investigar o efeito de uma sobrecarga aguda de lisina (injeção intraperitoneal

de 8 µmol/g de lisina) sobre as atividades dos complexos I-III, II, II-III e IV da cadeia respiratória, da CK e da Na^+, K^+ - ATPase em cérebro, coração e músculo esquelético de camundongos *Gcdh-/-* e WT com 15 dias de vida.

2. Investigar o efeito de uma sobrecarga de lisina na dieta (4,7 %) por 60 horas sobre as atividades dos complexos I-III, II, II-III e IV da cadeia respiratória, da CK e da Na^+, K^+ - ATPase, assim como sobre os parâmetros respiratórios (estados 3 e 4, RCR e estado desacoplado) em tecidos cerebrais (cérebro total, córtex cerebral, estriado e hipocampo) de camundongos *Gcdh-/-* e WT com 30 dias de vida.

3. Investigar o efeito de uma sobrecarga de lisina na dieta (4,7 %) por 60 horas sobre as atividades das enzimas do CAC CS, ACO, IDH, α-CGDH, SDH e MDH, assim como a liberação de lactato, os parâmetros respiratórios (estados 3 e 4, RCR e estado desacoplado) e o potencial de membrana mitocondrial na presença e ausência de Ca^{2+} em preparações mitocondriais de córtex cerebral e estriado de camundongos *Gcdh-/-* e WT com 30 dias de vida.

4. Investigar alterações histológicas em córtex cerebral e estriado de camundongos *Gcdh-/-* e WT com 30, 60 e 90 dias de vida sob o efeito de uma sobrecarga de lisina na dieta (4,7 %) por períodos variáveis (60 horas e 30 dias).

PARTE II

Artigos Científicos

Capítulo I

Marked reduction of Na^+, K^+-ATPase and creatine kinase activities induced by acute lysine administration in glutaryl-CoA dehydrogenase deficient mice

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Marked reduction of Na^+, K^+-ATPase and creatine kinase activities induced by acute lysine administration in glutaryl-CoA dehydrogenase deficient mice

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ABSTRACT

Glutaric acidemia type I (GA I) is an inherited neurometabolic disorder caused by a severe deficiency of the mitochondrial glutaryl-CoA dehydrogenase activity leading to accumulation of predominantly glutaric (GA) and 3-hydroxyglutaric (3HGA) acids in the brain and other tissues. Affected patients usually present with hypotonia and brain damage and acute encephalopathic episodes whose pathophysiology is not yet fully established. In this study we investigated important parameters of cellular bioenergetics in brain, heart and skeletal muscle from 15-day-old glutaryl-CoA dehydrogenase deficient mice (*Gcdh*$^{-/-}$) submitted to a single intra-peritoneal injection of saline (Sal) or lysine (Lys — 8 μmol/g) as compared to wild type (WT) mice. We evaluated the activities of the respiratory chain complexes II, II-III and IV, α-ketoglutarate dehydrogenase (α-KGDH), creatine kinase (CK) and synaptic Na^+, K^+-ATPase. No differences of all evaluated parameters were detected in the *Gcdh*$^{-/-}$ relatively to the WT mice injected at baseline (Sal). Furthermore, mild increases of the activities of some respiratory chain complexes (II-III and IV) were observed in heart and skeletal muscle *of Gcdh*$^{-/-}$ and WT mice after Lys administration. However, the most marked effects provoked by Lys administration were marked decreases of the activities of Na^+, K^+-ATPase in brain and CK in brain and skeletal muscle of *Gcdh*$^{-/-}$ mice. In contrast, brain α-KGDH activity was not altered in WT and *Gcdh*$^{-/-}$ injected with Sal or Lys. Our results demonstrate that reduction of Na^+, K^+-ATPase and CK activities may play an important role in the pathogenesis of the neurodegenerative changes in GA I.

1. Introduction

Glutaryl-CoA dehydrogenase (GCDH) deficiency or glutaric acidemia type I (GA I) (McKusick 231670) is an autosomal recessive neurometabolic disease biochemically characterized by the accumulation of glutaric acid (GA), and to a lesser extent 3-hydroxyglutaric acid (3HGA) and glutaconic acid in body fluids and tissues [1–3]. Clinically, the disease is characterized by macrocephaly with frontotemporal atrophy at birth and by marked dystonia and diskinesia, following encephalopathic episodes triggered by catabolic events, such as infections, fever and fasting, when the accumulating metabolites can reach millimolar concentrations [1,4,5]. However, progressive neurological symptoms with mental developmental delay and hypotonia without apparent acute episodes may also occur in a considerable number of patients [5–9]. Neuroradiological imaging shows, besides basal ganglia degeneration, widened Sylvian fissures, cortical atrophy with frontotemporal volume loss, delayed myelination, ventriculomegaly and subdural hemorrhages [1,5,6,8,10,11]

Although the pathogenesis of the brain damage in GA I is not fully established, accumulating evidence from in vitro and in vivo experiments performed in brain tissue and cultivated neural cells from

Abbreviations: CK, creatine kinase; DCIP, dichloroindophenol; EDTA, ethylenediaminetetracetic acid; GA, glutaric acid; GA I, glutaric acidemia type I; GCDH, glutaryl-CoA dehydrogenase; *Gcdh*$^{-/-}$, glutaryl-CoA dehydrogenase deficient mice; 3HGA, 3-hydroxyglutaric acid; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethane-sulfonic acid]; α-KGDH, α-ketoglutarate dehydrogenase; KO, knockout; Lys, lysine; Sal, saline; SPSS, Statistical Package for the Social Sciences; TCA, tricarboxylic acid; WT, wild type.

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rodents and chick suggest that excitotoxicity [6,12–21], oxidative stress [22–31] and cellular bioenergetic dysfunction [2,32–39] are involved in the brain damage of GA I patients. It is emphasized that these studies were carried out in animal tissues with normal GCDH activity.

A knockout (KO) model of GA I was developed in mice by replacing the GCDH gene with an in-frame beta-galactosidase cassette in order to investigate pathomechanisms underlying brain damage in this disorder [40]. Glutaryl-CoA dehydrogenase deficient (*Gcdh*$^{-/-}$) mice displayed vacuolization in the frontal cortex, but did not develop striatal damage typical of the human disease even when submitted to metabolic or infectious stress. It was subsequently found that exposure of these animals to high protein or lysine (Lys) intake resulted in striatal damage, besides neuronal loss, myelin disruption and gliosis mostly in the striatum and deep cortex [41].

Considering that various works have emphasized the role of bioenergetics dysfunction on the prominent neurologic findings of GA I [2,42] and that the toxic organic acids accumulating in this disorder are likely to induce secondary pathological changes in the brain [39,41], in the present study we further investigated the role of cellular bioenergetics alterations in the pathophysiology of GA I. We evaluated central components of mitochondrial energy production, transfer and utilization, by measuring the activities of the respiratory chain complexes II, II-III and IV, α-ketoglutarate dehydrogenase (α-KGDH), creatine kinase (CK) and Na^+,K^+-ATPase in brain, heart and skeletal muscle from 15-day-old *Gcdh*$^{-/-}$ mice on standard mouse chow, and after acute Lys administration in order to clarify whether disturbance of cellular bioenergetics is involved in the pathogenesis and more specifically in the brain damage of GA.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Solutions were prepared on the day of the experiments and the pH was adjusted to 7.2–7.4 with the appropriate buffers for each technique.

2.2. Animals

Fifteen-day-old *Gcdh*$^{-/-}$ and wild type (WT) mice littermate controls, both of 129SvEv background [40], were used in the experiments. We used 15-day-old animals because this age corresponds to very young animals, matching to humans of approximately 3–4 years of life. In addition, prior studies by Zinnanti et al. have shown that when fed a high lysine diet, 4-week-old Gcdh$^{-/-}$ mice accumulate high levels of glutaric acid and develop an acute brain injury, whereas 8-week old mice accumulate less glutaric acid and do not suffer from acute brain injury [39,41]. This age dependent sensitivity has been interpreted to indicate that the blood brain barrier is more permeable to Lys in younger animals, resulting in higher levels of GA and 3HGA being formed in the brain. The mice were generated from heterozygous and maintained at Unidade de Experimentação Animal (UEA) of the Hospital de Clínicas de Porto Alegre (HCPA). The animals were maintained on a 12:12 h light/dark cycle (lights on 07.00–19.00 h) in air conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil).

2.3. Ethical statement

This study was performed in strict accordance with the Principles of Laboratory Animal Care, National Institute of Health of United States of America, NIH, publication No. 85-23, revised in 1996 and approved by the Ethical Committee for the Care and Use of Laboratory Animals of HCPA. All efforts were also made to use the minimal number of animals necessary to produce reliable scientific data.

2.4. Lysine (Lys) administration

The WT and *Gcdh*$^{-/-}$ animals were administered with a single intraperitoneal injection of saline (Sal) or Lys (8 μmol/g) solution in order to investigate whether an acute Lys overload could disturb bionergetics in a model of GCDH deficiency. We have previously shown that this dose of lysine results in a significant increase in GA and 3HGA and induces an acute oxidative stress in the brains of *Gcdh*$^{-/-}$ mice [52]. Enzyme activities were measured in tissues collected 4 h after lysine injection. We observed that approximately 20% of Lys-treated *Gcdh*$^{-/-}$ mice became less active within 4 h of injection, and these mice were not used for analysis. On the other hand, we also observed in a distinct set of *Gcdh*$^{-/-}$ mice from the same background that approximately the same percentage of animals became hypoactive and died 12 h after Lys injection. These animals were also not used in the assays, so that the survival rate of the mice used for the biochemical analyses was 100%.

2.5. Tissue preparation

The mice were anesthetized with a mixture of ketamine (90 mg/kg) and xilazine (10 mg/kg) and intracardiacally perfused during 5 min with Sal solution. After perfusion, brain, heart and skeletal muscle were rapidly removed and placed on a Petri dish on ice.

For the determination of respiratory chain complexes, α-KGDH and total CK activities, the olfactory bulb, pons, medulla, and cerebellum were discarded, and the forebrain, as well as the heart and skeletal muscle, were used. The tissues were homogenized in 19 vol (1:20, w/v) of SETH buffer, pH 7.4 (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 UI mL^{-1} heparin). Homogenates were centrifuged at 800 × *g* for 10 min at 4 °C to discard nuclei and cell debris. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and used to measure these parameters.

For the determination of Na^+, K^+-ATPase activity, the forebrain was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. Synaptic plasma membranes were then prepared according to the method of Jones and Matus (1974) [43] using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000 × *g* for 2 h, the fraction at the 0.8–1.0 mM sucrose interface was taken as the synaptic membrane enzyme preparation.

2.6. Spectrophotometric analysis of the respiratory chain complexes I–IV activities

The activities of the various complexes of the respiratory chain were measured in the presence of approximately 30 μg of protein. Succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate:cytochrome *c* oxidoreductase (complex II–III) activities were determined according to Fischer et al. (1985) [44]. The cytochrome *c* oxidase (complex IV) was assayed according to Rustin et al. (1994) [45]. The activities of the respiratory chain complexes were calculated as nmol.min^{-1}.mg $protein^{-1}$.

2.7. Spectrophotometric analysis of creatine kinase (CK) activity

CK activity was measured in total homogenates according to Hughes (1962) [46] with slight modifications. 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. 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) [46]. The color was developed by the addition of 0.1 mL 20% α-naphtol and 0.1 mL 20% diacetyl in a final volume of 1.0 mL and read after 20 min at λ = 540 nm. Results were calculated as μmol of creatine.min^{-1}.mg protein^{-1}.

2.8. Spectrophotometric analysis of Na^+, K^+-ATPase activity

The reaction mixture for the Na^+, K^+-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, and purified synaptic membranes (approximately 3 μg of protein) in a final volume of 200 μL. The enzymatic assay occurred at 37 °C during 5 min and started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. The reaction was stopped by the addition of 200 μL of 10% trichloroacetic acid. Mg^{2+}-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays [47]. Released inorganic phosphate was measured by the method of Chan et al. (1986) [48]. Enzyme-specific activities were calculated as nmol Pi released^{-1}.min^{-1}.mg protein.

2.9. Fluorimetric analysis of α-ketoglutarate dehydrogenase (α-KGDH) activity

The activity of α-KGDH was evaluated according to Tretter and Adam-Vizi (2000) [49]. The reduction of NAD^+ was recorded in a Hitachi F-4500 spectrofluorometer at wavelengths of excitation and emission of 340 and 466 nm, respectively. This activity were calculated as nmol.min^{-1}.mg protein^{-1}.

2.10. Protein determination

Protein levels were measured by the method of Lowry et al. (1951) [50] using bovine serum albumin as standard.

2.11. Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in triplicate and the mean was used for statistical calculations. Data were analyzed using the Student's *t* test for unpaired samples. Only significant *t* values are shown in the text. Differences between groups were rated significant at $P<0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

3.1. Respiratory chain activities were mildly increased in heart and skeletal muscle of lysine (Lys)-treated $Gcdh^{-/-}$ mice

The respiratory chain functioning was first tested as a measure of oxidative phosphorylation in brain, heart and skeletal muscle from 15-day-old $Gcdh^{-/-}$ mice submitted to an acute intraperitoneal injection of Sal or Lys (8 μmol/g). We therefore determined the activities of the respiratory chain complexes II, II–III and IV in $Gcdh^{-/-}$ and WT animals receiving Sal or Lys. It was verified that the respiratory chain complex II–III activity was mildly and significantly increased in heart [$t_{(8)} = -2.446$; $P<0.05$] (Fig. 1B) and skeletal muscle [$t_{(8)} = -2.212$; $P<0.05$] (Fig. 1C) from Lys-treated $Gcdh^{-/-}$ mice as compared to Lys-treated WT. Furthermore, complex IV activity from the heart of Lys-treated WT mice (Fig. 1B) was also mildly increased when compared to Sal-treated WT [$t_{(8)} = -3.433$; $P<0.01$]. In contrast, no differences in the respiratory chain complex activities were observed in the brain of these animals (Fig. 1A).

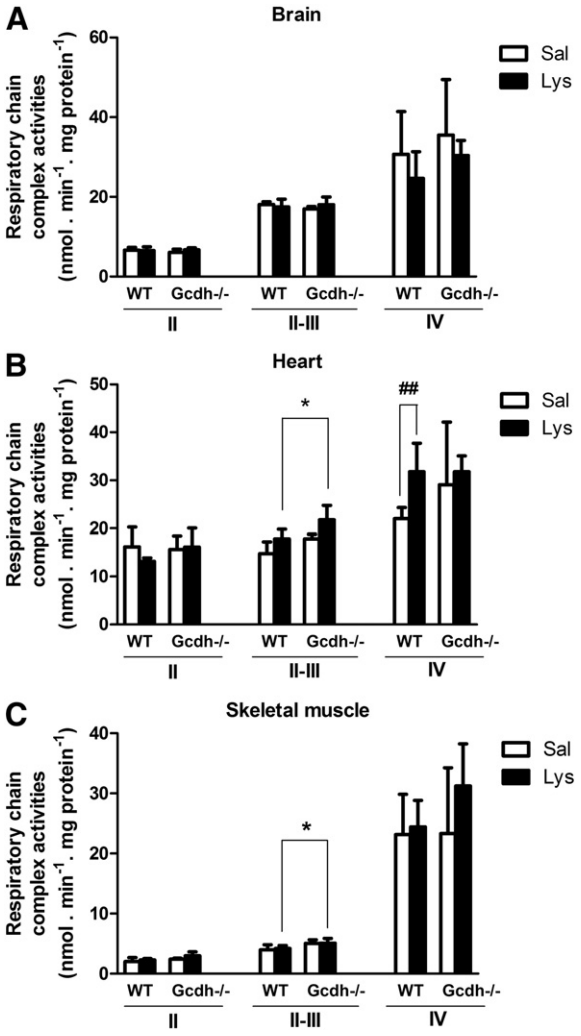

Fig. 1. Evaluation of the activities of the respiratory chain complexes II–IV in brain (A), heart (B) and skeletal muscle (C) from wild type (WT) and glutaryl-CoA dehydrogenase deficient mice ($Gcdh^{-/-}$) on a standard mouse chow injected with saline (Sal) or lysine (Lys — 8 μmol/g). The parameters were measured 4 h after injection. The activity of complex II is expressed as nmol DCIP reduced.min^{-1}.mg protein^{-1}, II–III as nmol cytochrome *c* reduced.min^{-1} and IV as nmol cytochrome *c* oxidized.min^{-1}.mg protein^{-1}. Values are mean ± standard deviation for three to five independent experiments (animals) performed in triplicate. $^{*}P<0.05$ for Lys-treated $Gcdh^{-/-}$ compared to Lys-treated WT mice; $^{\#\#}P<0.01$ for Lys-treated WT compared to Sal-treated WT mice (Student's *t* test for unpaired samples).

3.2. Creatine kinase (CK) activity was markedly inhibited in brain and skeletal muscle of lysine (Lys)-treated $Gcdh^{-/-}$ mice

Next we measured CK activity, a crucial enzyme involved in intracellular ATP transfer and buffering, in brain, heart and skeletal muscle from $Gcdh^{-/-}$ and WT mice, submitted to Sal or Lys acute treatment. A significant inhibition of CK was obtained in brain [$t_{(7)} = 3.024$; $P<0.05$] (Fig. 2A) and skeletal muscle [$t_{(5)} = 5.187$; $P<0.01$] (Fig. 2C), but not in heart (Fig. 2B), from Lys-treated $Gcdh^{-/-}$ mice, as compared to Lys-treated WT. Furthermore, Lys injection provoked a reduction of CK activity in brain [$t_{(6)} = 1.568$; $P<0.05$] (Fig. 2A) and skeletal muscle [$t_{(4)} = 3.732$; $P<0.05$] (Fig. 2C) of $Gcdh^{-/-}$ mice relatively to Sal-injected $Gcdh^{-/-}$ mice. Taken together, it seems that CK activity is more sensitive to Lys injection in $Gcdh^{-/-}$ mice than in WT mice.

