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EFEITOS DA GLICINA SOBRE A HOMEOSTASE ENERGÉTICA E REDOX, VIAS DE SINALIZAÇÃO DAS MAPK E MARCADORES DE DANO CELULAR EM CÉREBRO DE RATOS

Alana Pimentel Moura

ORIENTADOR: Prof. Dr. Guilhian Leipnitz

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"Clara manhã, obrigado,
o essencial é viver!"
Carlos Drummond de Andrade

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A hiperglicinemia não-cetótica (HNC) é um erro inato do metabolismo causado pela deficiência do sistema de clivagem da glicina (Gli), levando ao acúmulo tecidual e plasmático desse aminoácido. Apesar de a fisiopatologia do dano neurológico apresentado pelos pacientes afetados por HNC ainda não estar bem esclarecida, tem sido sugerido que o acúmulo de Gli é neurotóxico. O presente trabalho investigou inicialmente os efeitos *ex vivo* da administração intracerebroventricular (ICV) de Gli sobre o metabolismo energético e o imunoconteúdo das proteínas cinases dependentes de mitógenos (MAPK) p38, ERK1/2 e JNK, sinaptofisina e proteína Tau em córtex cerebral e estriado de ratos de 30 dias de vida. Verificamos que a injeção ICV de Gli reduziu a produção de CO_2 a partir de glicose e inibiu as atividades das enzimas citrato sintase e isocitrato desidrogenase em estriado. A Gli também diminuiu as atividades dos complexos da cadeia transportadora de elétrons I-III em estriado e do IV em córtex cerebral. Além disso, a Gli diminuiu a atividade da enzima creatina cinase (CK) e de sua isoforma mitocondrial em córtex cerebral e estriado. O tratamento com os antioxidantes melatonina (MEL) e creatina e com o antagonista do receptor NMDA (MK-801) preveniu a diminuição das atividades da CK e dos complexos I-III e IV causado pela administração de Gli. Ainda verificamos que a Gli diminuiu o conteúdo de sinaptofisina, e a fosforilação da proteína Tau e das MAPK p38, ERK1/2 e JNK. O tratamento com MK-801 preveniu a diminuição da fosforilação da p38 causada pela Gli. O próximo passo foi estudar o efeito *ex vivo* da injeção ICV de Gli sobre o metabolismo energético, a homeostase redox e parâmetros gliais em córtex cerebral de ratos neonatos. Foi demonstrado que a injeção de Gli em ratos de 1 dia de vida diminuiu a atividade do complexo IV da cadeia transportadora de elétrons e da CK, além de induzir aumento da geração de espécies reativas, diminuir as concentrações de glutationa reduzida

(GSH), aumentar os níveis de malondialdeído (MDA) e modular a atividade das enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). A injeção de Gli ainda foi capaz de induzir reatividade glial demonstrada pelo aumento da marcação de S100B em córtex cerebral e estriado, e GFAP em corpo caloso. Além disso, o tratamento com MEL preveniu a inibição do complexo IV e da enzima CK, o aumento dos níveis de MDA, a diminuição de GSH e o aumento de S100B causada pela Gli. Na última parte de nossa investigação foi examinado o efeito *in vitro* da Gli sobre a homeostase energética e redox em cerebelo de ratos de 30 dias de idade. A Gli aumentou a produção de espécies reativas, os níveis de MDA e diminuiu as concentrações de GSH, sem alterar o metabolismo energético no cerebelo. Além disso, a adição de MEL, trolox (análogo hidrossolúvel da vitamina E) e MK-801 preveniram os efeitos deletérios da Gli sobre os níveis de MDA e GSH em cerebelo. Como conclusão, o nosso estudo mostra que a Gli causa um prejuízo na homeostase energética e induz estresse oxidativo em cérebro de ratos jovens e neonatos, bem como provoca reatividade glial em cérebro de neonatos. A Gli também altera as vias de sinalização das MAPK e causa dano neuronal em córtex cerebral e estriado, além de induzir estresse oxidativo *in vitro* em cerebelo. Portanto, alterações na homeostase redox e energética e excitotoxicidade parecem contribuir, ao menos em parte, para a fisiopatologia do dano neurológico apresentado pelos portadores da HNC.

ABSTRACT

Nonketotic hyperglycinemia (NKH) is an inborn error of metabolism caused by a deficiency of the glycine (GLY) cleavage system, leading to tissue accumulation of this amino acid. Although the pathophysiology of neurological damage presented by patients affected by NKH is not yet well understood, it has been suggested that increased concentrations of GLY are toxic for the central nervous system. Therefore, we initially investigated the *ex vivo* effect of a single intracerebroventricular (ICV) administration of GLY on energy metabolism and immunocontent of the mitogen-activated protein kinases (MAPK) p38, ERK1/2 and JNK, synaptophysin, and Tau protein in cerebral cortex and striatum of thirty-days-old rats. We verified that the administration of GLY decreased CO_2 production using glucose as substrate, and inhibited the activities of citrate synthase and isocitrate dehydrogenase in striatum. GLY also decreased the activities of the respiratory chain complexes I-III in striatum and IV in cerebral cortex. Furthermore, GLY decreased the activity of creatine kinase (CK) and of its mitochondrial isoform in cerebral cortex and striatum. The antioxidants melatonin (MEL) and creatine, and the NMDA receptor antagonist MK-801 prevented the GLY-induced decrease of the activities of CK and of the complexes I-III and IV. It was further observed that GLY injection decreased synaptophysin immunocontent and the phosphorylation of Tau protein, p38, ERK1/2 e JNK in rat brain. MK-801 treatment prevented the decreased phosphorylation of p38 caused by GLY. Next, we evaluated the *ex vivo* effect of GLY ICV administration on energy and redox homeostasis and glial parameters in brain of neonatal rats. GLY injection decreased the activities of respiratory chain complex IV and CK, and also induced the generation of reactive species, decreased reduced glutathione (GSH) concentrations, increased malondialdehyde (MDA) levels and modulated the activities of the antioxidant enzymes