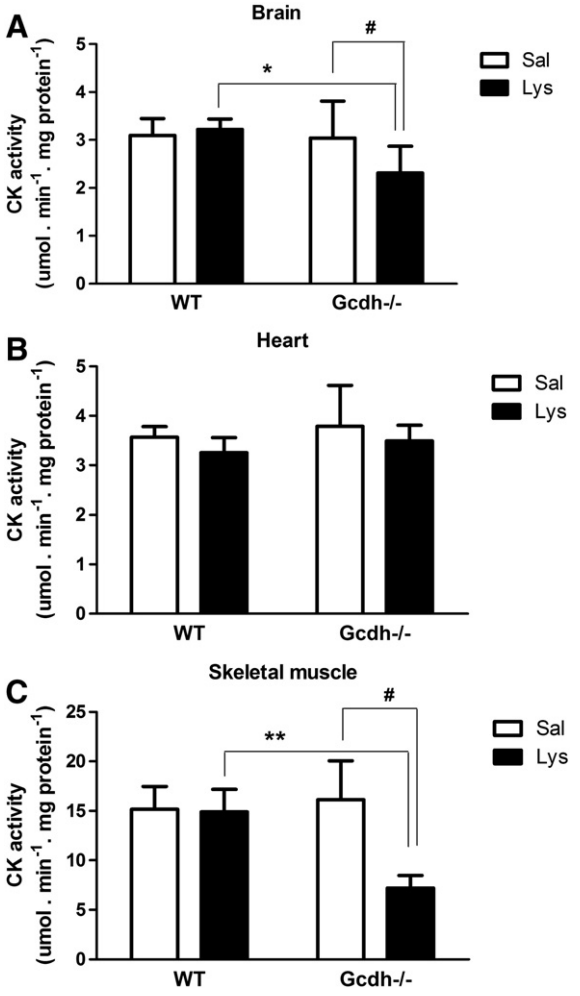

Fig. 2. Evaluation of creatine kinase (CK) activity in brain (A), heart (B) and skeletal muscle (C) from wild type (WT) and glutaryl-CoA dehydrogenase deficient mice (*Gcdh*−/−) on a standard mouse chow injected with saline (Sal) or lysine (Lys — 8 μmol/g). This parameter was measured 4 h after the injection. Values are mean ± standard deviation for three to five independent experiments (animals) performed in triplicate and are expressed as μmol creatine.min^{-1}.mg protein^{-1}. $^{*}P<0.05$, $^{**}P<0.01$ for Lys-treated *Gcdh*$^{-/-}$ compared to Lys-treated WT mice; $^{\#}P<0.05$ for Lys-treated *Gcdh*$^{-/-}$ compared to Sal-treated *Gcdh*$^{-/-}$ mice (Student's *t* test for unpaired samples).

3.3. Na^+, K^+-ATPase activity was significantly inhibited in brain of lysine (Lys)-treated *Gcdh*$^{-/-}$ mice

It was also observed that synaptic membrane Na^+, K^+-ATPase activity, which is important for neurotransmission, was markedly reduced in brain from Lys-treated *Gcdh*$^{-/-}$ mice as compared to Lys-treated WT [$t_{(7)} = 2.370$; $P<0.05$] and Sal-treated *Gcdh*$^{-/-}$ mice [$t_{(5)} = 2.351$; $P<0.05$] (Fig. 3). The data suggest that this enzyme activity is also more responsive to Lys treatment in *Gcdh*$^{-/-}$ mice as compared to normal mice.

3.4. α-Ketoglutarate dehydrogenase (α-KGDH) activity was not altered in *Gcdh*$^{-/-}$ mice

Finally, we verified that α-KGDH activity, which is a key and a rate-controlling enzyme of the tricarboxylic acid (TCA) cycle, did not change in *Gcdh*$^{-/-}$ and WT mice submitted to Sal or Lys acute administration (Table 1).

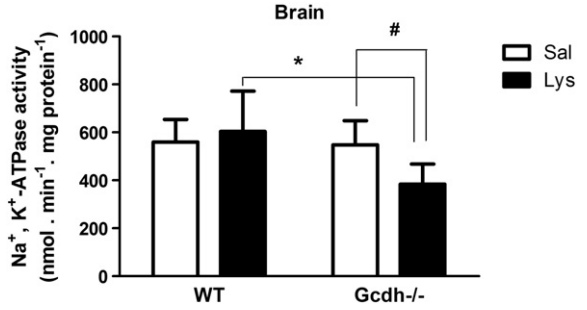

Fig. 3. Evaluation of Na^+, K^+-ATPase activity in brain from wild type (WT) and glutaryl-CoA dehydrogenase deficient mice (*Gcdh*$^{-/-}$) on a standard mouse chow injected with saline (Sal) or lysine (Lys — 8 μmol/g). This parameter was measured 4 h after the injection. Values are mean ± standard deviation for three to five independent experiments (animals) performed in triplicate and are expressed as nmol Pi.min^{-1}.mg protein^{-1}. $^{*}P<0.05$ for Lys-treated *Gcdh*$^{-/-}$ compared to Lys-treated WT mice; $^{\#}P<0.05$ for Lys-treated *Gcdh*$^{-/-}$ compared to Sal-treated *Gcdh*$^{-/-}$ mice (Student's *t* test for unpaired samples).

4. Discussion

Impairment of cellular bioenergetics has been proposed as an important pathomechanism of brain damage in GA I patients [2,6,36,39,51], but this is still being debated. However, this presumption was based on experiments performed on fresh cerebral cortex and striatum and on cell cultures from rat and chick brain embryo with normal GCDH activity, which makes the pathophysiological relevance of these works uncertain.

Therefore, the aim of the present investigation was to evaluate important parameters of mitochondrial metabolism regarding energy production, transfer and utilization namely the activities of the respiratory chain complexes, α-KGDH, CK and Na^+,K^+, ATPase in brain, skeletal muscle and heart of *Gcdh*$^{-/-}$ mice on a normal chow (0.9% Lys) and after Lys administration.

First, we observed no significant differences in the activity of respiratory chain enzymes, α-KGDH, CK, or Na^+, K^+-ATPase in mitochondrial-enriched tissues (brain, heart and skeletal muscle) between *Gcdh*$^{-/-}$ and WT mice fed with a standard mouse chow (Sal). This is in line with a study showing no alterations of the respiratory chain activities in brain, as well as in liver, skeletal and heart muscle of *Gcdh*$^{-/-}$ mice when compared to WT animals [36]. Next, we evaluated whether *Gcdh*$^{-/-}$ mice are more sensitive than WT mice to acute Lys overload by analyzing the same parameters of mitochondrial homeostasis.

Mild increases of the activities of some respiratory chain complexes (II–III and IV) in heart and skeletal muscle *of Gcdh*$^{-/-}$ and WT mice were observed after Lys administration. Considering that enhanced activity of the respiratory chain was previously reported in skeletal muscle from rats treated with GA [38], it is possible that Lys administration induced elevation of GA and 3HGA concentrations ultimately leading to mild elevation of these activities in cardiac and

Table 1
α-Ketoglutarate dehydrogenase (α-KGDH) activity in rat brain from wild type (WT) and glutaryl-CoA dehydrogenase deficient mice (*Gcdh*$^{-/-}$) on a standard mouse chow injected with saline (Sal) or lysine (Lys — 8 μmol/g).

α-KGDH activity		
	Sal	Lys
WT	15.15 ± 2.69	17.67 ± 5.07
Gcdh$^{-/-}$	16.33 ± 3.41	14.75 ± 3.42

Values are mean ± standard deviation of three to five independent experiments (animals) performed in triplicate and are expressed as nmol NADH.min^{-1}.mg.protein^{-1}. No significant differences were detected between the various groups (Student's *t* test for unpaired samples).

skeletal muscle of the animals. In this particular, we have recently observed that Lys injection leads to elevation of brain GA concentrations in *Gcdh*$^{-/-}$ mice [52].

We also found that the activity of endogenous brain α-KGDH, a rate-controlling enzyme of the TCA cycle, was not altered at baseline (Sal) in *Gcdh*$^{-/-}$ mice, or following Lys injection. These data are apparently in conflict with previous in vitro results showing that glutaryl-CoA strongly inhibits α-KGDH from porcine heart [36]. However, we must consider that the observed inhibitory effect was achieved in vitro and on a purified commercial α-KGDH preparation obtained from a distinct species and tissue [36]. We cannot also rule out the possibility that lower glutaryl-CoA concentrations were reached in brain of *Gcdh*$^{-/-}$ mice after Lys injection as compared to the doses of glutaryl-CoA tested (0.25–2.0 mM) by Sauer and collaborators (2005) [36].

A novel and interesting finding of the present investigation was that CK activity was markedly diminished in brain and skeletal muscle from Lys-treated *Gcdh*$^{-/-}$ mice, suggesting that intracellular ATP transfer and buffering is compromised in the *Gcdh*$^{-/-}$ mice that received Lys administration. Consistent with this observation, Zinnanti and colleagues found markedly decreased concentrations of phosphocreatine in the brains of *Gcdh*$^{-/-}$ mice on a high lysine diet [39]. Our results are also in accord with other studies showing an inhibition of CK by GA, which was prevented by glutathione (GSH), in rat midbrain in vitro [33] and in vivo in skeletal muscle of rats chronically-treated with GA [38]. Considering that CK plays an important role in cellular energy homeostasis, this observation suggests that the severe brain injury and hypotonia presented by GA I patients may in part be the result of decreased activity of this enzyme.

We also observed that Na^+, K^+-ATPase activity was also strongly inhibited in brain from Lys-treated *Gcdh*$^{-/-}$ mice. This enzyme is necessary to maintain neuronal excitability and cellular volume control through the generation and maintenance of the membrane potential by the active transport of sodium and potassium ions in the central nervous system. It is present at high concentrations in the brain, consuming about 40–50% of the ATP generated in this tissue, highlighting its importance for normal brain functioning. Indeed, reduction of this activity is related to neuronal damage in rat and human brain [53,54]. Furthermore, excitotoxicity and epilepsy have been related to a diminution of Na^+, K^+-ATPase activity [54,55]. Our present data, allied to previous works showing that GA and 3HGA inhibit in vitro this enzyme in primary neuronal cultures from chick embryo telencephalon and in rat brain [14,27], as well as alter glutamate uptake and induces glutamate receptor activation [13,15,17,19,20,27,56], reinforce the hypothesis that excitotoxicity may represent an important mechanism of brain damage in GA I.

Regarding to the mechanisms by which CK and Na^+, K^+-ATPase were found to be inhibited in *Gcdh*$^{-/-}$ mice after Lys overload, it has been extensively reported that these activities are very susceptible to free radical attack [53,57–63]. Furthermore, considering that previous studies demonstrated that the major metabolites accumulating in GA I provoke oxidative damage in the brain [23,25,26,28,36], we may presume that increased oxidative stress may underlie the inhibitions of CK and Na^+, K^+-ATPase activities in the *Gcdh*$^{-/-}$ animals submitted to Lys overload. This conclusion is in accordance with a recent publication showing that oxidative stress is elicited in the brain of *Gcdh*$^{-/-}$ after Lys supplementation [52].

Otherwise, it could be tentatively postulated that the results of the present study were achieved due to Lys accumulation. This is unlikely, since Lys administration did not change the mitochondrial parameters of homeostasis evaluated in WT mice. Therefore, we postulate that the observed effects were probably caused by the increase in tissue concentrations of GA and/or 3HGA, which take place only in *Gcdh*$^{-/-}$ mice [52].

Previous studies of the effect of GA and 3HGA on parameters of energy metabolism have generally reported in vitro effects of the addition of supraphysiologic levels of these metabolites to WT rodent tissues [32,33,35,37,64]. In contrast, our data were obtained in a knock out model of GA I submitted to a metabolic stress with Lys overload in which the concentrations of the major accumulating metabolites, especially GA, are similar to those observed in glutaric acidemia patients [40,52]. Consequently, it is likely that the data obtained in the present work better mimic the in vivo conditions in human GA I patients.

In conclusion, we report for the first time that acute Lys supplementation to developing *Gcdh*$^{-/-}$ mice provokes marked reduction in the activities of the important enzyme activities Na^+, K^+-ATPase (brain) and CK (brain and skeletal muscle), as well as mild increases in the activities of some complexes of the respiratory chain (heart and skeletal muscle). Our present data showing impairment of bioenergetics in the brain and skeletal muscle of *Gcdh*$^{-/-}$ mice following Lys administration may possibly explain the clinical observations that therapies aimed at reducing brain Lys uptake are effective for treatment of GA I. Finally, considering that CK and Na^+, K^+-ATPase activities are crucial for normal energy transfer and for the maintenance of membrane potential necessary for neurotransmission, respectively, these results indicate that disruption of intracellular energy transfer and neurotransmission may represent mechanisms responsible for the brain damage and neurologic abnormalities observed in patients affected by GA I.

5. Conclusions

Acute lysine overload in *Gcdh*$^{-/-}$ mice provokes a disturbance of cellular bioenergetics through inhibition of creatine kinase (brain and skeletal muscle) and Na^+, K^+-ATPase (brain) activities.

Acknowledgments

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Capítulo II

Reduction of Na^+, K^+-ATPase activity and expression in cerebral cortex of glutaryl-CoA dehydrogenase deficient mice: a possible mechanism for brain injury in glutaric aciduria type I

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Reduction of Na^+, K^+-ATPase activity and expression in cerebral cortex of glutaryl-CoA dehydrogenase deficient mice: A possible mechanism for brain injury in glutaric aciduria type I

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ABSTRACT

Mitochondrial dysfunction has been proposed to play an important role in the neuropathology of glutaric acidemia type I (GA I). However, the relevance of bioenergetics disruption and the exact mechanisms responsible for the cortical leukodystrophy and the striatum degeneration presented by GA I patients are not yet fully understood. Therefore, in the present work we measured the respiratory chain complexes activities I-IV, mitochondrial respiratory parameters state 3, state 4, the respiratory control ratio and dinitrophenol (DNP)-stimulated respiration (uncoupled state), as well as the activities of α-ketoglutarate dehydrogenase (α-KGDH), creatine kinase (CK) and Na+, K+-ATPase in cerebral cortex, striatum and hippocampus from 30-day-old *Gcdh* −/− and wild type (WT) mice fed with a normal or a high Lys (4.7%) diet. When a baseline (0.9% Lys) diet was given, we verified mild alterations of the activities of some respiratory chain complexes in cerebral cortex and hippocampus, but not in striatum from *Gcdh* −/− mice as compared to WT animals. Furthermore, the mitochondrial respiratory parameters and the activities of α-KGDH and CK were not modified in all brain structures from *Gcdh* −/− mice. In contrast, we found a significant reduction of Na^+, K^+-ATPase activity associated with a lower degree of its expression in cerebral cortex from *Gcdh* −/− mice. Furthermore, a high Lys (4.7%) diet did not accentuate the biochemical alterations observed in *Gcdh* −/− mice fed with a normal diet. Since Na^+, K^+-ATPase activity is required for cell volume regulation and to maintain the membrane potential necessary for a normal neurotransmission, it is presumed that reduction of this enzyme activity may represent a potential underlying mechanism involved in the brain swelling and cortical abnormalities (cortical atrophy with leukodystrophy) observed in patients affected by GA I.

1. Introduction

Glutaric acidemia type I (GA I, McKusick 23167; OMIM #231670) is an autosomal recessive neurometabolic disease caused by severe deficiency of the activity of the mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH) (EC 1.3.99.7), which is involved in the catabolic pathway of lysine (Lys), hydroxylysine and tryptophan [1]. This defect leads to increased concentrations of glutaric acid (GA), 3-hydroxyglutaric acid (3HGA), glutaconic acid and glutarylcarnitine in the body fluids and tissues [2,3]. GA I patients usually present macrocephaly and frontotemporal atrophy at birth and commonly develop acute bilateral striatal degeneration during catabolic events.

Abbreviations: CK, creatine kinase; DCIP, dichloroindophenol; DNP, dinitrophenol; EDTA, ethylenediaminetetracetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′, N′-tetraacetic acid; GA, glutaric acid; GA I, glutaric acidemia type I; GCDH, glutaryl-CoA dehydrogenase; *Gcdh* −/−, glutaryl-CoA dehydrogenase deficient mice; 3HGA, 3-hydroxyglutaric acid; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethane-sulfonic acid]; α-KGDH, α-ketoglutarate dehydrogenase; KO, knockout; Lys, lysine; RT-qPCR, quantitative real time polymerase chain reaction; SPSS, Statistical Package for the Social Sciences; TCA, tricarboxylic acid; WT, wild type.

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Progressive cortical leukodystrophy and striatal lesions without documented acute metabolic events with encephalopathy are also found in 10 to 20% of patients with neurological symptoms [4–7].

A great body of data have suggested that excitotoxicity [8–18], oxidative stress [19–28] and mitochondrial dysfunction [29–37] are involved in the brain injury of GA I. However, the relevance of energy homeostasis disruption in the brain damage in this disease is not yet established. Although some investigators proposed that this pathomechanism is crucial to explain the development of neurological symptoms and especially striatum degeneration in GA I patients [30,34], experimental studies revealed that GA and 3HGA caused only mild alterations of mitochondrial homeostasis in the brain [29,31–33,35,36].

Recently a knockout (KO) GA I model was developed in mice by replacing the glutaryl-CoA dehydrogenase (GCDH) gene with an in-frame beta-galactosidase cassette [38]. Glutaryl-CoA dehydrogenase deficient mice (*Gcdh* −/−) presented increased cerebral, blood and urine GA and 3HGA levels and displayed vacuolization in the frontal cortex (spongiform leukoencephalopathy). However, the animals did not develop striatal damage typical of the human disease even when submitted to metabolic or infectious stress. This model was later improved by exposing these mice to high protein or Lys intake, which provoked neuronal loss, myelin disruption and gliosis mostly in the striatum and deep cortex [37,39]. Oral Lys overload to weaning (4-week-old) *Gcdh* −/− mice resulted in a predominant increase of brain Lys and GA concentrations after 48 h of Lys exposure. It was also seen a simultaneous decrease of Lys and increase of brain GA levels, indicating GA formation from Lys in the brain. These investigators suggested that the cortical and particularly striatal lesions developed in the *Gcdh* −/− animals submitted to Lys overload during 48–72 h were probably due to the increase of brain GA concentrations [37,39].

The major aim of the present study was to evaluate important parameters of bioenergetics, such as the activities of the respiratory chain complexes I–III, II, II–III and IV, the respiratory parameters states 3 and 4 respiration, the respiratory control ratio (RCR) and dinitrophenol (DNP)-stimulated respiration (uncoupled state), as well as the key and regulatory activities of α-ketoglutarate dehydrogenase (α-KGDH), creatine kinase (CK) and Na^+,K^+-ATPase in cerebral cortex, striatum and hippocampus from 30-day-old *Gcdh* −/− and WT mice under a baseline diet (0.9% Lys) or a high Lys (4.7%) dietary intake. We also evaluated the expression of the catalytic subunits of Na^+,K^+-ATPase in cerebral cortex of *Gcdh* −/− and WT mice.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Solutions were prepared on the day of the experiments and the pH was adjusted to 7.2–7.4 with the appropriate buffers for each technique.

2.2. Animals

Gcdh −/− and WT mice littermate controls, both of 129SvEv background [38], were generated from heterozygous and maintained at the Unidade Experimental Animal, Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil). The animals were maintained on a 12:12 h light/dark cycle (lights on 07.00–19.00 h) in air conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and a normal chow diet containing 20% (w/w) protein and 0.9% Lys (NUVILAB). Thirty-day-old WT and *Gcdh* −/− mice from F1 and F2 generations were used in all experiments. A group of WT and *Gcdh* −/− animals were submitted to a 20% (w/w) protein diet containing 4.7% Lys.

2.3. Ethical statement

This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (Eighth edition, 2001) and approved by the Ethical Committee for the Care and Use of Laboratory Animals of Hospital de Clínicas de Porto Alegre. All efforts were also made to use the minimal number of animals necessary to produce reliable scientific data.

2.4. Tissue preparation

The mice were anesthetized with a mixture of ketamine (90 mg/kg) and xilazine (10 mg/kg) and intracardially perfused during 5 min with saline solution. After perfusion, the brain was rapidly removed and placed on a Petri dish on ice.

For the determination of the respiratory chain complexes, α-KGDH and total CK activities, the olfactory bulb, pons, medulla, and cerebellum were discarded, and the cerebral cortex, striatum and hippocampus dissected and weighed. The tissues were homogenized in 19 volumes (1:20, w/v) of SETH buffer, pH 7.4 (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 UI mL^{-1} heparin). Homogenates were centrifuged at 800 ×*g* for 10 min at 4 °C to discard nuclei and cell debris. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and used to measure these parameters.

For the determination of Na^+, K^+-ATPase activity, the cerebral cortex, striatum and hippocampus were homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. Synaptic plasma membranes were then prepared according to the method of Jones and Matus [40] using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000 ×*g* for 2 h, the fraction at the 0.8–1.0 mM sucrose interface was taken as the synaptic membrane enzyme preparation.

The expression of the catalytic subunits of Na^+, K^+-ATPase *α1*, *α2* and *α3* was determined in cerebral cortex of *Gcdh* −/− and WT mice. This structure was dissected and immediately frozen in the presence of Trizol® for isolation of total RNA.

Determination of the respiratory parameters was carried out in isolated mitochondrial preparations from forebrain. The olfactory bulb, pons, medulla, and cerebellum were discarded and forebrain mitochondria were isolated from rat brain as previously described [41]. The final pellet was gently washed and resuspended in isolation buffer devoid of EGTA, at an approximate protein concentration of 20 mg mL^{-1}. This preparation results in a mixture of synaptosomal and non-synaptosomal mitochondria similar to the general brain composition.

2.5. Spectrophotometric analysis of the respiratory chain complexes I-IV activities

The activity of NADH:cytochrome *c* oxidoreductase (complexes I–III) was assayed according to the method described by Schapira et al. [42]. The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate:cytochrome *c* oxidoreductase (complex II-III) were determined according to Fischer et al. [43]. Cytochrome *c* oxidase (complex IV) activity was assayed according to Rustin et al. [44]. The activities of the respiratory chain complexes were calculated as nmol min^{-1}. mg $protein^{-1}$ and expressed as percentage of controls.

2.6. Determination of mitochondrial respiratory parameters by oxygen consumption

Oxygen consumption rate was measured as described previously [41] using a Clark-type electrode in a thermostatically controlled (37 °C) and magnetically stirred incubation chamber using glutamate plus malate (2.5 mM each) or succinate (5 mM) plus rotenone (2 μg/mL) as substrates in a reaction medium containing the mitochondrial preparations (0.75 mg protein mL^{-1} using glutamate plus malate and 0.5 mg protein mL^{-1} using succinate). The respiration induced by the classical uncoupler DNP (150 μM with glutamate plus malate and 112.5 μM with succinate as substrate) was also measured. State 3, state 4 and DNP-stimulated respiration (uncoupled state) were calculated as nmol O_2 consumed min^{-1} mg of $protein^{-1}$.

2.7. Fluorimetric analysis of α-ketoglutarate dehydrogenase (α-KGDH) activity

The activity of α-KGDH was evaluated according to Tretter and Adam-Vizi [45]. The reduction of NAD^+ was recorded in a Hitachi F-4500 spectrofluorometer at wavelengths of excitation and emission of 340 and 466 nm, respectively. This activity was calculated as nmol min^{-1} mg $protein^{-1}$.

2.8. Spectrophotometric analysis of creatine kinase (CK) activity

CK activity was measured in total homogenates according to Hughes [46] with slight modifications. 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. 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 [46]. The color was developed by the addition of 0.1 mL 20% α-naphtol and 0.1 mL 20% diacetyl in a final volume of 1.0 mL and read after 20 min at $\lambda =$ 540 nm. Results were calculated as μmol of creatine. min^{-1} mg $protein^{-1}$.

2.9. Spectrophotometric analysis of Na^+, K^+-ATPase activity

The reaction mixture for the Na^+, K^+-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, and purified synaptic membranes (approximately 3 μg of protein) in a final volume of 200 μL. The enzymatic assay occurred at 37 °C for 5 min and started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. The reaction was stopped by the addition of 200 μL of 10% trichloroacetic acid. Mg^{2+}-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays [47]. Released inorganic phosphate (Pi) was measured as previously described [48]. Enzyme-specific activities were calculated as nmol Pi $released^{-1}$ min^{-1} mg protein and expressed as percentage of controls.

2.10. Gene expression analysis of the catalytic subunits α1, α2 and α3 of Na^+, K^+-ATPase by quantitative real time RT-PCR (RT-qPCR)

Gene expression analysis of *Na^+, K^+-ATPase* was carried out in cerebral cortex of *Gcdh* −/− and WT mice. For this analysis reagents were purchased from Invitrogen (Carlsbad, California, USA). Total RNA was isolated with Trizol® reagent in accordance with the manufacturer's instructions. Total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA. Quantitative PCR was performed using SYBR® Green I to detect double-strand cDNA synthesis. Reactions were carried out in a volume of 25 μL using 12.5 μL of diluted cDNA (1:50 for *Hprt1*, *α1*, *α2* and *α3*), containing a final concentration of 0.2× SYBR® Green I, 100 μM dNTP, 1× PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase and 200 nM of each reverse and forward primers (Table 1) [49]. The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C. Quantitative PCR reactions were performed on the 7500 Fast Real-Time System (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl). The stability of the reference gene *Hprt1* (*M*-value) and the optimal number of reference genes were carried out according to the pairwise variation (*V*) and were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$method [50].

2.11. Protein determination

Protein levels were measured by the method of Lowry et al. [51] or Bradford [52] using bovine serum albumin as standard.

2.12. Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in triplicate and the mean was used for statistical calculations. Data were analyzed using the Student's *t* test for unpaired samples. Only significant *t* values are shown in the text. Differences between groups were rated significant at $P<0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

3.1. Outcome of Gcdh −/− animals under a high Lys (4.7%) intake

In our study mice submitted to a baseline (0.9% Lys) or a high Lys (4.7%) diet were sacrificed at 60 h after Lys supplementation. Most animals were asymptomatic although a few (5–10%) *Gcdh* −/− mice presented hypotonia and/or moderate paralysis. We also verified in a different set of mice that approximately 20% of *Gcdh* −/− became hypoactive 72 h after Lys overload and this was followed by paralysis, seizures and death after 5–7 days of diet. These *Gcdh* −/− animals under high dietary Lys overload behaved similarly to those previously described by Zinnanti and collaborators [39].

Table 1
PCR primers design.

Enzymes	Primer sequences (5′–3′)	GenBank accession number (mRNA)	Amplicon length (bp)
Hprt1[a]	F-CTCATGGACTGATTATGGACAGGAC R-GCAGGTCAGCAAAGAACTTATAGCC	NM_013556	123
α1[b]	F-CCTTTGACAAGACGTCAGCCACCTG R-CCATCACGGAGCCGCAGCAGAC	BC042435	177
α2[b]	F-CATCTCCGTGTCTAAGCGGGACAC R-CTCTGGGGACTGTCTTCCCTCTCG	NM_178405	186
α3[b]	F-GGGTGGCCCTGTCCCACATCG R-AGCCACTTTCTTGTTCCGTTCTCG	BC037206	182

[a] According to Pernot et al. [49].
[b] Designed by authors.

3.2. Respiratory chain activities were slightly altered in cerebral cortex and hippocampus of Gcdh −/− mice

It is shown in Fig. 1 that complex I–III activity was mildly increased [$t_{(8)} = -2.960$; $P<0.05$] and complex IV activity decreased [$t_{(6)} = 2.511$; $P<0.05$] in cerebral cortex of *Gcdh* −/− mice as compared to WT mice under baseline diet (0.9% Lys). Furthermore, complex II [$t_{(8)} = 2.210$; $P<0.05$] and IV [$t_{(8)} = 2.239$; $P<0.05$] activities were diminished in hippocampus of *Gcdh* −/− mice under a baseline diet (0.9% Lys). On the other hand, no significant differences were found in all respiratory chain complex activities in the striatum of *Gcdh* −/− mice. Similar results were obtained in *Gcdh* −/− mice fed a high Lys (4.7%) diet, except for complexes I-III and IV, which were not changed with this diet.

3.3. Mitochondrial respiration was not altered in forebrain of Gcdh −/− mice

The next step was to evaluate the mitochondrial respiratory parameters states 3 and 4 respiration, RCR and DNP-stimulated respiration (uncoupled state) measured by oxygen consumption, in order to examine whether the mild alterations found in the respiratory chain complexes activities in brain of *Gcdh* −/− mice fed a normal or a high Lys (4.7%) diet were able to change mitochondrial respiration. We observed that none of the respiratory parameters analyzed were altered in forebrain of *Gcdh* −/− mice when compared to WT mice using glutamate plus malate or succinate as respiratory substrates (Tables 2 and 3).

3.4. α-Ketoglutarate dehydrogenase (α-KGDH) and creatine kinase (CK) activities were not changed in brain of Gcdh −/− mice

α-KGDH and CK activities were not changed in cerebral cortex, striatum and hippocampus of *Gcdh* −/− mice under baseline diet (0.9% Lys) as compared to WT animals (Table 4). Furthermore, no alteration of CK activity occurred in all brain structures from *Gcdh* −/− mice fed a high Lys (4.7%) diet, the same occurring for α-KGDH activity in the cerebral cortex (Table 5).

3.5. Na^+, K^+-ATPase activity and expression was significantly reduced in cerebral cortex of Gcdh −/− mice

Finally, it was found that synaptic membrane Na^+, K^+-ATPase activity was markedly reduced [$t_{(7)} = 2.460$; $P<0.05$] in cerebral cortex, but not in striatum and hippocampus from *Gcdh* −/− mice fed a baseline diet (0.9% Lys) in comparison to the WT mice (Figs. 2A and B). Furthermore, this activity was decreased to approximately the same degree in the cerebral cortex of *Gcdh* −/− mice fed with a high Lys (4.7%) dietary intake [$t_{(6)} = 2.041$; $P<0.05$] (Fig. 2A).

Since the reduced activity of Na^+, K^+-ATPase in cerebral cortex could be due to an altered transcriptional control, we determined the expression of the Na^+, K^+-ATPase catalytic subunits *α1*, *α2* and *α3* in this cerebral structure. Fig. 3 shows that only *α2* transcript levels were decreased in cerebral cortex of *Gcdh* −/− mice when compared to the WT mice [$t_{(6)} = 7.354$; $P<0.001$], with no alteration in the expression of *α1* and *α3*.

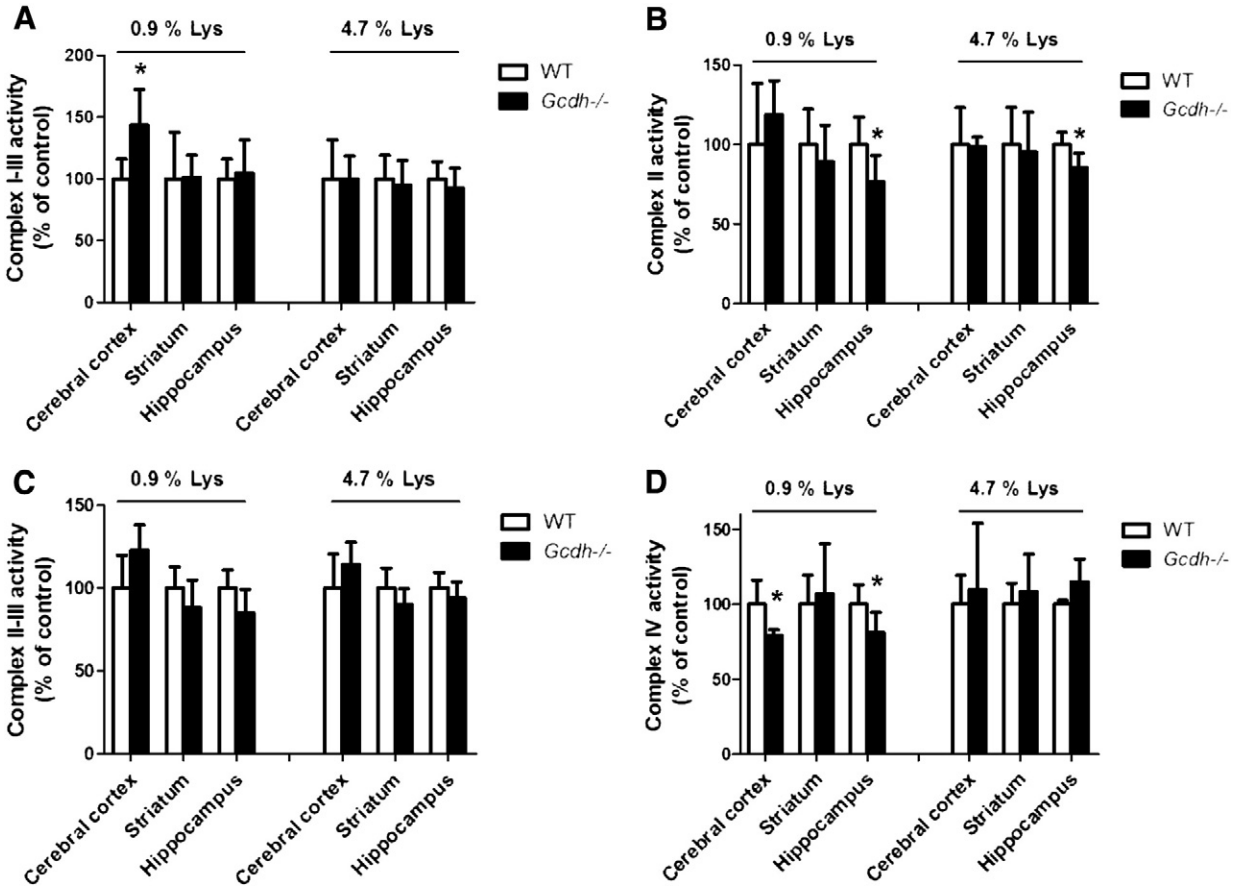

Fig. 1. Respiratory chain complexes I–IV activities in rat cerebral cortex, striatum and hippocampus from glutaryl-CoA dehydrogenase deficient knockout mice (*Gcdh* −/−) under a baseline (0.9% Lys) or a high Lys (4.7%) diet. The activities of complexes I-III (A) were calculated as nmol cytochrome *c* reduced min^{-1} mg $protein^{-1}$, II (B) as nmol DCIP reduced min^{-1} mg $protein^{-1}$, II–III (C) as nmol cytochrome *c* reduced min^{-1} and IV (D) as nmol cytochrome *c* oxidized min^{-1} mg $protein^{-1}$. Values are mean ± standard deviation of four to five independent experiments (animals) performed in triplicate and expressed as percentage of wild type (WT) values. *$P<0.05$ compared to WT (Student's *t* test for unpaired samples).

Table 2
Respiratory parameters measured by oxygen consumption in resting (state 4), ADP-stimulated (state 3), respiratory control ratio (RCR) and dinitrophenol (DNP)-stimulated (uncoupled state) respiration supported by glutamate plus malate or succinate using mitochondria from forebrain glutaryl-CoA dehydrogenase deficient knockout mice (*Gcdh* −/−) under a baseline diet (0.9% Lys).

	State 3	State 4	RCR	DNP
Glutamate/malate				
WT	82.7 ± 8.25	4.98 ± 0.87	16.8 ± 1.54	72.8 ± 8.17
Gcdh −/−	88.3 ± 12.9	4.98 ± 1.22	18.6 ± 5.28	75.2 ± 8.10
Succinate				
WT	98.3 ± 17.4	17.9 ± 3.32	5.51 ± 0.06	91.3 ± 16.3
Gcdh −/−	105 ± 19.4	17.4 ± 3.00	5.99 ± 0.12	96.4 ± 17.2

Values are means ± standard deviation of three independent experiments (animals) performed in triplicate and are expressed as nmol O_2 min^{-1} mg $protein^{-1}$. Protein concentrations used for experiments performed with glutamate/malate and succinate were respectively 0.75 mg protein mL^{-1} and 0.5 mg protein mL^{-1}. No significant differences were detected compared to wild type (WT) mice (Student's *t* test for unpaired samples).

4. Discussion

Mitochondrial dysfunction has been proposed to play an important role in the brain injury of patients affected by GA I [8,30,34]. However, this hypothesis is still under debate because experimental studies performed in vitro and in vivo in fresh brain and in cell cultures from rat and chick brain embryo revealed only mild impairment of mitochondrial functions caused by GA and 3HGA [29,31–33,35,36,53]. It is of note that these investigations were performed essentially in tissues with normal GCDH activity. The generation of *Gcdh* −/− mice via gene targeting in mouse embryonic stem cells produced a GA I genetic model with a similar biochemical and neuropathological phenotype (diffuse spongiform myelinopathy) to that found in the human condition [38]. This model was later improved by submitting the animals to high Lys (4.7%) or protein dietary intake resulting in striatal lesions [37,39]. The present study used *Gcdh* −/− mice in order to comprehensively investigate whether mitochondrial dysfunction represents a major underlying mechanism of cortical and striatal damage in this disorder. *Gcdh* −/− and WT mice were submitted to a baseline (0.9% Lys) or a high Lys (4.7%) supplementation and the evaluated parameters determined. Although most animals were asymptomatic, a few (5–10%) *Gcdh* −/− mice became hypotonic and/or had moderate paralysis.