superoxide dismutase, catalase and glutathione peroxidase. Moreover, GLY induced glial reactivity, as demonstrated by the increase in GFAP in corpus callosum and S100B in cerebral cortex and striatum. MEL treatment prevented GLY-induced inhibition of complex IV and CK activities, decrease of GSH, and increase of MDA levels and S100B staining. In the last part of our study we assessed the *in vitro* effects of GLY on energy and redox homeostasis in cerebellum of thirty-day-old rats. We verified that GLY increased reactive species generation and MDA levels, and decreased GSH concentrations in rat cerebellum. Furthermore, the addition of MEL, trolox (hydrosoluble analogue of vitamin E) and MK-801 prevented MDA levels increase and GSH concentrations decrease caused by GLY. In conclusion, the present study demonstrated that GLY alters the energy metabolism and induces oxidative stress in brain of thirty-day-old and neonatal rats, and provokes glial reactivity in neonatal rat brain. GLY also alters MAPK signaling pathways and causes neuronal damage in rat cerebral cortex and striatum, besides inducing oxidative stress in cerebellum. Therefore, it may be presumed that bioenergetics dysfunction, oxidative stress and excitotoxicity contribute to the pathophysiology of brain damage observed in patients affected by NKH.

LISTA DE ABREVIATURAS

AMT – aminometil transferase

CAT – catalase

CTE – cadeia transportadora de elétrons

CS – citrato sintase

Cr – creatina

CK – creatina cinase

DCFH – 2',7'-diclorofluoresceína

ERK – cinase regulada por sinalização extracelular

EIM – erro inato do metabolismo

ERN – espécies reativas de nitrogênio

ERO – espécies reativas de oxigênio

GCSH – proteína contendo ácido lipoico

GFAP – proteína ácida fibrilar glial

GLDC – glicina descarboxilase

Gli – glicina

GR – glutationa redutase

GSH – glutationa reduzida

GPx – glutationa peroxidase

HNC – hiperglicinemia não-cetótica

H_2O_2 – peróxido de hidrogênio

ICV – intracerebroventricular

IE – intraestriatal

IDH – isocitrato desidrogenase

IP – intraperitoneal

JNK – cinase C-Jun N-terminal

LCR – líquido cefalorraquidiano

MAPK – proteínas cinases dependentes de mitógenos

MDA – malondialdeído

MEL – melatonina

NH_4^+ – amônia

MK-801 – dizocilpina

NMDA – N-metil-D-aspartato

5,10-MeTHF – 5,10- metileno- tetrahidrofolato

NAC – N-acetilcisteína

NeuN – proteína nuclear neuronal

$NO^{\bullet}$ – óxido nítrico

O_2 – oxigênio molecular

$O_2^{\bullet -}$ – ânion superóxido

$^1O_2$ – oxigênio singlete

$OH^{\bullet}$ – radical hidroxila

$ONOO^-$ – peroxinitrito

p38 – p38 cinase

SCG – sistema de clivagem da glicina

Ser – serina

SHMT – serina hidroximetil transferase

SNC – sistema nervoso central

SOD – superóxido dismutase

THF – tetrahidrofolato

TRO – trolox

I.1. INTRODUÇÃO

I.1.1. Erros inatos do metabolismo

Sir Archibald E. Garrod usou o termo erros inatos do metabolismo (EIM) em 1908 para designar doenças como a alcaptonúria em que os indivíduos afetados excretam grandes quantidades de ácido homogentísico na urina. Garrod observou uma maior frequência desta doença em indivíduos de uma mesma família e maior incidência de consanguinidade entre os pais dos pacientes. Baseando-se nas leis de Mendel e no fato de que os pais dos indivíduos afetados não apresentavam a doença, Garrod propôs um modelo de herança autossômica recessiva para este distúrbio. Através da observação de que o ácido homogentísico presente em excesso na urina dos pacientes era um metabólito normal da degradação proteica, ele relacionou este acúmulo a um bloqueio na rota de catabolismo da tirosina. Com o surgimento de novos distúrbios relacionados a alterações genéticas e que envolviam o acúmulo de outras substâncias nos líquidos biológicos dos pacientes, postulou-se que estas doenças resultavam da síntese qualitativa ou quantitativamente anormal de uma proteína, enzimática ou não, pertencente ao metabolismo (Scriver et al., 2001). Dessa forma, pode ser presumido que em consequência deste bloqueio metabólico pode ocorrer o acúmulo de precursores da reação catalisada pela enzima deficiente, com o substrato sendo "dirigido" para rotas metabólicas alternativas, e a deficiência de produtos essenciais ao organismo.

Até o momento foram descritos mais de 600 EIM, sendo que a maioria deles envolve processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (Scriver et al., 2001). Embora individualmente raras, essas doenças em seu

conjunto afetam aproximadamente 1 a cada 500-2.000 recém-nascidos vivos (Baric et al., 2001; Mak et al., 2013).

I.1.2. Metabolismo da glicina (Gli)

A glicina (Gli), o menor e mais simples dos aminoácidos, é abundante em quase todas as proteínas animais e participa de muitas rotas biossintéticas. Além de ser um componente de proteínas, esse aminoácido não essencial está envolvido na síntese de moléculas, tais como as porfirinas e purinas. Outro importante papel fisiológico da Gli é o de atuar como neurotransmissor no sistema nervoso. A Gli atua tanto como um neurotransmissor inibitório no tronco encefálico e medula espinhal, quanto como um neurotransmissor excitatório no córtex cerebral, gânglios da base e cerebelo (Hara et al., 1993; Applegarth e Toone, 2001; Hamosh e Johnston, 2001; Katsuki et al., 2007; Hennermann et al., 2012).