We first observed that the activities of some respiratory chain complexes were mildly changed in cerebral cortex (I–III and IV) and in hippocampus (II and IV), but not in the striatum of *Gcdh* −/− mice under a baseline diet with 0.9% Lys, as compared to age-matched WT mice. However, resting (state 4) and ADP-stimulated (state 3) mitochondrial respiration, as well as RCR and DNP-stimulated respiration (uncoupled state) measured by oxygen consumption were not altered in the *Gcdh* −/− mice, strongly indicating that oxidative phosphorylation is not mainly disturbed in the brain of these animals. It is concluded that the weak inhibition of some complexes activities of the respiratory chain was not sufficient to compromise the mitochondrial oxidative metabolism estimated by oxymetry in brain of *Gcdh* −/− mice under a baseline diet (0.9% Lys). These results are in agreement with those of other investigators using brain, liver, skeletal and heart muscle from the same *Gcdh* −/− mice model fed a normal diet that showed no significant alterations of the endogenous activities of single respiratory chain complexes I-V and of the tricarboxylic acid (TCA) enzymes when compared to WT animals [34]. We also verified that Lys overload to these animals for 60 h produced similar effects on the mitochondrial parameters examined. In contrast, another study indicated mitochondrial disruption in cerebral cortex of *Gcdh* −/− mice exposed to high protein or Lys intake for 48–72 h, as determined by accumulation of acetyl coenzyme A, as well as a decrease of ATP, phosphocreatine, coenzyme A, alpha-ketoglutarate, glutamate, glutamine and GABA [37]. Unfortunately, the authors did not describe whether the alterations of energy homeostasis were obtained before or after the beginning of neurological symptoms, but reported cortical swelling and striatal and hippocampal histopathological alterations 24–48 h after high Lys (4.7%) intake. They also did not mention whether changes of biochemical energy parameters also occurred in the striatum, so that we cannot ascertain whether the mitochondrial dysfunction was a cause or a consequence of brain damage associated with neuronal loss and with the striatum morphological alterations observed.

Regarding to α-KGDH, a key and a rate-controlling enzyme of the TCA cycle, we found no significant differences in its activity in the brain structures (cerebral cortex, striatum and hippocampus) of the *Gcdh* −/− mice, as compared to WT animals. These data do not

Table 3
Respiratory parameters measured by oxygen consumption in resting (state 4), ADP-stimulated (state 3), respiratory control ratio (RCR) and dinitrophenol (DNP)-stimulated (uncoupled state) respiration supported by glutamate plus malate or succinate using mitochondria from forebrain glutaryl-CoA dehydrogenase deficient knockout mice (*Gcdh* −/−) under a high Lys (4.7%) diet.

	State 3	State 4	RCR	DNP
Glutamate/malate				
WT	55.0 ± 1.68	5.35 ± 1.30	10.7 ± 2.71	33.1 ± 7.68
Gcdh −/−	55.2 ± 6.20	5.99 ± 1.00	9.30 ± 0.85	36.1 ± 7.57
Succinate				
WT	80.5 ± 5.80	18.4 ± 2.16	4.37 ± 0.20	80.6 ± 6.92
Gcdh −/−	78.9 ± 7.60	18.2 ± 1.97	4.35 ± 0.03	79.9 ± 8.73

Values are means ± standard deviation of three independent experiments (animals) performed in triplicate and are expressed as nmol O_2. min^{-1} mg $protein^{-1}$. Protein concentrations used for experiments performed with glutamate/malate and succinate were respectively 0.75 mg protein mL^{-1} and 0.5 mg protein mL^{-1}. No significant differences were detected compared to wild type (WT) mice (Student's *t* test for unpaired samples).

Table 4
α-Ketoglutarate dehydrogenase (α-KGDH) and creatine kinase (CK) activities in rat cerebral cortex, striatum and hippocampus from glutaryl-CoA dehydrogenase deficient mice (*Gcdh* −/−) under a baseline diet (0.9% Lys).

	Cerebral cortex	Striatum	Hippocampus
α-KGDH activity			
WT	10.9 ± 1.27	20.7 ± 3.30	14.8 ± 2.33
Gcdh −/−	13.6 ± 1.30	26.4 ± 5.46	19.8 ± 5.19
CK activity			
WT	1.75 ± 0.41	2.47 ± 0.35	1.24 ± 0.15
Gcdh −/−	1.65 ± 0.27	2.42 ± 0.26	1.12 ± 0.14

Values are means ± standard deviation of four to five independent experiments (animals) performed in triplicate and are expressed as nmol NADH. min^{-1} mg $protein^{-1}$ (α-KGDH activity) and μmol creatine min^{-1} mg $protein^{-1}$ (CK activity). No significant differences were detected compared to wild type (WT) mice (Student's *t* test for unpaired samples).

Table 5
α-Ketoglutarate dehydrogenase (α-KGDH) and creatine kinase (CK) activities in rat cerebral cortex, striatum and hippocampus from glutaryl-CoA dehydrogenase deficient mice (*Gcdh* −/−) under a high Lys (4.7%) diet.

	Cerebral cortex	Striatum	Hippocampus
α-KGDH activity			
WT	6.42 ± 1.40	–	–
Gcdh −/−	7.58 ± 0.091	–	–
CK activity			
WT	2.02 ± 0.23	2.12 ± 0.24	1.00 ± 0.07
Gcdh −/−	1.89 ± 0.30	2.43 ± 0.22	1.00 ± 0.16

Values are means ± standard deviation of four to five independent experiments (animals) performed in triplicate and are expressed as nmol NADH. min^{-1} mg $protein^{-1}$ (α-KGDH activity) and μmol creatine. min^{-1} mg $protein^{-1}$ (CK activity). No significant differences were detected compared to wild type (WT) mice (Student's *t* test for unpaired samples).

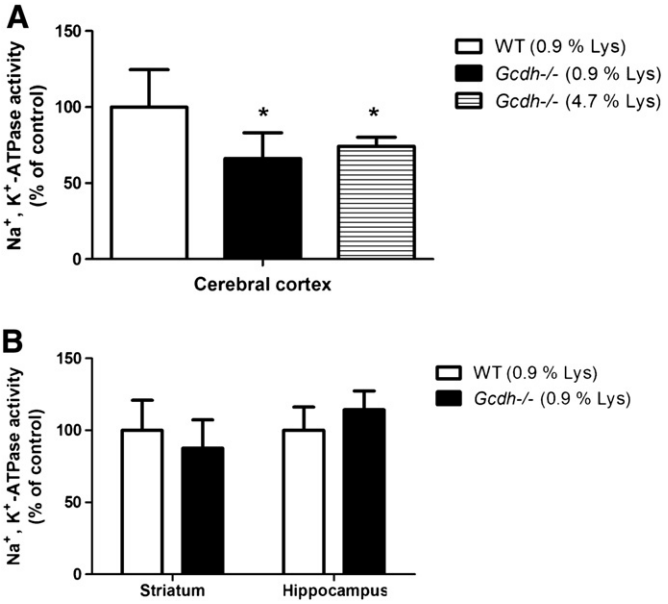

Fig. 2. Na^+, K^+-ATPase activity in rat cerebral cortex (A), striatum and hippocampus (B) from glutaryl-CoA dehydrogenase deficient knockout mice (*Gcdh* −/−). Animals received a baseline (0.9% Lys) or a high Lys (4.7%) diet. Values are mean ± standard deviation of four to five independent experiments (animals) performed in triplicate. They were calculated as mol Pi min^{-1} mg $protein^{-1}$ and expressed as percentage of wild type (WT) values. *$P<0.05$ compared to WT (Student's *t* test for unpaired samples).

support an important role of this enzyme in the pathogenesis of GA I, as previously suggested and based on the inhibition caused by glutaryl-CoA on purified α-KGDH obtained from porcine heart [34]. Therefore, it is feasible that the inhibition of α-KGDH activity by glutaryl-CoA (0.25–2.0 mM) reported *in vitro* in porcine heart does not occur in brain mice in the *in vivo* model of GA I.

It was also verified that the activity of CK, a crucial enzyme involved in intracellular ATP transfer and buffering, was not modified in *Gcdh* −/− mice. These data observed in GCDH deficient mice, allied to previous findings showing augmented or normal intracerebral creatine and phosphocreatine concentrations in cortical (periventricular) white matter and normal levels in the striatum from a late-onset glutaric acidemic patient [54], indicate that intracellular energy transfer is probably not affected in GA I. Zinnanti and colleagues [37] found a decrease of phosphocreatine concentrations in cerebral cortex of *Gcdh* −/− mice receiving high Lys (4.7%) diet for 48 h. However, it is emphasized that these findings were obtained in the presence of cortical swelling and structural changes with neuronal loss that may result in decreased cellular phosphate pool.

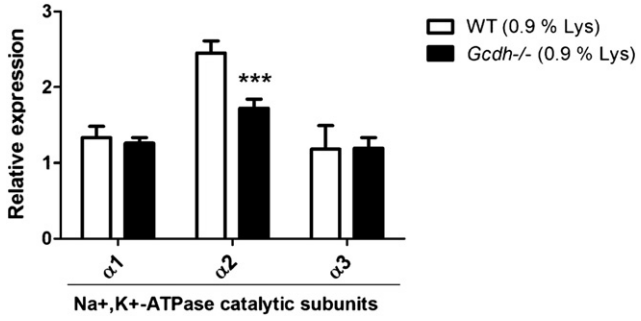

Fig. 3. Relative gene expression profile of the Na+,K+ −ATPase catalytic subunits in cerebral cortex from glutaryl-CoA dehydrogenase deficient knockout mice *(Gcdh −/−)* under baseline diet (0.9% Lys). RT-qPCR analysis was used for these experiments. Values are mean ± standard deviation for four independent experiments (animals) performed in triplicate and are expressed as relative gene expression. ***$P<0.001$ compared to wild type (WT) (Student's *t* test for unpaired samples).

Taken together, our present data obtained in 30-day-old *Gcdh* −/− mice under a basal or high Lys (4.7%) exposition indicate that disruption of mitochondrial oxidative metabolism is not mostly compromised in the brain of these animals and do not support an important role for bioenergetics disruption in the acute striatum degeneration of GA I, as previously hypothesized [8,30,34]. Interestingly, the measured parameters did not differ between asymptomatic and symptomatic *Gcdh* −/− mice presenting with hypotonia or moderate paralysis fed with a high Lys (4.7%) diet, suggesting that mitochondrial dysfunction was not correlated with the clinical outcome.

The most interesting finding of our investigation was that synaptic membrane Na^+, K^+-ATPase activity was markedly inhibited in cerebral cortex with no change in striatum and hippocampus of the *Gcdh* −/− mice. These results are in accordance with previous in vitro and in vivo experimental data showing that GA and 3HGA inhibit this enzyme activity in rat brain and in primary neuronal cultures from chick embryo telencephalons [12,23,55]. As regards to the mechanism by which Na^+, K^+-ATPase is inhibited, it is of note that this enzyme is highly vulnerable to free radical attack [56–60] and oxidative stress were recently shown to be elicited in brain of young *Gcdh* −/− mice submitted to a Lys overload [28], so that oxidative damage may represent a possible mechanism of Na^+, K^+-ATPase inhibition in this KO model. We also observed that high dietary Lys intake did not intensify the decreased activity of this enzyme in the cerebral cortex nor significantly altered the activities of brain α-KGDH and CK.

On the other hand, reduction of Na^+, K^+- ATPase activity could also be due to a lower expression of this enzyme protein. We showed here that the catalytic subunit *α2* of this enzyme was significantly less expressed in the cerebral cortex of *Gcdh* −/− mice, whereas no changes in levels of *α1* and *α3* genes were observed. Therefore, the decrease of *α2* transcript in cerebral cortex of *Gcdh* −/− mice suggests that the gene encoding this subunit could be involved in the reduction of Na^+, K^+- ATPase activity. It is of note that the *α2* subunit is well expressed in brain tissue [61], particularly in glial cells [62–64], whereas neurons are the principal source of the *α3* polypeptide [65,66]. Furthermore, the activity of Na^+, K^+- ATPase is critical for glutamate reuptake into astrocytes surrounding the nerve terminals, so that reduction of its activity is associated with pathological states involving excitotoxicity. In this context, a knockout murine model of the *α2* subunit was shown to have decreased re-uptake of glutamate and higher mortality, reflecting the importance of this subunit to keep synaptic glutamate concentrations within normal levels [67].

Na^+, K^+-ATPase is present at high concentrations in the brain and consumes about 40–50% of the ATP generated in this tissue, highlighting its importance for normal brain functioning. The enzyme is necessary to maintain neuronal excitability (neurotransmission) and cellular volume control through the generation and maintenance of the membrane potential by the active transport of sodium and potassium ions in the CNS [68–70]. Thus, it is not surprising that reduction of Na^+, K^+-ATPase activity was observed in patients and animal models of common neurodegenerative states and of various inherited metabolic disorders involving neurodegeneration [71–80].

We have recently reported mild alterations of cell bioenergetics evaluated by the respiratory chain complexes activities and inhibition of Na^+, K^+- ATPase activity in whole brain from 15-day-old *Gcdh* −/− mice [81]. The present study also evaluated mitochondrial oxidative metabolism by oxymetry and found that the small changes of the activities of the respiratory chain were not enough to alter resting and ADP-stimulated and uncoupled mitochondrial respiration. We also showed a selective and significant inhibition of the activity and expression of the catalytic *α2* subunit of Na^+, K^+-ATPase in the cerebral cortex, from *Gcdh* −/− mice at 30 days of life. However, this activity was not changed in the striatum and hippocampus from *Gcdh* −/− mice.

In summary, the present findings demonstrate for the first time a marked inhibition of synaptic Na^+, K^+-ATPase activity and expression in the cerebral cortex of young *Gcdh* −/− mice. Since Na^+, K^+-ATPase activity is crucial for normal brain development and function, it is conceivable that reduction of this activity may be relevant to explain at least in part the cortical swelling observed in *Gcdh* −/− mice [37] and the focal edema of affected patients [82]. A persistent decrease of this activity might also contribute in the chronic progressive changes with leukoencephalopathy and cortical atrophy observed in glutaric acidemic patients [4,7]. Finally, the present study does not support an important role of bioenergetics dysfunction in the striatum damage in GA I since *Gcdh* −/− mice under baseline (0.9% Lys) or high Lys (4.7%) intake did not show any significant alteration of mitochondrial homeostasis in this cerebral structure. We cannot however exclude the possibility that other pathomechanisms of brain damage occur in this disorder, including oxidative stress and excitotoxicity. The later mechanism may be triggered or accentuated by the inhibition of Na^+, K^+- ATPase activity causing an impairment of glutamate reuptake by astrocytes leaving more of this excitatory neurotransmitter in the synaptic cleft. The presence of cysts resembling lesions caused by excitotoxicity in the cerebral cortex of glutaric acidemic patients supports this hypothesis [2].

5. Conclusions

The activity and $\alpha 2$ transcript levels of synaptic Na^+, K^+- ATPase are significantly reduced in cerebral cortex of *Gcdh* −/− mice. It is presumed that decrease of this crucial enzyme activity may represent a relevant pathomechanism of the cortical abnormalities observed in GA I.

Conflicts of interest statement

There are no conflicts of interest between the authors.

Acknowledgments

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Capítulo III

Histological alterations and mild disruption of mitochondrial energy homeostasis in striatum of glutaryl-CoA dehydrogenase deficient mice submitted to lysine overload

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Life Sciences

Histological alterations and mild disruption of mitochondrial energy homeostasis in striatum of glutaryl-CoA dehydrogenase deficient mice submitted to lysine overload

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Abstract

Bioenergetics dysfunction has been postulated as an important pathomechanism of brain damage in glutaric aciduria type I (GA I), but this is still under debate. Therefore, the present work investigated a large spectrum of important parameters of mitochondrial energy homeostasis, namely the activities of key enzymes of the citric acid cycle, including citrate synthase (CS), aconitase, isocitrate dehydrogenase (IDH), α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase, lactate release, the respiratory parameters states 3 and 4, respiratory control ratio and CCCP-stimulated state and the membrane potential (ΔΨm) in purified mitochondrial preparations from cerebral cortex and striatum of adolescent glutaryl-CoA dehydrogenase deficient (*Gcdh-/-*) and wild type mice fed a normal or a high lysine (Lys, 4.7 %) chow for 60 hours. Histological analysis was also evaluated in the brain of these animals. A moderate reduction of CS and IDH activities (20-30 %) and a very mild (10 %) increase of lactate release were observed in striatum from *Gcdh-/-* animals submitted to a high Lys chow. In contrast, the respiratory parameters and ΔΨm were not altered in these animals. Histological analysis revealed the presence of a few vacuoles in the cerebral cortex, but not in striatum from *Gcdh-/-* mice exposed for a short time to a normal or a high Lys show. However, intense vacuolation was found in the cerebral cortex of 60 and 90-day-old *Gcdh-/-* mice fed a baseline chow and in the striatum of *Gcdh-/-* mice fed a high Lys chow for 30 days. Taken together, the present data demonstrate very mild impairment of bioenergetics homeostasis in striatum from adolescent *Gcdh-/-* mice under a short exposition to a high Lys chow and important histological alterations in this cerebral structure when these animals were

submitted to this chow for a long period.

Keywords: glutaric acidemia type I; glutaric acid; *Gcdh-/-* mice; citric acid cycle; mitochondrial homeostasis, brain histology

Conflicts of interest

There are no conflicts of interest between the authors.

1. Introduction

Accumulation of glutaric acid (GA), 3-hydroxyglutaric acid (3HGA) and glutarylcarnitine in the body fluids and tissues are the biochemical hallmark of patients affected by glutaric aciduria type I (GA I, McKusick 23167; OMIM #231670) (Goodman et al., 1977; Kolker et al., 2006). This neurometabolic disease is caused by a deficiency in the activity of the mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH) (EC 1.3.99.7), which is involved in the catabolic pathway of lysine (Lys) and tryptophan (Goodman and Frerman, 2001). Untreated patients present with acute bilateral striatal degeneration that follows catabolic events occurring from 6 months to four years of age. A chronic presentation of demyelination of the central nervous system, resulting in progressive cortical atrophy, may be seen as well. (Funk et al., 2005; Harting et al., 2009; Strauss et al., 2007). Neurological symptoms including developmental delay, dystonia, dyskinesia, hypotonia, seizures and spasticity start especially after the encephalopathic crises commonly found in these patients (Hoffmann and Zschocke, 1999; Neumaier-Probst et al., 2004).

Excitotoxicity (Dalcin et al., 2007; Kolker et al., 1999; Kolker et al., 2004; Kolker et al., 2002a; Kolker et al., 2002b; Magni et al., 2009; Porciuncula et al., 2000; Porciuncula et al., 2004; Rosa et al., 2007; Rosa et al., 2004; Wajner et al., 2004), oxidative stress (de Oliveira Marques et al. 2003; Fighera et al., 2006; Latini et al., 2002; Latini et al., 2007; Latini et al., 2005b; Magni et al., 2007; Seminotti et al., 2013) and mitochondrial dysfunction (Ferreira et al., 2005a; Ferreira et al., 2005b; Ferreira et al., 2007a; Ferreira et al., 2007b; Latini et al., 2005a; Olivera et al., 2008; Sauer et al., 2005; Silva et al., 2000; Strauss and Morton, 2003; Zinnanti

et al., 2007) have been proposed to be involved in GA I neuropathology, but the contribution of each pathomechanism to the brain injury of affected patients is still under discussion and needs further investigation. Mild alterations in mitochondrial homeostasis are provoked by GA and 3HGA in brain of rodents with normal GCDH activity (Ferreira et al., 2005a; Ferreira et al., 2005b; Latini et al., 2005a).

A model for the study of GA I was developed in mice by replacing most of the GCDH gene with an in-frame beta-galactosidase cassette (Koeller et al., 2002). Glutaryl-CoA dehydrogenase deficient mice (*Gcdh-/-*) had increased cerebral, blood and urine GA and 3HGA levels and displayed vacuolation in the frontal cortex (spongiform leukoencephalopathy). However, the animals did not develop striatal damage spontaneously, as is typical of the human disease. Exposing these mice to a high protein or Lys chow provoked neuronal loss, myelin disruption, and gliosis, mostly in the striatum and deep cortex (Zinnanti et al., 2007; Zinnanti et al., 2006). Oral Lys overload in weaning (4-week-old) *Gcdh-/-* mice resulted in increased brain Lys and GA concentrations, followed by a simultaneous decrease of Lys and further increase of brain GA levels, indicating GA formation from Lys catabolism in the brain. These investigators suggested that the cortical and striatal lesions developed in the *Gcdh-/-* animals submitted to Lys overload were probably due to the increase of brain GA concentrations (Zinnanti et al., 2007; Zinnanti et al., 2006).

Our group recently demonstrated mild impairment of the respiratory chain and reduced activities of Na^+, K^+ - ATPase and creatine kinase in brain and skeletal muscle of 15 and 30-day-old *Gcdh-/-* mice (Amaral et al., 2012a; Amaral et al., 2012b). The purpose of the present study was to evaluate a large spectrum of

parameters of energy homeostasis in mitochondria obtained from cerebral cortex and striatum of *Gcdh-/-* mice under distinct metabolic conditions.

2. Materials and Methods

2.1. Animals and reagents

Gcdh-/- and wild type (WT) mice littermate controls, both of 129SvEv background (Koeller et al., 2002), were generated from heterozygotes and maintained at the Unidade Experimental Animal, Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil). This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (Eighth edition, 2001) and approved by the Ethical Committee for the Care and Use of Laboratory Animals of Hospital de Clínicas de Porto Alegre. All efforts were also made to use the minimal number of animals necessary to produce reliable scientific data.

The animals were maintained on a 12:12 h light/dark cycle (lights on 07.00-19.00 h) in air conditioned constant temperature (22 ± 1 ºC) colony room, with free access to water and a normal chow containing 20% (w/w) protein (NUVILAB). Mitochondrial preparations obtained from cerebral cortex and striatum of 30-day-old WT and *Gcdh-/-* mice submitted to a normal Lys (0.9 %) or high Lys (4.7 %) chow for 60 hours were used for the biochemical determinations. Histological analysis were carried out in the brain from these animals and also in 60-day-old WT and *Gcdh-/-* animals under a normal diet and in 90-day-old animals fed a normal or a high Lys (4.7 %) chow for 30 days.

All chemicals were of analytical grade and purchased from Sigma (St Louis,

MO, USA) unless otherwise stated. Solutions were prepared on the day of the experiments and the pH was adjusted to 7.2-7.4 with the buffers used in each technique.

2.2. Tissue Preparation and general experimental conditions

Determination of the respiratory parameters, the ΔΨm and the activities of citric acid cycle (CAC) enzymes were carried out in mitochondrial preparations from cerebral cortex and striatum of 30-day-old WT and *Gcdh-/-* mice, as previously described (Rosenthal et al., 1987) with minor modifications (Mirandola et al., 2008). Digitonin was used to permeabilize synaptosomal plasma membranes. The final pellet was gently washed and resuspended in isolation buffer devoid of EGTA, at an approximate protein concentration of 6-8 mg . mL^{-1}. This preparation results in a mixture of synaptosomal and non-synaptosomal mitochondria similar to the general brain composition.

The expression of the catalytic subunit of NAD-dependent isocitrate dehydrogenase (*Idh3α*) was determined in striatum of 30-day-old *Gcdh-/-* and WT mice. These structures were dissected and immediately frozen in the presence of Trizol® for isolation of total RNA.

For the determination of lactate release, cerebral cortex and striatum from 30-day-old WT and *Gcdh-/-* mice were cut into two perpendicular directions to produce 400 mM-wide prisms using a Mcllwain chopper.

2.3. Citric acid cycle (CAC) enzyme activities

Citrate synthase (CS) activity was measured according to Srere (1969), by

determining DTNB reduction at λ= 412 nm; aconitase (ACO) according to Morrison (1954); isocitrate dehydrogenase (IDH) activity by the method of Plaut (1969); α-ketoglutarate dehydrogenase (αKGDH) complex according to Lai and Cooper (1986) and Tretter and Adam-Vizi (2000), with slight modifications (Amaral et al., 2010); succinate dehydrogenase (SDH) as described by Fischer et al. (1985); and malate dehydrogenase (MDH) activity according to Kitto (1969). The activities of the CAC enzymes were calculated as nmol . min^{-1}. mg $protein^{-1}$.

2.4. Gene expression analysis of the catalytic subunit of NAD-dependent isocitrate dehydrogenase (Idh3α) by quantitative real time (RT-qPCR)

Total RNA was isolated with Trizol® reagent purchased from Invitrogen (Carlsbad, California, USA) in accordance with the manufacturer's instructions. RNA concentrations were evaluated at 260 and 280 nm absorbances in a NanoDrop 1000 (Thermo Scientific, San Jose, CA, USA). cDNA was synthesized by reverse transcription (RT) using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, Grand Island, NY, USA). RT reactions contained 1 µg of RNA, 1 uL of oligo dT(20), 1 µL of annealing buffer, 10 µL of 2X First-Strand Reaction Mix and 2 µL of SuperScript® III/RNaseOUT™ Enzyme Mix in a total reaction volume of 20 µL. Reactions were performed for 5 min at 65°C, 50 min at 50°C and terminated with 5 min at 85°C. Subsequently, cDNA was kept at -20°C until PCR quantitation. A 1:20 dilution of cDNA solution was prepared in water for quantitative real-time polymerase chain reaction (RT-qPCR).

Messenger RNA (mRNA) expression was measured by RT-qPCR using

gene-specific TaqMan FAM/MGB inventoried assays (Applied Biosystems, Foster City, CA, EUA) (*Idh3α,* assay number Mm00499674_m1). Expression of the targed gene was normalized to the expression of endogenous control *Hprt*, a gene with low expression variability in the central nervous system (Pernot et al., 2010), using another TaqMan probe (assay number Mm00446968_m1). Reactions were carried out in a Stratagene MX3000p qPCR System (Stratagene, GE Healthcare Life Sciences, Piscataway, NJ, USA). The cDNA of each mouse (n=4) was used separately in RT-qPCR reactions. Reactions were carried out in a total volume of 12 µL using 5 µL of cDNA solution, 0.5 µL of gene specific TaqMan assay, 1.5 µL of water milli-Q and 5 µL of Master Mix (Applied Biosystems), containing ROX, Amplitaq Gold DNA polymerase, AmpErase UNG, dATP, dCTP, dGTP, dUTP, and $MgCl_2$. The cycling program was 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Reactions were performed in duplicate. Target transcripts' relative expression levels were determined by the DDCt method (Livak and Schmittgen, 2001), using WT mice at each time point as calibrators.

2.5. Lactate release

Cortical prisms were initially pre-incubated at 37 ºC for 15 min in Krebs-Ringer bicarbonate buffer, pH 7.0, followed by the addition of 5 mM glucose. After 60 min incubation at 37 ºC 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 x g, lactate was measured in the supernatant by the lactase-peroxidase method (Shimojo et al., 1989). Results were expressed as mmol of lactate h^{-1} . g $tissue^{-1}$.

2.6. Determination of mitochondrial respiratory parameters by oxygen consumption

Oxygen consumption rate was measured as described previously (Amaral et al., 2010) using a Clark-type electrode in a thermostatically controlled (37ºC) and magnetically stirred incubation chamber using pyruvate plus malate (2.5 mM each) as substrates in a reaction medium containing the striatum mitochondrial preparations (0.4 mg protein . mL^{-1}) and 300 mM sucrose, 5 mM potassium phosphate, 1 mM EGTA, 5 mM MOPS and 0.1 % BSA. We measured state 3 (ADP stimulated), state 4 (oligomycin stimulated) and carbonyl cyanide m-chlorophenyl hydrazone (CCCP)-stimulated respiration (uncoupled state), which were calculated as nmol O_2 consumed . min^{-1} . mg of $protein^{-1}$. The RCR (state 3 / state 4) was also determined. Only mitochondrial preparations with RCR higher than 4 were used in the experiments.

2.7. Determination of mitochondrial membrane potential (ΔΨm)

The ΔΨm was estimated according to (Akerman and Wikstrom, 1976; Figueira et al., 2012) on a temperature-controlled Hitachi F-4500 spectrofluorometer with magnetic stirring operating at excitation and emission of 495 and 586 nm, respectively, slit width of 10 nm, using 2.5 mM pyruvate plus 2.5 mM malate as substrates and supplemented with 5 µM safranine O. Striatal

mitochondrial preparations (0.4 mg protein . mL^{-1}) were incubated at 37 ^{o}C in 5 mM HEPES buffer, pH 7.2, containing 150 mM potassium chloride, 5 mM magnesium chloride, 0.015 mM EGTA, 5 mM potassium phosphate, 0.01 % BSA and 1 µg . mL^{-1} oligomycin A. $CaCl_2$ (10 µM) was added 100 s afterwards. In the end of the measurements maximal depolarization was induced by 1 µM CCCP. Data were expressed as fluorescence arbitrary units (FAU).

2.8. Histological analysis

In order to evaluate morphological changes, we performed histological analysis in cerebral cortex and striatum from WT and *Gcdh-/-* mice using hematoxylin and eosin (HE) staining. For each group four mice were used. Image analysis (100 and 400 x magnification) was done using Q Capture Pro Software (Olympus). Whole brains from 30, 60 and 90-day- old WT and *Gcdh-/-* mice were removed and postfixed in 10% formaldehyde buffered solution (pH 7.00 - 7.05) for 48 h at room temperature and processed for paraffin embedded sectioning. Cerebral cortex and striatum were sectioned (three micrometers) on a Microtome (MICROM HM 360) and slices were collected for HE staining.

2.9. Protein Determination

Protein levels were measured by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard.

2.10. Statistical analysis

Results are presented as mean ± standard deviation. Assays were

performed in triplicate and the mean was used for statistical calculations. Data were analyzed using ANOVA followed by the post-hoc Duncan multiple range test (one-way ANOVA) or Student´s *t* test for unpaired samples. Only significant *t* values are shown in the text. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

3.1. Glutaryl-CoA dehydrogenase deficient (Gcdh-/-) animals fed a high Lys (4.7 %) chow

The effect of a high lysine diet on *Gcdh-/-* mice depends on both the age of the mice and the length of treatment. In these studies, we used 30-day-old (adolescent) mice exposed for a short time (60 hours) to a baseline (0.9 % Lys) or a high Lys (4.7 %) chow to determine bioenergetics parameters and to perform histological analysis. Most *Gcdh-/-* mice fed a high Lys chow were asymptomatic, although a few (5-10 %) showed symptoms of hypotonia and hypoactivity. Symptomatic mice were not used for the biochemical measurements. We also verified that 60-day-old *Gcdh-/-* mice given the high Lys (4.7 %) chow for thirty days were asymptomatic and presented no mortality.

3.2. The activities of citric acid cycle (CAC) enzymes

The activities of CS and IDH were reduced in mitochondrial preparations from striatum of *Gcdh-/-* mice submitted to a short-term high Lys chow as

compared to WT fed the same chow and *Gcdh-/-* fed normal chow (CS: [$F_{(2,18)}$=8.215, P < 0.01]; IDH: [$F_{(2,16)}$ = 3.183; P < 0.05]) (Figure 1). Furthermore, there was a no statistically reduction in the activity of IDH (15 %) in the cerebral cortex of *Gcdh-/-* mice (Figure 1). In contrast, no differences in the activities of αKGDH, SDH and MDH were found in the cerebral cortex and striatum of *Gcdh-/-* mice under baseline or high Lys chow (Figure 1). We assessed the expression of the catalytic subunit of NAD-dependent isocitrate dehydrogenase (*Idh3α*) in order to determine whether the reduction of IDH activity could be due to transcriptional control. Figure 2 shows that *Idh3α* expression was not reduced, but increased in the striatum of *Gcdh-/-* mice on high Lys chow for 60 hours when compared to WT on high Lys chow and *Gcdh-/-* mice on baseline chow [$F_{(2,9)}$=9.118, P < 0.01].

We also found a very mild increase (10 %) of lactate release in cerebral cortex and striatum slices obtained from *Gcdh-/-* animals treated with high Lys chow (Table 1).

3.3. Mitochondrial respiration and membrane potential

We evaluated the mitochondrial respiratory parameters states 3 and 4, RCR and CCCP-stimulated respiration (uncoupled state) measured by oxygen consumption of mitochondria from striatum of *Gcdh-/-* mice fed a high Lys chow. Although several CAC enzymes had mild reductions in activity, none of the respiratory parameters analyzed in intact mitochondria were altered (Table 2). The mitochondrial membrane potential (ΔΨm) was also measured (Figure 3), and was unaffected by genotype.

3.4. Brain histology

Mice of different ages were fed normal chow or high Lys chow for 60 hours. In 30-day-old *Gcdh-/-* mice, slight vacuolation was seen in the cortex (Figure 4A-D), while the striatum appeared normal (Figure 5A-D), regardless of the composition of the chow. Furthermore, intense vacuolation was found in the cerebral cortex of 60- and 90-day-old *Gcdh-/-* mice fed normal chow (Figure 6A-D), which was not intensified in 60-day-old *Gcdh-/-* mice by exposure to high Lys chow for 30 days (Figure 6E and F). On the other hand, striatum from *Gcdh-/-* mice aged 60 and 90 days fed a normal chow showed normal histology (Figure 7A-D); but high Lys chow provoked vacuolation in the striatum (Figure 7E and F). These data indicate a progressive vacuolation in the cerebral cortex from *Gcdh-/-* mice, independent of the diet, as age advanced. In contrast, striatum only showed vacuolation when the knockout mice were fed high Lys chow for a long period.

4. Discussion

The present investigation shows moderate inhibitions (20-30 %) of the activities of CS and IDH in the striatum of 30-day-old *Gcdh-/-* mice submitted to a high Lys chow for 60 hours. Since these activities are critical to CAC functioning, it is presumed that this cycle is at least partially blocked in these animals. Interestingly, a very mild (10 %) increase of lactate release was also observed in the cerebral cortex and striatum of WT and *Gcdh-/-* fed a high Lys chow.

We also found an increase of *Idh3α* expression in the striatum of the *Gcdh-/-* submitted to Lys overload. In this regard, it is well described that mRNA does not necessarily correlates with protein and enzyme activity (de Sousa Abreu et al.,

2009; Griffin et al., 2002; Gygi et al., 1999; Tian et al., 2004), which could be caused by posttranscriptional regulation and differences in mRNA and protein turnover rates (Cox et al., 2005; Hack, 2004). In this case, the observed *in vivo* increase of striatum *Idh3α* expression from *Gcdh-/-* mice exposed to a high Lys chow may represent a compensatory mechanism in response to the persistent reduction in this enzyme activity.

These results are possibly related to previous findings showing a decrease in ATP, CoA, α-ketoglutarate, glutamate and GABA levels, as well as an increase of acetyl-CoA in brain of 4-week-old *Gcdh-/-* submitted to a high Lys chow (Zinnanti et al., 2007). Another work demonstrated that the succinate transport from astrocytic to neuronal cells was compromised in primary astrocytic and neuronal culture of *Gcdh-/-* mice, leading to the loss of CAC intermediates necessary for neuronal functions (Lamp et al., 2011). Taken these data together, it is presumed that the CAC activity is compromised in brain from *Gcdh*-/- mice under Lys overload.

In order to further evaluate bioenergetics in these animals, we tested the resting (state 4) and ADP-stimulated (state 3) states, as well as RCR and CCCP-stimulated respiration (uncoupled state) supported by pyruvate plus malate and measured by oxygen consumption in striatum mitochondrial preparations from 30-day-old WT and *Gcdh*-/- mice fed a high Lys diet for a short period. No significant differences in these respiratory parameters were observed when comparing WT and *Gcdh-/-* mice under Lys overload, indicating that these substrates were sufficiently oxidized to support mitochondrial respiration. The observations of normal respiratory parameters in *Gcdh*-/- mice were expected since only strong inhibitions of the CAC and respiratory chain enzymes are necessary to significantly

alter these parameters (Brand and Nicholls, 2011).

ΔΨm was also not changed in striatum of *Gcdh-/-* fed a high Lys chow, even when mitochondria were challenged by Ca^{2+}, indicating that the mitochondrial capacity to maintain the ion balance necessary to keep this potential, as well as Ca^{2+} buffering were preserved in the striatum of *Gcdh-/-* mice.

We emphasize that our present data showing very mild alterations of mitochondrial bioenergetics in striatum from 30-day-old *Gcdh-/-* mice occurred only when these animals were exposed to a high Lys chow for 60 hours, mimicking the human condition of catabolic stress leading to brain accumulation of GA and 3HGA and simultaneous brain damage, especially in the striatum (Funk et al., 2005; Harting et al., 2009; Hoffmann and Zschocke, 1999; Sauer et al., 2006). Therefore, it is conceivable that Lys-induced effects were dependent on the increased brain production of GA and 3HGA from Lys which easily cross the blood brain barrier in *Gcdh-/-* mice (Zinnanti et al., 2007; Zinnanti et al., 2006).

In order to evaluate brain damage, we submitted *Gcdh-/-* mice to a short (60 hours) or a long (30 days) Lys (4.7 %) overload and performed histological analysis in cerebral cortex and striatum. We verified a low degree of vacuolation in the cerebral cortex, but not in the striatum of 30-day-old *Gcdh-/-* mice exposed to a normal or a high Lys chow for 60 hours, as compared to WT mice. Furthermore, a large number of vacuoles were verified in the cerebral cortex, but not in the striatum, of 60- and 90-day-old *Gcdh-/-* mice fed a normal chow. Finally, when 60-day-old *Gcdh-/-* mice were fed for 30 days a high Lys chow, we found intense vacuolation in the striatum, but vacuolation in the cereral cortex did not change. Our results are in agreement with previous findings showing minor microscopic

changes with vacuolation in the cerebral cortex of 4-week-old *Gcdh-/-* mice exposed for a short time with high Lys (Zinnanti et al., 2006). The same investigators observed severe histological alterations in the striatum and cerebral cortex including neuronal loss and astrocyte activation in adult *Gcdh-/-* mice exposed for a long time with a high Lys chow. These data, allied to a recent study showing delayed onset of striatum degeneration caused by early GA treatment in rat pups (Olivera-Bravo et al., 2011), suggest that striatum can also be progressively damaged by the persistent increase of the accumulating metabolites of GA I, in particular GA.