A Gli pode ser sintetizada a partir do aminoácido serina (Ser) pela ação da enzima serina hidroximetil transferase (SHMT), ou sintetizada de novo a partir de dióxido de carbono (CO_2) e amônia (NH_4^+) pelo sistema de clivagem da glicina (SCG). Já o catabolismo da Gli pode ocorrer por diversas vias metabólicas, dentre as quais a da SCG é a mais importante. A SCG é um complexo multienzimático intramitocondrial composto por 4 proteínas denominadas P (glicina descarboxilase dependente de piridoxal fosfato), H (proteína contendo ácido lipoico), T (aminometil transferase dependente de tetrahidrofolato) e L (lipoamida desidrogenase) (Kikuchi et al., 2008), e está presente no cérebro, rins, fígado e placenta (Sakata et al., 2001). O SCG degrada a Gli em NH_4^+ e CO_2, convertendo simultaneamente tetrahidrofolato (THF) em 5,10-metileno-tetrahidrofolato (5,10-MeTHF) (Figura 1). A Gli também pode ser degradada através da sua interconversão

à Ser pela ação da SHMT (Hamosh e Johnston, 2001), ou através de outras rotas menos utilizadas, como a que catalisa a sua conversão a glioxalato, ou a que realiza a sua depuração através de acilação com benzoil-CoA formando o produto N-benzoilglicina (ácido hipúrico) que, por sua vez, é excretado na urina.

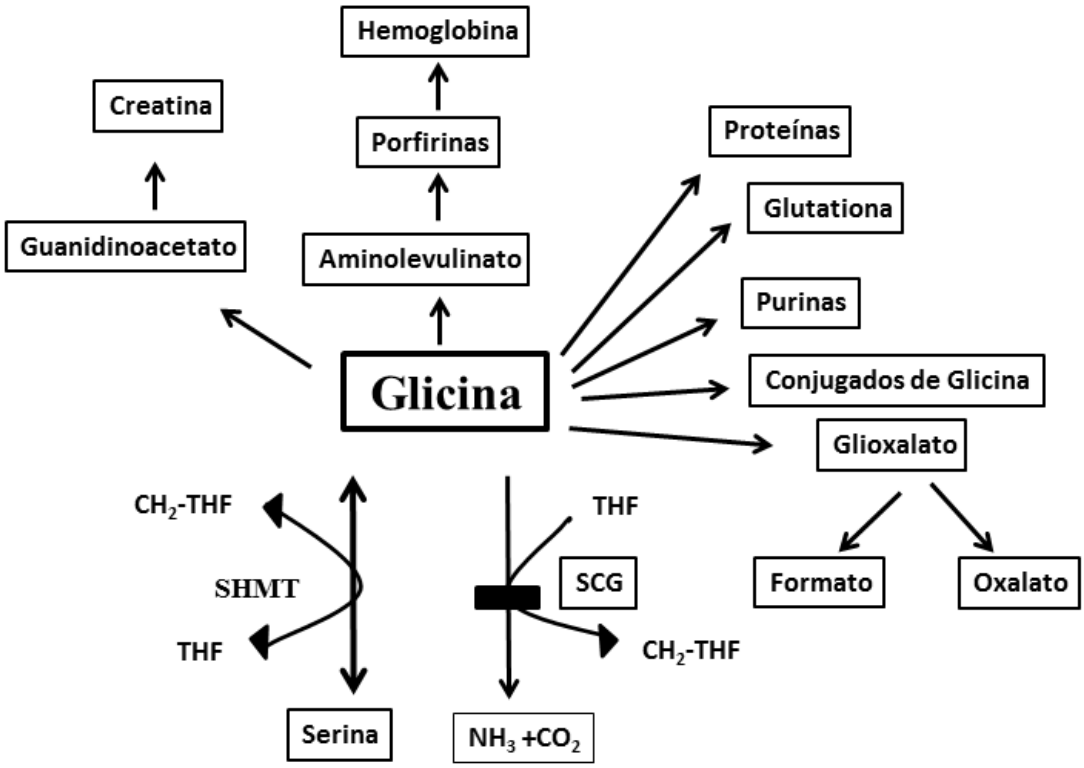

Figura 1. Representação da rota do metabolismo da glicina. CH_2-THF, 5,10-metileno tetrahidrofolato; SCG, sistema de clivagem da glicina; SHMT, serina hidroximetil transferase; THF, tetrahidrofolato. O defeito enzimático na hiperglicinemia não-cetótica (HNC) está representado pela barra preta na reação catabólica da SCG (Figura adaptada de Fernandes e Saudubray, 2006).

I.1.3. Hiperglicinemia não-cetótica

A hiperglicinemia não-cetótica (HNC), também conhecida como encefalopatia por glicina (EG), é um erro inato do metabolismo de herança autossômica recessiva causada pela deficiência do SCG (Hamosh e Johnston, 2001) (Figura 1). Dados da literatura mostram que mais de 80 % dos pacientes afetados pela HNC possuem um defeito no gene que codifica a proteína P (gene *GLDC*), enquanto que em torno de 20 % apresentam alteração no gene da proteína T (gene *AMT*), e apenas uma pequena parcela dos pacientes tem deficiência na proteína H (gene *GCSH*). A proteína L parece não ser afetada nessa aminoacidopatia (Applegarth e Toone, 2001). A prevalência estimada da HNC é de 1:60.000 nascidos vivos no Canadá e 1:12.000 na Finlândia, com prevalências ainda maiores observadas em Israel e na Holanda (Applegarth e Toone, 2001; Hoover-Fong et al., 2004; Boneh, 2005).

O defeito do SCG leva os pacientes portadores de HNC a apresentarem um aumento nas concentrações de Gli no plasma, urina e líquido cefalorraquidiano (LCR), bem como o acúmulo nos tecidos, especialmente no cérebro (Hamosh e Johnston, 2001). Na forma clássica da doença, os níveis plasmáticos de Gli podem atingir 2 mM, sendo que no LCR as concentrações chegam a 0,5 mM (valores normais de Gli para um recém-nascido giram em torno de 104-254 μM no plasma e 3-7 μM no LCR) (Tekinalp et al., 1995). Já no cérebro, estudos realizados com espectroscopia por ressonância magnética de prótons revelaram que as concentrações de Gli podem chegar a 7,3 mM em momentos de crises de descompensação metabólica, com uma média que varia de 4,0 a 4,8 mM (Hamosh e Johnston, 2001).