In conclusion, the present study demonstrated a very mild bioenergetics dysfunction in the brain of adolescent *Gcdh-/-* mice exposed for a short time to a high Lys chow. Furthermore, our histological findings clearly show important striatum alterations in these animals only when submitted for a long period with Lys overload, in contrast to the cerebral cortex where vacuolation was not increased by this treatment. These data are in accordance with recent publications showing that, apart from the progressive cortical injury, chronic striatum injury also takes place in GA I patients (Neumaier-Probst et al., 2004). Therefore, we believe that disturbance of bioenergetics possibly does not contribute to the chronically progressive striatal and cortical damage found in *Gcdh-/-* mice. In case these data can be extrapolated to the human condition, it is presumed that mitochondrial dysfunction does not play a decisive role in the pathogenesis of the brain damage in GA I. However, we cannot rule out the possibility that this pathomechanism, acting synergistically with others such as oxidative stress (Latini et al., 2007; Seminotti et al., 2013) and excitotoxicity (Kolker et al., 2002b; Wajner et al., 2004),

may underlie the neurological injury of this disorder.

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Legends to Figures

Figure 1. Citric acid cycle (CAC) enzyme activities in enriched mitochondrial fractio ns of cerebral cortex and striatum from adolescent (30-day-old) glutaryl-CoA dehyd rogenase deficient mice (*Gcdh-/-*) fed a baseline (0.9 % lysine - Lys) or a high Lys (4.7 %) chow for 60 hours. The activity of citrate synthase (CS) is expressed as nm ol TNB . min^{-1}. mg $protein^{-1}$; aconitase (ACO) as nmol NADPH . min^{-1}. mg $protein^{-1}$; isocitrate dehydrogenase (IDH), α-ketoglutarate dehydrogenase (αKGDH) and mal ate dehydrogenase (MDH) as nmol NADH. min^{-1}. mg $protein^{-1}$; and succinate dehy drogenase (SDH) as nmol DCIP. min^{-1}. mg $protein^{-1}$. Values are mean ± standard d eviation of four to eight independent experiments (animals) performed in triplicate. **P*<0.05; ***P*<0.01 compared between the groups (Duncan multiple range test).

Figure 2. Relative gene expression profile of the catalytic subunit of NAD-dependent isocitrate dehydrogenase (*Idh3α*) in striatum from adolescent (30-day-old) glutaryl-CoA dehydrogenase deficient mice *(Gcdh-/-)* fed a baseline (0.9 % lysine - Lys) or a high Lys (4.7 %) chow for 60 hours. Quantitative real time (RT-qPCR) analysis was used for these experiments. Values are mean ± standard deviation for four independent experiments (animals) performed in duplicate and are expressed as relative gene expression. $^{**}P$<0.01 compared between the groups (Duncan multiple range test).

Figure 3. Mitochondrial membrane potential (ΔΨm) in the absence or presence of

Ca^{2+} using mitochondrial preparations obtained of striatum from adolescent (30-day-old) glutaryl-CoA dehydrogenase deficient mice (*Gcdh-/-*) fed a high lysine (Lys; 4.7 %) chow for 60 hours. 10 µM Ca^{2+} were added 100 seconds after the beginning of incubation to the reaction medium containing the mitochondrial preparations (0.4 mg protein . mL^{-1} supported by pyruvate plus malate). CCCP (1 µM) was added at the end of the measurements. Traces are representative of three independent (animals) experiments performed in duplicates and were expressed as fluorescence arbitrary units (FAU).

Figure 4. Light microscopic images of cerebral cortex from adolescent (30-day-old) glutaryl-CoA dehydrogenase deficient mice (*Gcdh-/-*) fed a baseline (0.9 % lysine - Lys) or a high Lys (4.7 %) chow for 60 hours. (A) Wild type (WT) fed a baseline chow; (B) *Gcdh-/-* fed a baseline chow; (C) WT fed a high Lys chow; (D) *Gcdh-/-* fed a high Lys chow. The arrows in panels B and D show the presence of mild vacuolation. Representative images were obtained from three independent animals per group. Hematoxylin and eosin (HE) staining with magnification of ×100 and x400.

Figure 5. Light microscopic images of striatum from adolescent (30-day-old) glutaryl-CoA dehydrogenase deficient mice (*Gcdh-/-*) fed a baseline (0.9 % lysine - Lys) or a high Lys (4.7 %) chow for 60 hours. (A) Wild type (WT) fed a baseline chow; (B) *Gcdh-/-* fed a baseline chow; (C) WT fed a high Lys chow; (D) *Gcdh-/-* fed a high Lys chow. No histological abnormalities were identified. Representative images were obtained from three independent animals per group. Hematoxylin and

eosin (HE) staining with magnification of ×100 and x400.

Figure 6. Light microscopic images of cerebral cortex from adult glutaryl-CoA dehydrogenase deficient mice (*Gcdh-/-*) fed a baseline (0.9 % lysine - Lys) or a high Lys (4.7 %) chow for 30 days. (A) 60-day-old wild type (WT) fed a baseline chow; (B) 60-day-old *Gcdh-/-* fed a baseline chow; (C) 90-day-old WT fed a baseline chow; (D) 90-day-old *Gcdh-/-* fed a baseline chow; (E) 90-day-old WT fed a high Lys chow; (F) 90-day-old *Gcdh-/-* fed a high Lys chow. The arrows in panels B, D and F show the presence of intense vacuolation. Representative images were obtained from three independent animals per group. Hematoxylin and eosin (HE) staining with magnification of ×100 and x400.

Figure 7. Light microscopic images of striatum from adult glutaryl-CoA dehydrogenase deficient mice (*Gcdh-/-*) fed a baseline (0.9 % lysine - Lys) or a high Lys (4.7 %) chow for 30 days. (A) 60-day-old wild type (WT) fed a baseline chow; (B) 60-day-old *Gcdh-/-* fed a baseline chow; (C) 90-day-old WT fed a baseline chow; (D) 90-day-old *Gcdh-/-* fed a baseline chow; (E) 90-day-old WT fed a high Lys chow; (F) 90-day-old *Gcdh-/-* fed a high Lys chow. The arrow in panel F shows the presence of intense vacuolation. Representative images were obtained from three independent animals per group. Hematoxylin and eosin (HE) staining with magnification of ×100 and x400.

Figure 1

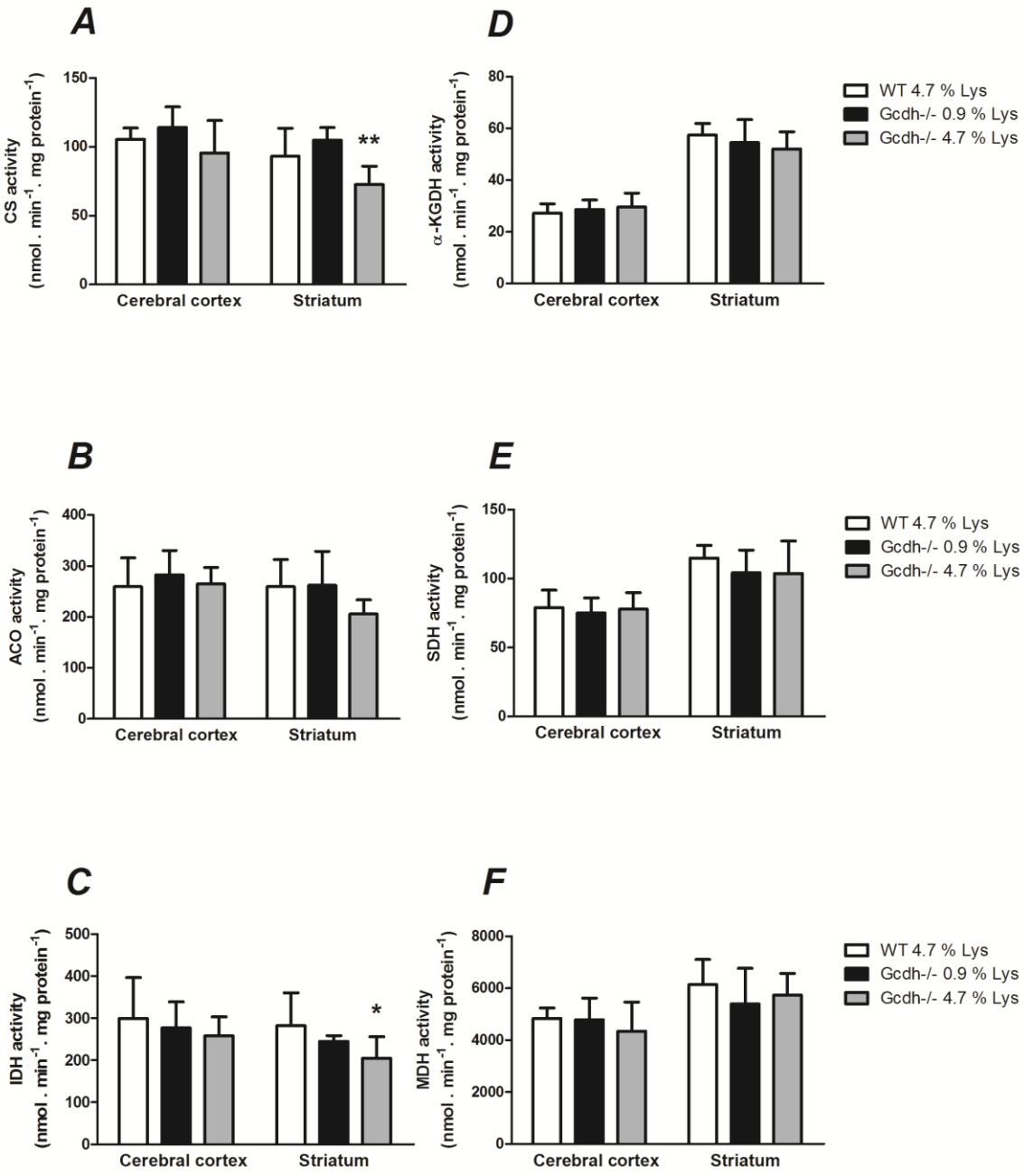
A
CS activity
(nmol . min-1 . mg protein-1)
150
100
50
0
**
Cerebral cortex
Striatum
D
α-KGDH activity
(nmol . min-1 . mg protein-1)
80
60
40
20
0
Cerebral cortex
Striatum
WT 4.7 % Lys
Gcdh-/- 0.9 % Lys
Gcdh-/- 4.7 % Lys
B
ACO activity
(nmol . min-1 . mg protein-1)
400
300
200
100
0
Cerebral cortex
Striatum
E
SDH activity
(nmol . min-1 . mg protein-1)
150
100
50
0
Cerebral cortex
Striatum
WT 4.7 % Lys
Gcdh-/- 0.9 % Lys
Gcdh-/- 4.7 % Lys
C
IDH activity
(nmol . min-1 . mg protein-1)
500
400
300
200
100
0
*
Cerebral cortex
Striatum
F
MDH activity
(nmol . min-1 . mg protein-1)
8000
6000
4000
2000
0
Cerebral cortex
Striatum
WT 4.7 % Lys
Gcdh-/- 0.9 % Lys
Gcdh-/- 4.7 % Lys

Figure 2

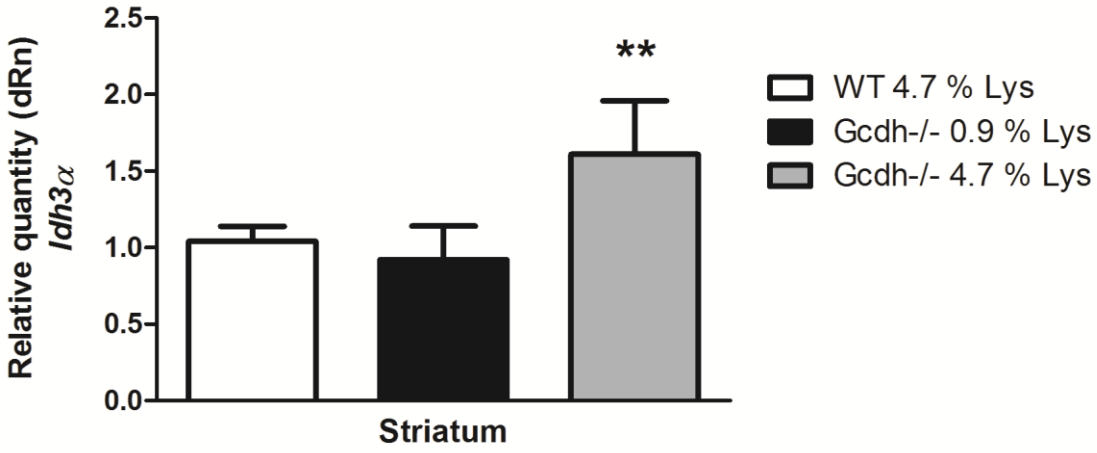
Relative quantity (dRn)
Idh3α
2.5
2.0
1.5
1.0
0.5
0.0
**
Striatum
WT 4.7 % Lys
Gcdh-/- 0.9 % Lys
Gcdh-/- 4.7 % Lys

Figure 3

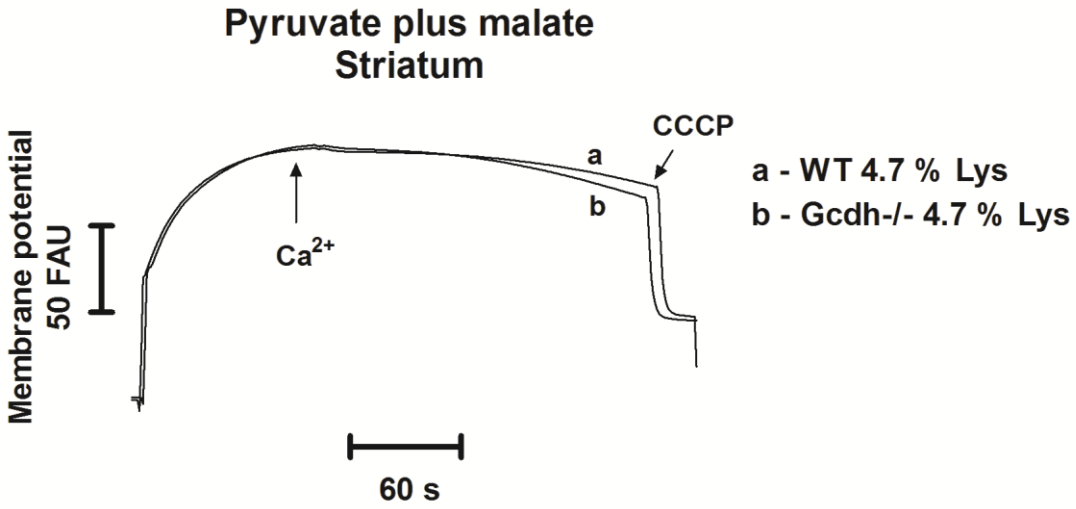
Pyruvate plus malate
Striatum
Membrane potential
50 FAU
Ca2+
CCCP
a
b
60 s
a - WT 4.7 % Lys
b - Gcdh-/- 4.7 % Lys

Figure 4

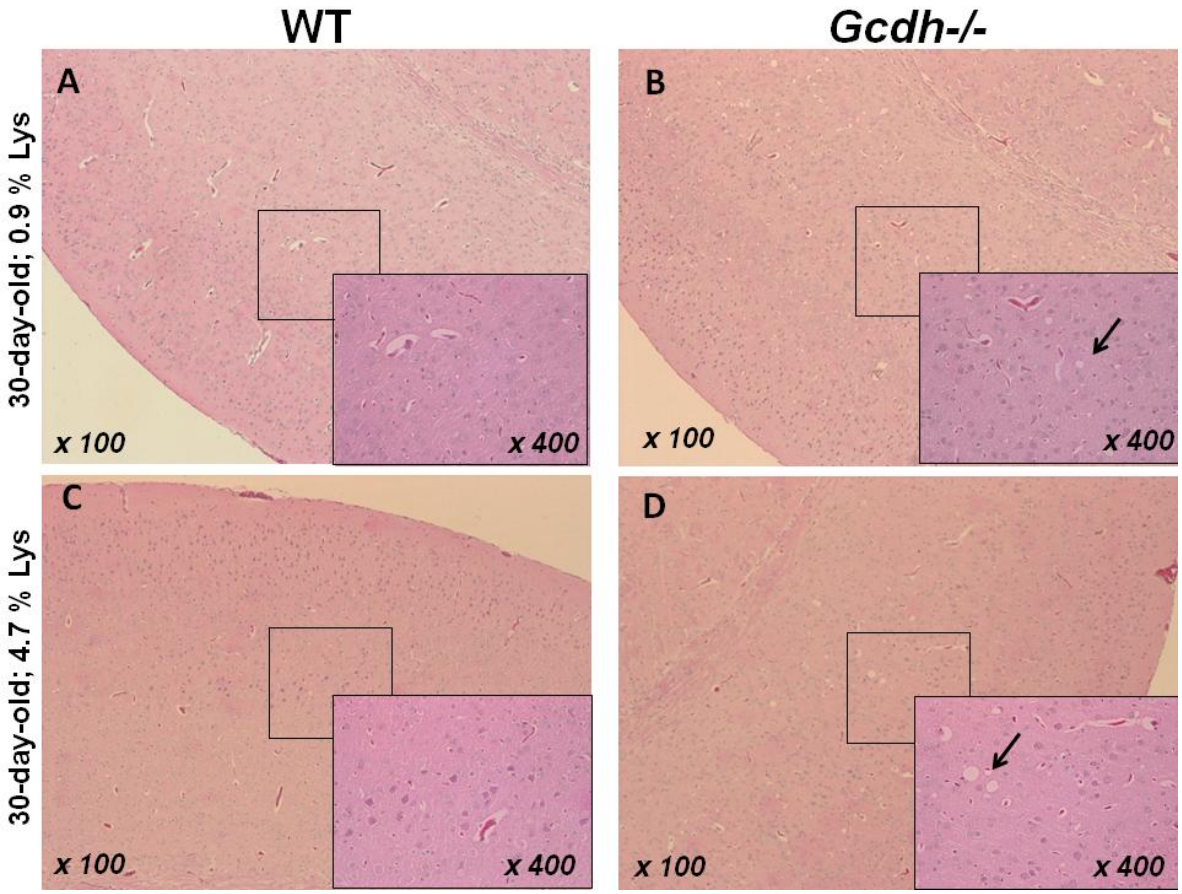
WT
Gcdh-/-
30-day-old; 0.9 % Lys
30-day-old; 4.7 % Lys
A
B
C
D
x 100
x 400
x 100
x 400
x 100
x 400
x 100
x 400