Visto que a Gli é um co-agonista do receptor glutamatérgico N-metil-D-aspartato (NMDA), principalmente no córtex cerebral, ela exerce, dessa forma, efeitos excitatórios

sobre esse receptor. Portanto, é sugerido que a superestimulação do receptor NMDA pelas altas concentrações cerebrais de Gli verificadas em pacientes com HNC seja possivelmente o evento responsável pelas graves convulsões e anormalidades no sistema nervoso central (SNC) observadas nesses indivíduos. Já a atuação da Gli como um neurotransmissor inibitório, que acontece na medula espinhal e no tronco encefálico através dos receptores glicinérgicos, causa apneia neonatal e hipotonia nos indivíduos com HNC (Shin et al., 2011; Hennermann et al., 2012; Huisman et al., 2012; Kanekar e Byler, 2013).

A HNC apresenta cinco fenótipos diferentes: neonatal, infantil, de sintomatologia moderada, de início tardio e uma forma denominada transitória. A forma neonatal da doença, também chamada de forma clássica, é a mais frequente e grave, onde os pacientes possuem uma atividade muito baixa do SCG. Indivíduos do sexo feminino parecem ter pior prognóstico, com maior índice de mortalidade no período neonatal e complicações neurológicas mais graves do que os pacientes do sexo masculino (Hoover-Fong et al., 2004).

Nos outros fenótipos da HNC os sintomas aparecem mais tardiamente e os pacientes possuem uma atividade residual do SCG. Os achados clínicos estão mais relacionados com comprometimento motor e cognitivo, além de problemas comportamentais. Dessa forma, as formas tardias são consideradas mais brandas quanto à sintomatologia e de melhor prognóstico para o paciente (Steiner et al. 1996; Hamosh e Johnston, 2001).

I.1.3.1. Sintomas e achados neuropatológicos da HNC neonatal

Os sintomas da forma neonatal da HNC incluem hipotonia, convulsões, apneia, letargia progressiva, insuficiência respiratória e encefalopatia que pode levar ao coma. A maioria dos pacientes morre nas primeiras semanas após o nascimento, enquanto que os

que sobrevivem geralmente apresentam um grave retardo psicomotor e convulsões (Sehgal e Ramji, 1998; Hamosh e Johnston, 2001).

Estudos de ressonância nuclear magnética revelam que pacientes com HNC neonatal apresentam atrofia cortical e cerebelar progressiva, hipoplasia ou agenesia do corpo caloso, degeneração espongiforme e vacuolização difusa da mielina (Press et al., 1989). Em uma análise *postmortem* de pacientes entre 15 e 36 meses de idade também foi encontrada degeneração na substância branca com variados graus de gliose (Shuman et al., 1978). Além disso, existem evidências de que os pacientes apresentam dano cerebral ainda no período fetal (Rogers et al., 1991; Rice e Barone, 2000; Avishai-Eliner et al., 2012; Hennermann, et al., 2012; Pai et al., 2015).

I.1.3.2. Diagnóstico

O diagnóstico da HNC é baseado na análise dos níveis de aminoácidos no LCR e plasma, e na posterior determinação da razão das concentrações de Gli no LCR e no plasma. Essa razão pode chegar a valores maiores do que 0,08 em portadores da forma neonatal da HNC (valores normais < 0,02), enquanto que no fenótipo de início tardio os valores são mais moderados ou até mesmo normais (Steiner et al., 1996; Hamosh e Johnston, 2001). Também deve ser feita a análise de ácidos orgânicos na urina através de cromatografia gasosa associada à espectroscopia de massas. Se for encontrada uma elevação isolada da Gli sem aumento dos níveis de outros ácidos orgânicos, deve-se descartar uma acidemia orgânica com hiperglicinemia cetótica (Hamosh e Johnston, 2001). A confirmação do diagnóstico pode ser realizada pela determinação da atividade do SCG em fígado e por análise mutacional. Contudo, esses testes são procedimentos invasivos e nem sempre indicados (Applegarth e Toone, 2001). O diagnóstico pré-natal também é

possível pela determinação da atividade do SCG nas vilosidades coriônicas (Tada e Kure, 1993; Sehgal e Ramji, 1998).

I.1.3.3. Tratamento

O tratamento da doença é baseado na redução dos níveis plasmáticos e teciduais de Gli através de dietas livres desse aminoácido. Contudo, já foi relatado que esse tratamento não tem efeito sobre a frequência das convulsões ou sobre o retardo no desenvolvimento (Hamosh e Johnston, 2001). Também é geralmente feita a administração de benzoato de sódio, o qual forma um conjugado com a Gli plasmática excedente gerando ácido hipúrico, que por sua vez é excretado na urina (Hamosh e Johnston, 2001; Chien et al., 2004). Em alguns casos tem sido proposta a administração de ácido pantotênico, visto que ele é o precursor da coenzima A necessária para a ativação do benzoato de sódio (Palekar, 2000).

O tratamento ainda consiste em bloquear a ação da Gli como neurotransmissor excitatório através do uso de antagonistas do receptor NMDA, tais como o dextrometorfano e cetamina, o que leva a uma melhora parcial na frequência das convulsões na forma clássica da doença (Hamosh e Johnston, 2001; Boneh et al., 2008). Os medicamentos antiepilépticos como os benzodiazepínicos também são utilizados para amenizar as convulsões (Applegarth e Toone, 2001). As estratégias terapêuticas supracitadas nem sempre são eficazes para todos os indivíduos portadores da HNC e, por isso, o diagnóstico e acompanhamento precoce do paciente melhoram a escolha da abordagem que será mais adequada para cada caso.

I.1.3.4. Fisiopatologia

Os mecanismos fisiopatogênicos responsáveis pelo dano neurológico apresentado pelos pacientes portadores de HNC ainda não estão bem estabelecidos. Entretanto, tem sido sugerido que o acúmulo de Gli contribui para a fisiopatologia dessa doença. Nesse contexto, tem sido proposto que o início dos sintomas na forma neonatal da HNC pode ocorrer ainda no período fetal, já que foram observadas altas concentrações de Gli no LCR dos pacientes logo após o nascimento (Von Wendt et al., 1981; Rogers et al., 1991; Korman, 2006; Pai et al., 2015). Essa hipótese está de acordo com o fato de que foi observada hipoplasia do corpo caloso em alguns pacientes já na primeira semana de vida (Press et al., 1989). Além disso, em um estudo realizado em camundongos mutantes para o gene da glicina descaboxilase (gene *GLDC*) foram encontradas anormalidades estruturais no cérebro desses animais no estágio fetal (Pai et al., 2015).

Os mecanismos pelos quais as altas concentrações de Gli levam ao aparecimento e/ou progressão dos sintomas neurológicos nos pacientes têm sido estudados por alguns grupos de pesquisa. O primeiro mecanismo proposto é de que a Gli induz excitotoxicidade, visto que esse aminoácido é um conhecido co-agonista do receptor NMDA e, portanto, é capaz de superestimular esse receptor (McNamara e Dingledine, 1990; Patel et al., 1990; Hara et al., 1993; Kure et al., 1997; Applegarth e Toone, 2001; Hamosh e Johnston, 2001; Katsuki et al., 2007; Kono et al., 2007). Além disso, estudos *in vitro* e *ex vivo* realizados em roedores evidenciaram que a Gli induz dano oxidativo lipídico e proteico, e diminui as defesas antioxidantes não enzimáticas *in vitro* em córtex cerebral e *ex vivo* em estriado de ratos (Leipnitz et al., 2009; Seminotti et al., 2011). Também já foi visto *in vitro* que a Gli provoca disfunção bioenergética por inibir o ciclo do ácido cítrico, a cadeia respiratória

mitocondrial e as atividades das enzimas Na^+,K^+-ATPase e creatina cinase (CK) (Busanello et al., 2010).

I.1.4. Metabolismo energético cerebral

O cérebro é um dos órgãos mais ativos metabolicamente apesar de possuir reservas energéticas extremamente pequenas em relação à sua alta taxa metabólica (Dickinson, 1996; Rooijackers et al., 2015). A glicose é o principal substrato energético no cérebro em condições normais (Sokoloff, 1993; Erecinska e Silver, 1994; Rooijackers et al., 2015). A oxidação da glicose no cérebro ocorre mais rapidamente do que em outros órgãos, como fígado, coração e rins e, diferentemente de outros tecidos, o cérebro não necessita de insulina para captar e oxidar esse substrato (Dickinson, 1996; McKenna et al., 2012). A oxidação da glicose através da via glicolítica forma piruvato, que é convertido a CO_2 e H_2O pelo ciclo do ácido cítrico (CAC) e pela cadeia transportadora de elétrons (CTE). O acoplamento entre a CTE e a fosforilação oxidativa gera grande parte do ATP necessário ao cérebro (Erecinska e Silver, 1994; McKenna et al., 2012).

Já está bem descrito que no cérebro a fosforilação oxidativa fornece em torno de 95 % de todo o ATP sintetizado. Parte dos 5 % do ATP que não é gerado pela cadeia respiratória é produzido pela enzima CK. A CK está presente tanto no citosol quanto ligada às membranas mitocondriais e catalisa a transferência reversível de um fosfato da fosfocreatina para o ADP produzindo ATP. O alto fluxo da reação na direção da síntese de ATP no citosol em situações de consumo de energia indica que a reação da CK é crucial para a manutenção de concentrações constantes dos substratos energéticos nesse compartimento. Assim, o sistema creatina/fosfocreatina/CK está envolvido na transferência de ATP de sítios de produção para outros sítios de consumo (Erecinska e Silver, 1994),

visto que a CK mitocondrial (mCK) catalisa a geração de fosfocreatina a partir de creatina e ATP, sendo essa molécula então transportada para o citosol onde a CK citosólica (cCK) poderá utilizá-la a fim de gerar ATP. Tal mecanismo evita grandes variações nos níveis celulares de ATP e ADP (Wyss et al., 1992; O'Gorman et al., 1996).

I.1.5. Estresse oxidativo

I.1.5.1. Radicais livres

Radicais livres são espécies químicas com um ou mais elétrons desemparelhados que possuem a capacidade de existir de forma independente (Southorn e Powis, 1988; Halliwell e Gutteridge, 2007). Essas espécies químicas podem ser átomos, metais de transição ou uma molécula que possua um ou mais elétrons desemparelhados no orbital externo. Este elétron confere uma alta reatividade à molécula, pois pode perder ou adquirir um segundo elétron para esse orbital (Halliwell, 2006).

Os radicais livres podem ser formados pela perda de um elétron de um não-radical ou pelo recebimento de um elétron por um não-radical. Radicais também podem ser formados em um processo de fissão homolítica, no qual uma ligação covalente é quebrada e cada elétron do par anteriormente compartilhado permanece com cada um dos átomos envolvidos (Halliwell e Gutteridge, 2007). Quando um radical livre reage com um composto não-radical, outro radical livre pode ser formado; assim, a presença de um único radical pode iniciar uma sequência de reações em uma cadeia de transferência de elétrons (redox) (Maxwell, 1995). Nas reações em cadeia induzidas pelos radicais livres, um radical reativo leva à formação de um produto que também é um radical livre e que, por sua vez, reage produzindo um terceiro radical, e assim sucessivamente.

Em condições fisiológicas do metabolismo celular aeróbio, o oxigênio molecular (O_2) sofre redução tetravalente por receber quatro elétrons, resultando na formação de água (H_2O). No entanto, aproximadamente 3 % do oxigênio utilizado na cadeia de transporte de elétrons mitocondrial não é completamente reduzido à água, podendo ser convertido a intermediários reativos como os radicais superóxido ($O_2^{\bullet-}$) e hidroxila ($OH^{\bullet}$), e também o peróxido de hidrogênio (H_2O_2), processo esse que pode ser exacerbado em condições patológicas (Boveris e Chance, 1973; Roede e Jones, 2010).