Figure 5

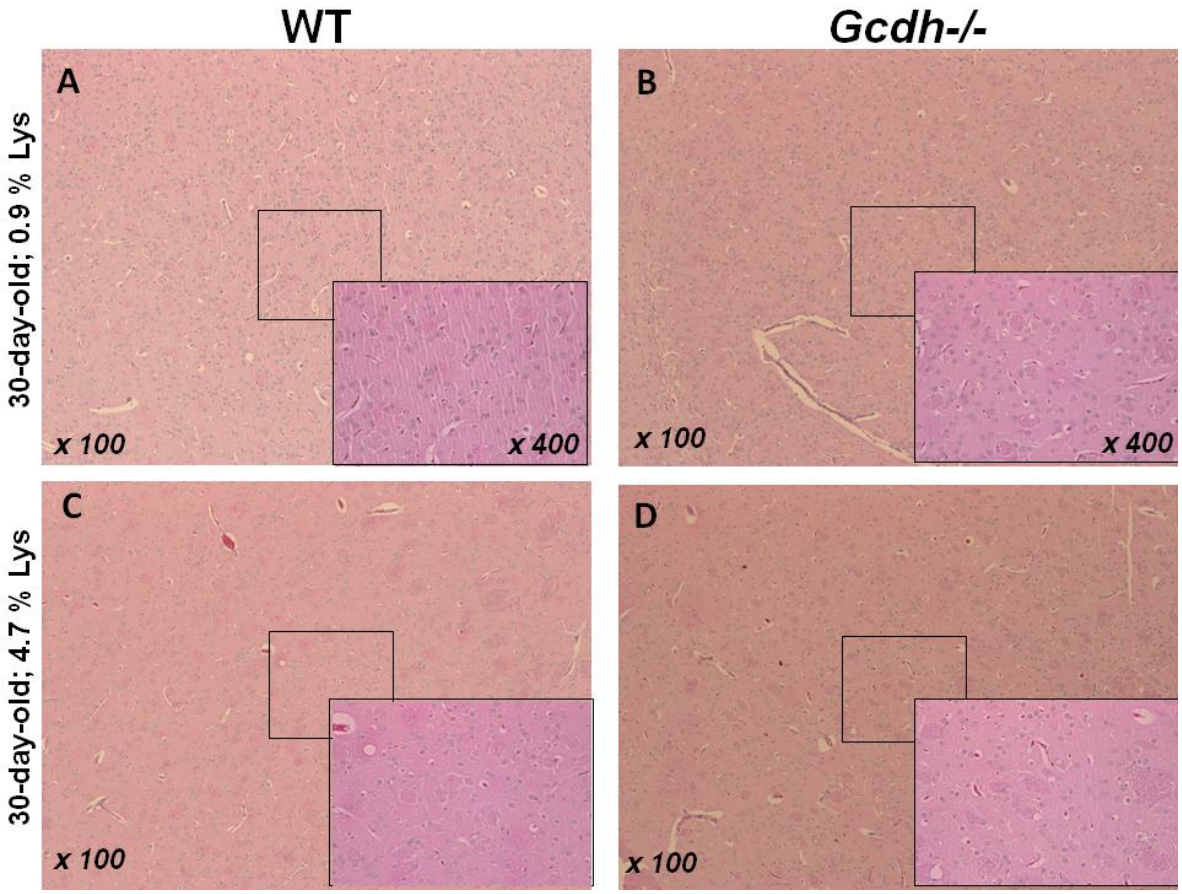
WT
Gcdh-/-
30-day-old; 0.9 % Lys
30-day-old; 4.7 % Lys
A
B
C
D
x 100
x 400
x 100
x 400
x 100
x 100

Figure 6

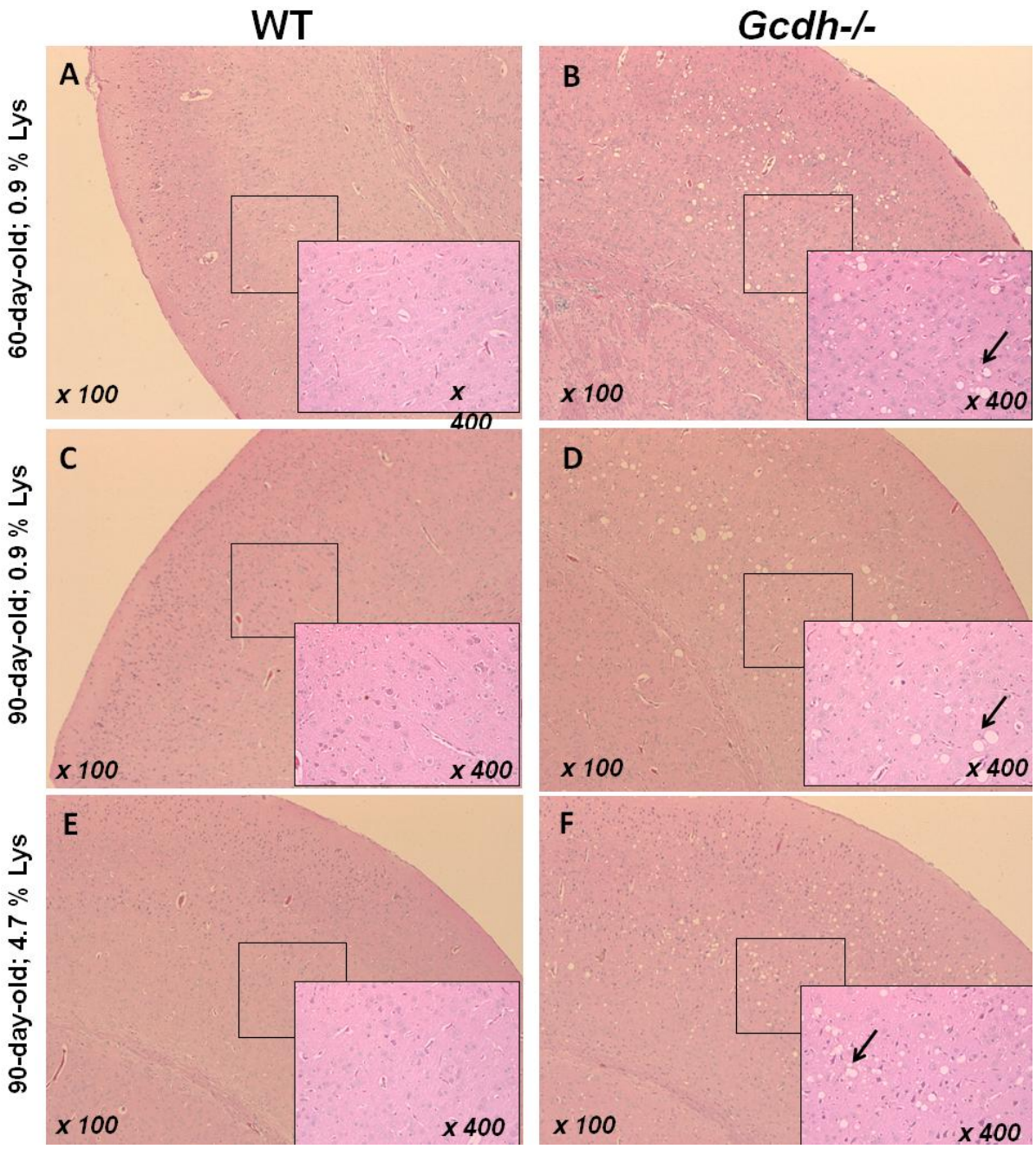
WT
Gcdh-/-
60-day-old; 0.9 % Lys
90-day-old; 0.9 % Lys
90-day-old; 4.7 % Lys
A
B
C
D
E
F
x 100
x 400

Figure 7

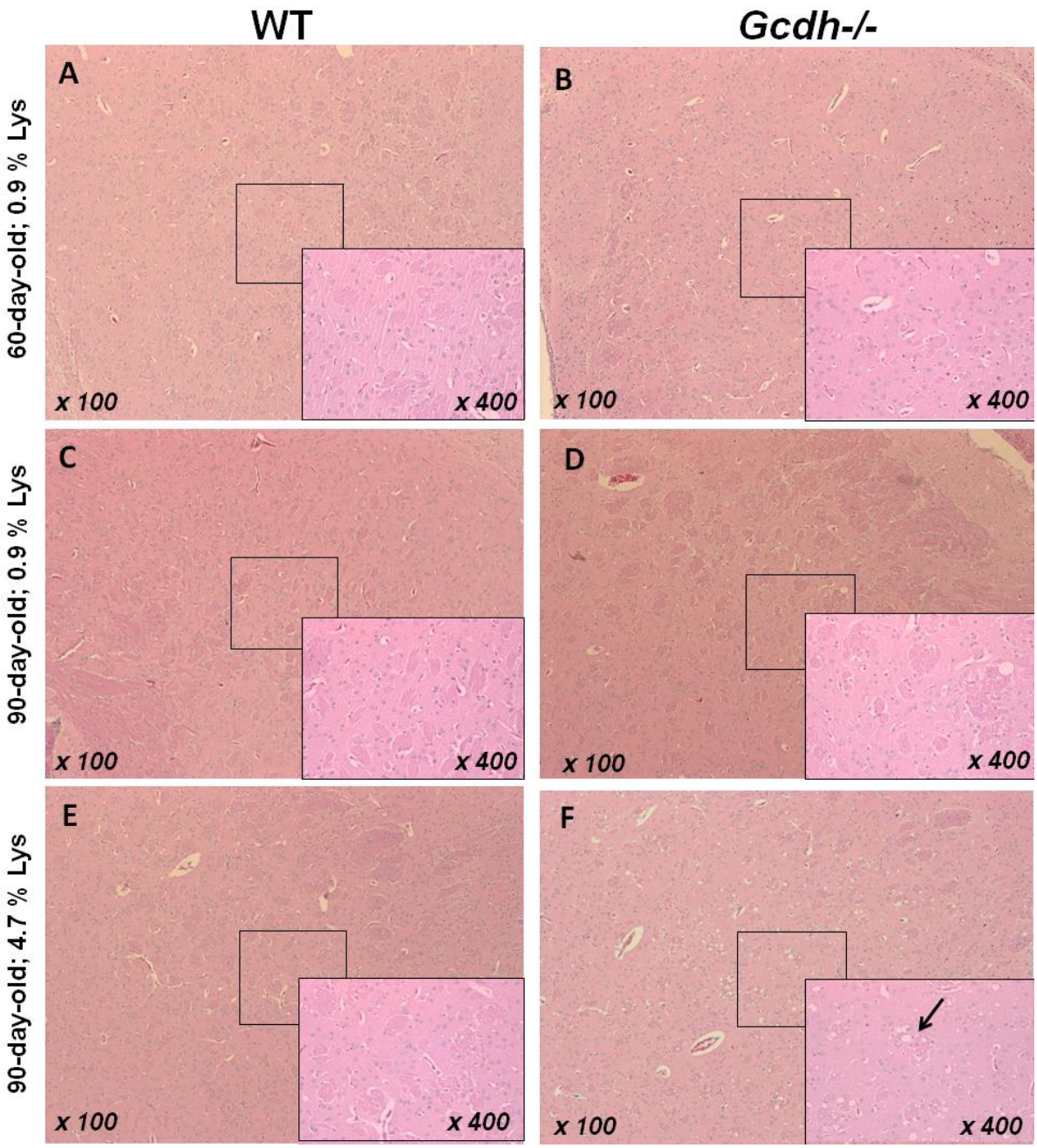
WT
Gcdh-/-
60-day-old; 0.9 % Lys
90-day-old; 0.9 % Lys
90-day-old; 4.7 % Lys
A
B
C
D
E
F
x 100
x 400

Table 1. Lactate release in cerebral cortex and striatum prisms from adolescent (30-day-old) glutaryl-CoA dehydrogenase deficient mice (*Gcdh-/-*) fed a baseline (0.9 % lysine - Lys) or a high Lys (4.7 %) chow for 60 hours.

	Lactate release		
	WT 4.7 % Lys	*Gcdh-/-* 0.9 % Lys	*Gcdh-/-* 4.7 % Lys
Cerebral cortex	28.4 ± 3.43	27.6 ± 1.71	30.2 ± 5.40
Striatum	31.7 ± 6.80	31.5 ± 4.00	33.7 ± 1.97

Values are mean ± standard deviation of five independent experiments (animals) performed in triplicate. Lactate release is expressed as µmol lactate . h^{-1} . g $tissue^{-1}$. No significant differences were detected between the groups (Duncan multiple range test).

Table 2. Respiratory parameters measured by oxygen consumption in resting (state 4), ADP-stimulated (state 3), respiratory control ratio (RCR) and CCCP-stimulated (uncoupled state) respiration supported by pyruvate plus malate using mitochondrial preparations obtained of striatum from adolescent (30-day-old) glutaryl-CoA dehydrogenase deficient mice (*Gcdh-/-*) fed a high lysine (Lys; 4.7 %) chow for 60 hours.

	Respiratory parameters			
	State 3	State 4	RCR	CCCP
WT 4.7 % Lys	84.6 ± 9.10	14.3 ± 3.30	6.10 ± 1.10	97.8 ± 11.1
Gcdh-/- 4.7 % Lys	88.5 ± 22.5	15.3 ± 1.40	5.80 ± 1.24	118 ± 24.1

Values are means ± standard deviation of three independent experiments (animals) performed in triplicate and are expressed as nmol O_2 . min^{-1}. mg . $protein^{-1}$. Protein concentrations used for experiments were respectively 0.4 mg protein . mL^{-1}. No significant differences were detected between the groups (Student's *t* test for unpaired samples).

PARTE III

Discussão e Conclusões

III.1. DISCUSSÃO

Pacientes com AG I apresentam caracteristicamente destruição estriatal aguda que ocorre durante as crises encefalopáticas, bem como leucoencefalopatia progressiva que abrange principalmente o córtex cerebral. Embora a etiopatogenia do dano cerebral nessa doença não esteja bem esclarecida, várias hipóteses tem sido levantadas para explicar as manifestações neurológicas dessa doença, incluindo excitotoxicidade, estresse oxidativo, dano na barreira hematoencefálica e alterações da produção de energia (Latini et al., 2007; Strauss e Morton, 2003; Wajner et al., 2004). Neste particular, um comprometimento importante da homeostase bioenergética tem sido proposto como um mecanismo fundamental da injúria cerebral apresentada pelos pacientes acometidos por essa doença (Kolker et al., 2004; Strauss e Morton, 2003). No entanto, essa hipótese ainda não está comprovada, visto que esses estudos experimentais realizados *in vitro* e *in vivo* em cérebro e cultura de células neurais demonstraram efeitos moderados dos principais metabólitos acumulados nesta doença (AG e 3HG) sobre parâmetros da função mitocondrial (Das et al., 2003; Ferreira et al., 2005a; Ferreira et al., 2005b; Ferreira et al., 2007b; Kolker et al., 2002a; Kolker et al., 2002b; Latini et al., 2005a; Silva et al., 2000; Ullrich et al., 1999). Além disso, quase todos os estudos prévios foram feitos em tecidos de animais com atividade normal da enzima GCDH. O desenvolvimento de camundongos geneticamente modificados com atividade nula da GCDH (*Gcdh-/-*) reproduziu o fenótipo bioquímico e neuropatológico dos pacientes, especialmente a leucoencefalopatia (Koeller et al., 2002), se tornando um bom modelo animal para o estudo da AG I. Esse modelo foi mais tarde aperfeiçoado pela suplementação de uma dieta rica em lisina (4,7 %) aos animais

Gcdh-/-, resultando em um aumento nas concentrações teciduais de AG e 3HG, além de provocar o aparecimento de lesões no estriado desses animais, semelhantes as que ocorrem em seres humanos afetados pela AG I (Zinnanti et al., 2006).

A presente tese foi desenvolvida utilizando cérebro, músculo esquelético e coração de camundongos WT e *Gcdh-/-* com 15 e 30 dias de vida no intuito de investigar a homeostase energética nesses animais submetidos a uma sobrecarga de lisina através de uma injeção aguda intraperitoneal de lisina (8 µmol/g) em camundongos com 15 dias de vida, bem como por uma dieta rica em lisina (4,7 %) por 60 horas para camundongos com 30 dias de vida. Foram avaliados os seguintes parâmetros da bioenergética celular: atividades dos complexos I-III, II, II-III e IV da cadeia respiratória, os parâmetros respiratórios (estados 3 e 4, RCR e o estado desacoplado), atividades das enzimas do CAC CS, ACO, IDH, α-CGDH, SDH e MDH, a liberação de lactato e o potencial de membrana mitocondrial com ou sem a adição exógena de Ca^{2+}, assim como as atividades da CK e da Na^{+}, K^{+} - ATPase. Estudos histológicos também foram feitos para determinar alterações na arquitetura do córtex cerebral e estriado dos camundongos WT e *Gcdh-/-* em diferentes idades (30 a 90 dias) submetidos por um curto (60 horas) ou longo (30 dias) período com dieta com alta concentração de lisina (4,7 %).

Determinamos inicialmente as atividades dos complexos da cadeia respiratória e da α-CGDH em cérebro, coração e músculo esquelético de camundongos WT e *Gcdh-/-* com 15 dias de vida submetidos a uma injeção intraperitoneal de solução salina (NaCl 0,9 %) ou lisina (8 µmol/g). Também investigamos a influência de uma dieta com alta concentração de lisina (4,7 %) por

60 horas em camundongos WT e *Gcdh-/-* com 30 dias de vida sobre as mesmas medidas em córtex cerebral, estriado e hipocampo desses animais, bem como sobre os parâmetros respiratórios estados 3 e 4, RCR e estado desacoplado. Com relação aos animais *Gcdh-/-* com 15 dias de vida que receberam uma injeção intraperitoneal de solução salina, não verificamos alteração em nenhum dos parâmetros de bioenergética acima mencionados quando comparados aos animais WT. Entretanto observamos um aumento na atividade do complexo II-III em músculo esquelético e coração, mas não em cérebro total dos camundongos *Gcdh-/-* submetidos à administração aguda de lisina.

Já nos camundongos *Gcdh-/-* de 30 dias de vida com dieta normal (0,9 % de lisina), encontramos pequenas alterações na atividade de alguns complexos da cadeia respiratória em córtex cerebral (I-III e IV) e hipocampo (II e IV). No entanto, os parâmetros respiratórios (estados 3 e 4, RCR e estado desacoplado) não foram alterados em mitocôndrias isoladas de cérebro total utilizando glutamato/malato ou sucinato como substratos. Observamos também uma diminuição na atividade dos complexos II e II-III no músculo esquelético desses animais. Nossos resultados indicam que as pequenas alterações na atividade da cadeia transportadora de elétrons não foram suficientes para comprometer o consumo de oxigênio no estado não fosforilante (estado 4) e fosforilante (estado 3), bem como no estado desacoplado no cérebro dos animais nocautes. Sabe-se que uma inibição importante da atividade da cadeia transportadora de elétrons é necessária para alterar a respiração mitocondrial (Brand e Nicholls, 2011). Finalmente, verificamos também que uma sobrecarga de lisina na dieta não acentuou significativamente as diferenças observadas.