O termo genérico espécies reativas de oxigênio (ERO) é usado para incluir não só os radicais formados pela redução do O_2 ($O_2^{\bullet-}$ e $OH^{\bullet}$), mas também alguns não-radicais derivados do O_2, como o H_2O_2 e o oxigênio *singlet* ($^1O_2$) (Halliwell e Guteridge, 2007). Além dessas, existem ainda as espécies reativas de nitrogênio (ERN), sendo o óxido nítrico ($NO^{\bullet}$) e o peroxinitrito ($ONOO^-$) os principais representantes.

I.1.5.2. Defesas antioxidantes

Antioxidantes são substâncias endógenas ou exógenas que reduzem a formação de radicais livres ou reagem com os mesmos, neutralizando-os (Halliwell e Gutteridge, 2007). Embora diferindo na composição, as defesas antioxidantes estão amplamente distribuídas no organismo e compreendem agentes que removem cataliticamente os radicais livres, como as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa redutase (GR), dentre outras; proteínas que minimizam a disponibilidade de pró-oxidantes (íons de ferro, por exemplo), ao se ligarem aos mesmos, como as transferrinas; proteínas que protegem biomoléculas de danos (incluindo dano oxidativo) por

outros mecanismos; agentes de baixo peso molecular que aprisionam ERO e ERN, como glutationa reduzida (GSH) e α-tocoferol (Halliwell e Gutteridge, 2007).

I.1.5.3. Definição de estresse oxidativo

Espécies reativas são necessárias para a função normal da célula, servindo como moléculas de sinalização para importantes papéis fisiológicos. Elas são continuamente produzidas e neutralizadas pelos sistemas de defesa antioxidante. Contudo, quando produzidos em altas concentrações ou quando as defesas antioxidantes estão deficientes, as espécies reativas podem causar dano celular. Se o aumento de espécies reativas é relativamente pequeno, a resposta antioxidante será suficiente para compensar esse aumento. No entanto, sob certas condições patológicas, a produção de espécies reativas aumenta de forma tão intensa que as defesas antioxidantes podem ser insuficientes para restabelecer a homeostase redox.

O rompimento entre o equilíbrio pró-oxidante e antioxidante é descrito como estresse oxidativo, e pode representar um mecanismo fundamental envolvido em doenças humanas (Sies, 1985; Halliwell e Gutteridge, 2007). O estresse oxidativo pode resultar tanto de uma diminuição das defesas antioxidantes quanto de uma produção aumentada de oxidantes, bem como da liberação de metais de transição ou a combinação de quaisquer desses fatores (Halliwell, 2001).

I.1.6. Via das proteínas cinases dependentes de mitógenos (MAPK)

As proteínas cinases dependentes de mitógenos (MAPK) são enzimas responsáveis pela fosforilação de diversas proteínas que desencadeiam sinalizações associadas a

diferentes respostas celulares, tais como proliferação, sobrevivência, diferenciação, apoptose, dentre outras (Haddad, 2005; Wang et al., 2007). Existem quatro vias principais mediadas pelas MAPK: a da cinase regulada por sinalização extracelular (ERK1/2), a cinase C-Jun N-terminal (JNK), a p38 cinase (p38) e a cinase regulada por sinalização extracelular 5 (ERK5). Geralmente, a rota da ERK responde à sinalização de fatores de crescimento, enquanto que as vias da p38 e JNK respondem tipicamente a uma variedade de sinais de estresse extracelular (Yang et al., 2013). Contudo, já foram evidenciadas alterações nas vias de sinalização das MAPK em diversas doenças neurodegenerativas que envolvem superestimulação do receptor NMDA e/ou estresse oxidativo, incluindo as doenças de Parkinson e Alzheimer, esclerose lateral amiotrófica e erros inatos do metabolismo (Kim e Choi, 2010; Poddar e Paul, 2013).

I.1.7. Proteína Tau e sinaptofisina

A proteína Tau é considerada uma marcadora de dano neuronal, pois é um membro da família das fosfoproteínas associadas aos microtúbulos que tem papel essencial na regulação e na estabilização da montagem dos microtúbulos neuronais, no transporte axonal e no desenvolvimento dos neuritos (Terry, 1998; Kuszczyk et at., 2009). O grau de fosforilação dessa proteína pode ser alterado pela modulação da atividade de enzimas, tais como as MAPK e/ou fosfatases, o que modifica a sua afinidade de ligação e dinâmica com os microtúbulos. Dados da literatura têm demonstrado que tais alterações na fosforilação da Tau mediada pelas MAPK podem ocorrer com o envolvimento do receptor NMDA (Amadoro et al., 2006; Kuszczyk et al., 2009). Outros estudos também mostraram que a hiperfosforilação da Tau é um importante passo para a degeneração celular, uma vez que ela diminui a ligação dessa proteína aos microtúbulos resultando na despolarização desses

(Lee et al., 2001; Yi e Simpkins, 2008), ao passo que a diminuição da fosforilação pode indicar uma resposta inicial celular contra insultos oxidativos (Davis et al., 1995; Ko et al., 1997; Mills et al., 1998; LoPresti e Konat, 2001).

A sinaptofisina também é uma marcadora de dano neuronal, mais especificamente um indicativo de sinaptotoxicidade, visto que é a principal proteína integrante da membrana das vesículas pré-sinápticas de neurônios. Dessa forma, a sinaptofisina está envolvida na regulação da liberação de neurotransmissores e na plasticidade sináptica, bem como na biogênese e reciclagem das vesículas sinápticas (Alder et al., 1995; Janz et al., 1999).

Alterações na fosforilação da Tau e diminuição no conteúdo de sinaptofisina foram relatados em alguns EIM. Trabalhos realizados em camundongos nocautes para o gene *Niemann-Pick tipo C1* (modelo da doença de Niemann-Pick) ou para o gene *HGSNAT* (modelo de mucopolissacaridose III tipo C) mostraram alterações na fosforilação da Tau no cérebro desses animais (Nunes et al., 2011; Martins et al., 2015). Além disso, foi verificada em modelos nocautes para a fenilcetonúria (deficiência da fenilalanina hidroxilase) e mucopolissacaridose III tipo B (deficiência da N-acetilglicosaminidase) desenvolvidos em camundongos uma redução do conteúdo de sinaptofisina (Vitry et al., 2009; Horling et al., 2015). Tais trabalhos sugerem que alterações nessas proteínas contribuem para a neurodegeneração observada nessas doenças.

I.1.8. Patomecanismos envolvidos na neurodegeneração

Dados da literatura têm sugerido diferentes patomecanismos para explicar a fisiopatologia do dano cerebral observado em vários EIM, bem como em doenças neurodegenerativas comuns, tais como as de Alzheimer e Parkinson. Dentre esses

mecanismos, os mais comumente citados são excitotoxicidade, deficiência no metabolismo energético e estresse oxidativo (Rose e Henneberry, 1994; Koutsilieri e Riederer, 2007; Ribas et al., 2011; Huo et al., 2014; Kamat et al., 2014;Leipnitz et al., 2015).

No que se refere à excitotoxicidade, sabe-se que quando há um estímulo do receptor glutamatérgico NMDA, ocorre aumento no influxo celular de Ca^{2+} levando à formação de NO pela ativação da óxido nítrico sintase e, possivelmente, à morte celular. Um dos EIM no qual o papel da excitotoxicidade tem sido mais intensamente estudado é a acidemia glutárica tipo I. Estudos mostram que os ácidos glutárico e 3-hidroxiglutárico, metabólitos acumulados nessa acidemia, atuam como agonistas do receptor NMDA induzindo excitotoxicidade em culturas de neurônios de galinha e em culturas mistas de hipocampo de ratos (Kölker et al., 2004; Jafari et al., 2011). No caso da doença de Alzheimer, já foi relatado que os peptídeos Aβ são capazes de superestimular os receptores NMDA (Ong et al., 2013; Black et al., 2014), ao passo que na doença de Parkinson foi observado que um aumento nos níveis de glutamato extracelular contribui para disfunção e morte celular dopaminérgica na substância *nigra* (Chan et al., 1993; Meredith et al., 2009; Finlay e Duty, 2014; Gonzalez et al., 2015)

Também sugere-se que alterações no metabolismo energético seja um evento deletério envolvido na maioria das doenças neurodegenerativas (Parker et al., 1990; Swerdlow et al., 1998; Wajner e Goodman, 2011). O cérebro é altamente dependente de energia para seu funcionamento normal e a mitocôndria é a estrutura intracelular que mantém os suprimentos de energia para o cérebro. Uma alteração funcional nessa estrutura pode levar, portanto, a alterações patológicas em neurônios e astrócitos (Beal, 1995; Bowling e Beal, 1995; Davis et al., 1995; Browne, 2008). Nesse particular, trabalhos

demonstraram disfunção energética em diferentes modelos *in vitro* e *in vivo* de EIM caracterizada por inibição das atividades de enzimas do ciclo de Krebs e/ou dos complexos da cadeia respiratória, e diminuição na síntese de ATP (Wajner e Goodman, 2011). Com relação à doença de Parkinson, um dos mecanismos clássicos envolvidos nesse distúrbio é a inibição do complexo I da cadeia respiratória (Schapira et al., 1989; Schapira et al., 1990a,b; Papa e De Rasmo, 2013; Grünewald et al., 2015), sendo que tal evidência já foi reportada até mesmo em cérebros *postmortem* de pacientes (Mizuno et al., 1990; Schapira et al., 1990a). Por outro lado, na doença de Alzheimer, é comumente evidenciada uma redução na atividade do complexo IV em diferentes modelos da doença (Maurer et al., 2000; Ferrer, 2009), enquanto que estudos em cérebros de pacientes *postmortem* também demonstraram diminuição nas atividades dos complexos enzimáticos da piruvato desidrogenase e da α-cetoglutarato desidrogenase (Perry et al., 1980; Gibson et al., 1988; Mastrogiacomo et al., 1993).

O estresse oxidativo é outro mecanismo com papel chave na morte neuronal observada em casos de neurodegeneração. Um crescente número de relatos da literatura tem mostrado que os metabólitos acumulados nos EIM aumentam a produção de espécies reativas, induzem dano lipídico e proteico, e diminuem as defesas antioxidantes em modelos animais (Latini et al., 2007; Ribeiro et al., 2007; Feksa et al., 2008; Kessler et al., 2008; Zugno et al., 2008; Leipnitz et al., 2015) e em tecidos de pacientes (Sitta et al., 2006; Deon et al., 2007; Barschak et al., 2008a,b; Deon et al., 2008; Wajner e Goodman, 2011). No que diz respeito às doenças de Alzheimer e Parkinson, trabalhos evidenciaram dano oxidativo em cérebros humanos *postmortem* com esses distúrbios através da observação de dano ao DNA e proteico, bem como aumento de marcadores de peroxidação lipídica (Smith

et al., 1991; Markesbery e Carney, 1999; Nourooz-Zadeh et al., 1999; Lovell et al., 2000; Kim et al., 2015).

I.2. OBJETIVOS

I.2.1. Geral

Visto que a HNC é caracterizada pelo acúmulo tecidual de Gli e que a fisiopatologia dessa doença ainda não está totalmente estabelecida, o presente trabalho investigou os efeitos *ex vivo* da administração intracerebroventricular (ICV) da Gli sobre parâmetros de metabolismo energético, estresse oxidativo, funcionalidade neuro-glial e vias de sinalização em cérebro de ratos.