Resultados anteriores mostraram que os complexos isolados da cadeia respiratória não estão alterados em cérebro de camundongos *Gcdh-/-* submetidos a uma dieta normal, o que reforça a hipótese de que não há um comprometimento importante da cadeia respiratória nesses animais (Sauer et al., 2005). Esses autores verificaram, no entanto, que o glutaril-CoA inibe significativamente a atividade da α-CGDH purificada comercial. Em nossos ensaios realizados com homogeneizados e com mitocôndrias purificadas de cérebro total, córtex cerebral, estriado e hipocampo dos camundongos *Gcdh-/-* com 15 e 30 dias de vida submetidos ou não a uma sobrecarga de lisina não identificamos qualquer inibição desse complexo enzimático. Assim, é possível que em nosso modelo animal *in vivo* concentrações intracerebrais de glutaril-CoA foram menores do que as testadas por Sauer e colaboradores (2005) e necessárias para causar a inibição *in vitro* observada por esses pesquisadores.

O próximo passo de nossa investigação foi o de verificar a função do CAC através da determinação da atividade das enzimas CS, ACO, IDH, SDH e MDH, bem como a liberação de lactato, os parâmetros respiratórios estados 3 e 4, RCR e estado desacoplado, e o potencial de membrana em preparações mitocondriais obtidas de córtex cerebral e estriado de camundongos *Gcdh-/-* de 30 dias de vida submetidos a uma dieta padrão (0,9 % lisina) ou rica em lisina (4,7 %) por 60 horas.

Observamos uma diminuição moderada nas atividades da CS e IDH em mitocôndrias de estriado de camundongos *Gcdh-/-* submetidos a uma dieta rica em lisina. Considerando a importância dessas enzimas, podemos presumir que o funcionamento do CAC está parcialmente prejudicado. Verificamos também um

pequeno aumento na liberação de lactato em córtex cerebral e estriado dos camundongos *Gcdh-/-* suplementados com uma dieta rica em lisina.

Também encontramos um aumento acentuado na expressão gênica da subunidade catalítica da IDH (*Idh3α*) no estriado de camundongos *Gcdh-/-* expostos à dieta rica em lisina, não correlacionando com a atividade diminuída dessa enzima que encontramos no estriado desses animais. Neste particular, está descrito na literatura que a expressão do gene que codifica uma enzima não necessariamente se correlaciona com a sua atividade (de Sousa Abreu et al., 2009; Griffin et al., 2002; Gygi et al., 1999; Tian et al., 2004), a qual pode ser dependente de alterações pós-transcricionais e muitas vezes existir uma diferença na taxa de conversão de mRNA em proteína (Cox et al., 2005; Hack, 2004). Assim, a expressão aumentada da *Idh3α* pode ocorrer como um mecanismo compensatório em resposta à inibição da atividade da IDH.

Esses resultados estão possivelmente relacionados com um estudo prévio que demonstrou uma diminuição nas concentrações de ATP, CoA, α-cetoglutarato, glutamato e GABA, assim como com um aumento nos níveis de acetil-CoA, em cérebro de camundongos *Gcdh-/-* submetidos a uma dieta com alta concentração de lisina (Zinnanti et al., 2007). Um outro estudo também demonstrou um bloqueio no transporte de sucinato dos astrócitos para os neurônios em culturas primárias de animais *Gcdh-/-* (Lamp et al., 2011), impedindo a reposição de intermediários do CAC para os neurônios.

Além disso, o fato de que a redução nas atividades da CS e IDH foi encontrada quando os camundongos *Gcdh-/-* foram submetidos a uma sobrecarga de lisina faz com que seja razoável associar esses efeitos ao aumento da

concentração de AG e 3HG que são derivados da lisina no cérebro desses animais (Zinnanti et al., 2006; Zinnanti et al., 2007).

No intuito de avaliar outros parâmetros da bioenergética mitocondrial no estriado desses animais, medimos os estados 3 e 4, RCR e estado desacoplado da respiração mitocondrial pelo consumo de oxigênio. Não verificamos alterações significativas em nenhum dos parâmetros respiratórios quando foram comparadas preparações mitocondriais obtidas de estriado de camundongos WT e *Gcdh-/-* expostos a uma dieta rica em lisina e utilizando piruvato/malato como substratos respiratórios. Esses resultados indicam que os substratos estão sendo suficientemente oxidados no CAC para abastecer a respiração mitocondrial. No entanto, como mencionado anteriormente, alterações leves na atividade de enzimas do CAC e da cadeia respiratória não são suficientes para causar alterações nos diferentes estados da respiração mitocondrial (Brand e Nicholls, 2011).

Observamos também que o potencial de membrana mitocondrial não se alterou em preparações mitocondriais de estriado de camundongos *Gcdh-/-* submetidos a uma sobrecarga de lisina, mesmo com suplementação exógena de Ca^{2+}. Esses dados sugerem que a função mitocondrial de formação e sustentação do potencial de membrana e a capacidade de tamponamento de Ca^{2+} não foram alteradas nesses animais.

Nosso estudo também demonstrou uma atividade reduzida da CK em cérebro e músculo esquelético de camundongos *Gcdh-/-* com 15 dias de vida injetados agudamente com lisina, indicando um comprometimento no processo de transferência e tamponamento intracelular de ATP. Estudos anteriores mostraram

um efeito inibitório causado pelo AG *in vitro* na atividade da CK em cérebro (Ferreira et al., 2005b) e *in vivo* em músculo esquelético de ratos (Ferreira et al., 2007b). Essas observações corroboram com nossos achados presentes e anteriores, demonstrando uma diminuição nos níveis de ATP e fosfocreatina em camundongos *Gcdh-/-* expostos a uma sobrecarga dietética de lisina (Zinnanti et al., 2007). Tendo em vista que a administração intraperitoneal de lisina resulta no aumento das concentrações cerebrais de AG e 3HG no cérebro de camundongos *Gcdh-/-* (Seminotti et al., 2012), é razoável postular que os efeitos encontrados em nossa investigação sejam decorrentes do acúmulo tecidual desses ácidos orgânicos. O significado desses achados é ainda desconhecido, mas possivelmente pode estar ligado à disfunção neurológica e à hipotonia que acomete os pacientes com AG I.

Um aspecto interessante de nossa investigação foi que não encontramos inibição da CK no cérebro de animais *Gcdh-/-* de mais idade (30 dias) quando submetidos à dieta rica em lisina por 60 horas. Neste particular, é possível que a barreira hematoencefálica esteja menos permeável à passagem da lisina em animais de 30 dias de vida com posterior conversão no cérebro em AG e 3HG (Zinnanti et al., 2007).

Possivelmente, o achado mais importante do presente estudo foi uma diminuição significativa da atividade da Na^+, K^+ - ATPase em membranas sinápticas de cérebro dos camundongos *Gcdh-/-* com 15 e 30 dias de vida. Verificamos ainda que essa inibição ocorreu no córtex cerebral, mas não em estriado e hipocampo dos camundongos *Gcdh-/-* com 30 dias de vida. Esses resultados corroboram com trabalhos anteriores que demonstram uma inibição

dessa atividade enzimática pelo AG *in vitro* (Kolker et al., 2002b) e *in vivo* (Fighera et al., 2006; Rodrigues et al., 2013).

Com relação ao mecanismo pelo qual a CK e a Na^+, K^+ - ATPase estão inibidas, sabe-se que essas enzimas são suscetíveis ao ataque oxidativo (Konorev et al., 1998; Kurella et al., 1997; Lees, 1993; Stachowiak et al., 1998; Wallimann et al., 1998). Neste contexto, trabalhos anteriores mostraram indução de estresse oxidativo em cérebro de camundongos *Gcdh-/-* submetidos a uma sobrecarga de lisina (Seminotti et al., 2012; Seminotti et al., 2013) e que o AG e 3HG induzem estresse oxidativo *in vitro* (de Oliveira Marques et al., 2003; Kolker et al., 2001; Latini et al., 2002; Latini et al., 2005b) e *in vivo* (Latini et al., 2007).

Alternativamente, a inibição da Na^+, K^+ - ATPase pode estar relacionada a uma reduzida expressão gênica de alguma de suas subunidades. Neste particular, encontramos uma diminuição na expressão da subunidade catalítica α2 da Na^+, K^+ - ATPase em córtex cerebral de animais *Gcdh-/-* com 30 dias de vida, sem qualquer alteração nas isoformas α1 e α3. Esses resultados indicam que possivelmente a diminuição da expressão gênica da isoforma α2 dessa subunidade esteja relacionada com a inibição da atividade da Na^+, K^+ - ATPase. É importante ressaltar que a isoforma α2 é bastante expressa em cérebro, particularmente nos astrócitos (Brines e Robbins, 1993; Cameron et al., 1994; Chen et al., 2013; Kawakami e Ikeda, 2006), e que a atividade da Na^+, K^+ - ATPase nessas células auxilia na recaptação do glutamato liberado na fenda sináptica (Rose et al., 2009). Neste contexto, um modelo de camundongos nocaute para a subunidade α2 da Na^+, K^+ - ATPase revelou haver um prejuízo na captação de glutamato, culminando numa alta mortalidade dos animais (Kawakami

e Ikeda, 2006). Dessa forma, nossos resultados mostrando uma redução na expressão dessa isoforma e redução da atividade da Na^+, K^+ - ATPase pode comprometer a captação do glutamato da fenda sináptica pelos astrócitos, resultando em aumento extracelular desse neurotransmissor, levando a um quadro de excitotoxicidade (Veldhuis et al., 2003).

Portanto, a inibição da atividade da Na^+, K^+ - ATPase cerebral pode levar à excitotoxicidade secundária e ser um fator importante no dano cerebral característico dos pacientes com AG I. Além disso, estudos anteriores mostram uma possível interação dos AG e 3HG com receptores e transportadores glutamatérgicos em culturas de células e cérebro de ratos (Bjugstad et al., 2001; de Mello et al., 2001; Flott-Rahmel et al., 1997; Kolker et al., 1999; Kolker et al., 2000; Kolker et al., 2002a; Kolker et al., 2002b; Porciuncula et al., 2000; Porciuncula et al., 2004; Rosa et al., 2004; Wajner et al., 2004), o que indica que a excitotoxicidade pode de fato representar um mecanismo patogênico na AG I.

Também não podemos desconsiderar o papel fundamental da Na^+, K^+ - ATPase para o cérebro na manutenção do potencial de membrana da célula no controle dos fluxos de sódio e potássio, mantendo a excitabilidade neuronal (neurotransmissão) e controlando o volume celular. Aproximadamente 50 % do ATP consumido pelo cérebro é gasto na atividade dessa enzima, o que implica em sua importância para o funcionamento do SNC (Erecinska et al., 2004; Erecinska e Silver, 1994; Satoh e Nakazato, 1992). Neste contexto, inibições da Na^+, K^+ - ATPase tem sido associadas a diversas doenças neurodegenerativas e também em doenças metabólicas hereditárias (Bagh et al., 2008; Busanello et al., 2011;

Cousin et al., 1995; Ellis et al., 2003; Lees e Leong, 1995; Moura et al., 2012; Vignini et al., 2007).

A última etapa deste trabalho foi investigar anormalidades histológicas em cérebro de camundongos *Gcdh-/-* com 30, 60 e 90 dias de vida submetidos a diferentes períodos (60 horas ou 30 dias) com dieta rica em lisina. Observamos a presença de alguns vacúolos no córtex cerebral, mas não no estriado de camundongos *Gcdh-/-* com 30 dias de vida submetidos a uma dieta normal ou rica em lisina por 60 horas. No entanto, animais *Gcdh-/-* com 60 e 90 dias de vida em dieta normal apresentaram uma intensa vacuoalização no córtex cerebral. Além disso, verificamos a presença de um grande número de vacúolos no estriado dos camundongos *Gcdh-/-* com 90 dias de vida que foram alimentados por 30 dias com uma dieta rica em lisina, enquanto que a vacuoalização cortical não foi aumentada. Alguns autores demonstraram a presença de vacuolização em estudos *postmortem* de cérebro de pacientes afetados pela AG I (Bergman et al., 1989; Forstner et al., 1999; Goodman et al., 1977; Hoffmann e Zschocke, 1999; Soffer et al., 1992).

Nossos resultados corroboram com um estudo anterior que descreve pequenas alterações microscópicas no cérebro de camundongos *Gcdh-/-* jovens expostos por um curto período (60 horas) a dieta com alta concentração de lisina, bem como importantes alterações histológicas com uma pequena perda neuronal e ativação astrocitária em córtex cerebral e estriado de camundongos *Gcdh-/-* adultos tratados com sobrecarga de lisina na dieta por pelo menos 45 dias (Zinnanti et al., 2006). Portanto, nossos dados indicam que o estriado dos camundongos *Gcdh-/-* pode estar sendo progressivamente lesado pelo aumento

persistente dos metabólitos acumulados na AG I, particularmente o AG. Neste sentido, um estudo recente observou uma degeneração estriatal tardia em ratos tratados com AG no primeiro dia de vida (Olivera-Bravo et al., 2011).

Caso nossos resultados pudessem ser extrapolados para a condição humana de AG I, acreditamos que as alterações leves da homeostase energética mitocondrial aqui detectadas não desempenham um papel decisivo para o dano neurológico severo que acomete principalmente o córtex cerebral e o estriado de pacientes com AG I. Não podemos, no entanto, excluir que esse mecanismo, associado a outros, como o estresse oxidativo e a excitotoxidade que são deletérios para o SNC, possam contribuir para explicar a fisiopatogenia da injúria cerebral na AG I. Por outro lado, considerando a importância da CK e Na^+, K^+-ATPase para a transferência energética intracelular e manutenção do potencial de membrana necessário para a neurotransmissão, respectivamente, inibições dessas atividades enzimáticas podem representar mecanismos responsáveis pelo dano cerebral, principalmente cortical, e pelas manifestações neurológicas apresentadas pelos pacientes afetados pela AG I.

Finalmente, nossos achados histológicos claramente mostram que apenas o estriado de camundongos *Gcdh-/-* adultos foi lesado após uma sobrecarga de lisina por um longo período (30 dias), enquanto que as lesões encontradas no córtex cerebral parecem não ser acentuadas por esse tratamento. Esses achados estão de acordo com estudos neurorradiológicos feitos em pacientes com AG I, demonstrando que lesão estriatal crônica também acomete pacientes com essa doença (Neumaier-Probst et al., 2004).

III.2. CONCLUSÕES

- Observamos pequenas alterações nos complexos da cadeia respiratória em cérebro, músculo e coração de camundongos *Gcdh-/-* com 15 e 30 dias de vida submetidos ou não a uma sobrecarga de lisina através de uma injeção intraperitoneal desse aminoácido ou por uma sobrecarga de lisina na dieta; entretanto essas alterações não foram suficientes para comprometer os parâmetros respiratórios medidos pelo consumo de oxigênio (estado 3, estado 4, RCR e estado desacoplado) em mitocôndrias de cérebro total de camundongos *Gcdh-/-* com 30 dias de vida utilizando glutamato/malato ou sucinato como substrato.

- Verificamos uma redução moderada na atividade das enzimas CS e IDH em mitocôndrias de estriado de camundongos *Gcdh-/-* com 30 dias de vida submetidos a uma dieta rica em lisina associada com um pequeno aumento na liberação de lactato.

- A respiração mitocondrial avaliada pelos parâmetros respiratórios e o potencial de membrana na presença ou ausência de Ca^{2+} permaneceram normais em mitocôndrias de estriado de camundongos *Gcdh-/-* com 30 dias de vida submetidos a uma dieta rica em lisina, utilizando piruvato/malato como substratos respiratórios.

- Verificamos uma atividade reduzida da CK em cérebro e músculo esquelético de camundongos *Gcdh-/-* com 15 dias de vida injetados agudamente com lisina, indicando um comprometimento no processo de transferência e tamponamento intracelular de ATP.

- Uma diminuição significativa da atividade da Na^+, K^+ - ATPase também foi demonstrada em membranas sinápticas de cérebro dos camundongos *Gcdh-/-* com 15 e 30 dias de vida. Verificamos ainda que essa inibição ocorreu no córtex cerebral dos camundongos *Gcdh-/-* com 30 dias de vida e estava associada a uma reduzida expressão da subunidade catalítica *α2* da Na^+, K^+ - ATPase.

- Finalmente, observamos a presença de vacuoalização no córtex cerebral de camundongos *Gcdh-/-* que foi mais intensa em animais adultos (60 e 90 dias de vida) e independentes de tratamento com lisina. Por outro lado, verificamos uma intensa vacuoalização no estriado desses animais apenas quando camundongos *Gcdh-/-* de 90 dias de vida foram alimentados por 30 dias com dieta rica em lisina.

- Acreditamos que um comprometimento da homeostase energética mitocondrial não contribua significativamente para o dano neurológico observado nos animais *Gcdh-/-*.

- Por outro lado, as inibições encontradas das atividades da CK e Na^+, K^+-ATPase nos camundongos *Gcdh-/-* podem implicar que o dano cerebral observado nesses animais e possivelmente nos pacientes com AG I, pode ser ao menos parcialmente devido a essas alterações, levando-se em consideração a importância dessas enzimas para a transferência energética intracelular e a manutenção do potencial de membrana necessário para a neurotransmissão.

III.3. PERSPECTIVAS

- Avaliar as atividades da cadeia respiratória, CK e Na^+, K^+-ATPase, bem como os parâmetros respiratórios estado 3, estado 4, RCR e estado desacoplado

em cérebro (córtex cerebral e estriado) de camundongos WT e *Gcdh-/-* com 30, 60 e 90 dias de vida submetidos a longos períodos (30, 45 e 60 dias) com dieta rica em lisina.

- Avaliar o potencial de membrana, inchamento, conteúdo de NAD(P)H e a capacidade de retenção de Ca^{2+} em preparações mitocondriais de cérebro (córtex cerebral e estriado) de camundongos WT e *Gcdh-/-* com 30, 60 e 90 dias de vida, na presença ou ausência de Ca^{2+}, submetidos a longos períodos (30, 45 e 60 dias) com dieta rica em lisina.

- Fazer estudos de imunoistoquímica (anti-S100β, anti-GFAP, anti-NeuN) e imunoblotting (anti-α-sinucleína, anti-sinaptofisina, anti-caspase-3) para melhor avaliar as alterações histológicas observadas no cérebro dos camundongos *Gcdh-/-* com 30, 60 e 90 dias de vida submetidos a curto (60 horas) e longos períodos (30, 45 e 60 dias) com dieta rica em lisina.

- Buscar a prevenção das alterações bioquímicas e histológicas encontradas nos camundongos *Gcdh-/-* com substâncias neuroprotetoras (gangliosídeo GM1, guanosina), antioxidantes (N-acetilcisteína e melatonina), substratos energéticos (creatina) e moduladores do sistema glutamatérgico (MK-801).

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