I.2.2. Específicos

a) Avaliar os efeitos *ex vivo* de uma única injeção ICV de Gli sobre a produção de CO_2, as atividades dos complexos da CTE, das enzimas do CAC, da CK e da Na^+,K^+-ATPase em córtex cerebral e estriado de ratos de 30 dias de idade 30 min ou 24 h após a administração de Gli;

b) Estudar os efeitos *ex vivo* da injeção ICV de Gli sobre o imunoconteúdo proteico da sinaptofisina, e fosforilação das MAPK (p38, ERK1/2 e JNK) e da proteína Tau 30 min ou 24 h após a injeção. Também foi avaliado o efeito da Gli sobre a marcação de GFAP e fluoromielina em córtex cerebral e estriado de ratos de 30 dias de idade 30 min após a injeção.

c) Investigar os efeitos *ex vivo* de única injeção ICV de Gli sobre as atividades enzimáticas dos complexos da CTE e da CK, dano a lipídeos, oxidação da 2',7'-diclorofluoresceína (DCFH), concentrações de glutationa (GSH), atividades da SOD,

CAT e GPx e redução do MTT em córtex cerebral de ratos neonatos de 1 ou 5 dias de vida eutanasiados 1, 5 ou 10 dias após a injeção. Além disso, foi avaliado o efeito da injeção da Gli sobre a imunomarcação da GFAP, S100β e NeuN em córtex cerebral, estriado e corpo caloso de ratos de 1 dia de idade eutanasiados 5 dias após a administração.

e) Estudar os efeitos da Gli *in vitro* em cerebelo quanto ao dano a lipídeos e proteínas, oxidação da DCFH e produção de peróxido de hidrogênio, concentrações de glutationa e atividades da SOD e CAT. Determinamos ainda a influência *in vitro* da Gli sobre as atividades dos complexos da CTE e da CK.

PARTE II

Artigos Científicos

Capítulo I

Glycine intracerebroventricular administration disrupts mitochondrial energy homeostasis in cerebral cortex and striatum of young rats

Alana Pimentel Moura, Mateus Grings, Belisa dos Santos Parmeggiani, Gustavo Flora Marcowich, Anelise Miotti Tonin, Carolina Maso Viegas, Ângela Zanatta, César Augusto João Ribeiro, Moacir Wajner, Guilhian Leipnitz

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ORIGINAL ARTICLE

Glycine Intracerebroventricular Administration Disrupts Mitochondrial Energy Homeostasis in Cerebral Cortex and Striatum of Young Rats

Alana Pimentel Moura · Mateus Grings · Belisa dos Santos Parmeggiani · Gustavo Flora Marcowich · Anelise Miotti Tonin · Carolina Maso Viegas · Ângela Zanatta · César Augusto João Ribeiro · Moacir Wajner · Guilhian Leipnitz

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Abstract High tissue levels of glycine (GLY) are the biochemical hallmark of nonketotic hyperglycinemia (NKH), an inherited metabolic disease clinically characterized by severe neurological symptoms and brain abnormalities. Considering that the mechanisms underlying the neuropathology of this disease are not fully established, the present work investigated the in vivo effects of intracerebroventricular administration of GLY on important parameters of energy metabolism in cerebral cortex and striatum from young rats. Our results show that GLY reduced CO_2 production using glucose as substrate and inhibited the activities of citrate synthase and isocitrate dehydrogenase in striatum, whereas no alterations of these parameters were verified in cerebral cortex 30 min after GLY injection. We also observed that GLY diminished the activities of complex IV in cerebral cortex and complex I–III in striatum at 30 min and inhibited complex I–III activity in striatum at 24 h after its injection. Furthermore, GLY reduced the activity of total and mitochondrial creatine kinase in both brain structures 30 min and 24 h after its administration. In contrast, the activity of Na^+, K^+-ATPase was not altered by GLY. Finally, the antioxidants *N*-acetylcysteine and creatine, and the NMDA receptor antagonist MK-801 attenuated or fully prevented the inhibitory effects of GLY on creatine kinase and respiratory complexes in cerebral cortex and striatum. Our data indicate that crucial pathways for energy production and intracellular energy transfer are severely compromised by GLY. It is proposed that bioenergetic impairment induced by GLY in vivo may contribute to the neurological dysfunction found in patients affected by NKH.

Keywords Nonketotic hyperglycinemia · Glycine · Energy metabolism · Cerebral cortex · Striatum

A. P. Moura · M. Grings · B. dos Santos Parmeggiani · G. F. Marcowich · A. M. Tonin · C. M. Viegas · Â. Zanatta · C. A. J. Ribeiro · M. Wajner · G. Leipnitz (✉)
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul, Rua Ramiro Barcelos N° 2600-Anexo, Porto Alegre, RS CEP 90035-003, Brazil
e-mail: guilhian@ufrgs.br

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

Introduction

Nonketotic hyperglycinemia (NKH), also termed glycine encephalopathy, is an autosomal recessive inborn error of glycine (GLY) catabolism with a prevalence estimated in 1:60,000 newborns (Applegarth et al. 2000). This disorder is caused by a deficiency in the GLY cleavage system, a multienzyme complex present in the inner mitochondrial membrane of brain, liver, kidney, and placenta (Hamosh and Johnston 2001). A defect in this system results in elevated GLY levels in all tissues and biological fluids, with increased cerebrospinal fluid (CSF)/plasma ratio (Applegarth and Toone 2001). Proton magnetic resonance spectroscopy has revealed that brain GLY concentrations may be as high as 7.3 mM in this pathological condition (Heindel et al. 1993; Hamosh and Johnston 2001).

Clinically, most patients have the classical neonatal form which appears in the first days after birth with spasticity, apnea, seizures, pronounced psychomotor development delay, and coma. Patients with late-onset variant forms exhibit a variable degree of mental retardation and seizures (Steiner et al. 1996). Cerebral MRI findings include progressive brain atrophy, decreased or absent myelination in the white matter, and hypoplasia of the corpus callosum

(Bekiesiniska-Figatowska et al. 2001; Hennermann et al. 2012; Tsuyusaki et al. 2012).

Although the pathogenesis of the brain lesions found in NKH is not fully established, it is long recognized that an increase of blood GLY concentrations leads to worsening of neurological symptoms (Frazier et al. 1978) and the higher the CSF/plasma ratio, the more severe is the disease (Hamosh and Johnston 2001). These data strongly indicate that elevated GLY concentrations are toxic to the brain and are probably involved in the pathophysiology of the brain injury found in this disease. In this regard, since GLY acts as an excitatory neurotransmitter via the *N*-methyl-D-aspartate glutamate (NMDA) receptor, it has been proposed that the neurologic damage associated with NKH is in part attributed to NMDA receptor overstimulation leading to excitotoxicity (Hara et al. 1993; Kure et al. 1997; Applegarth and Toone 2001; Kono et al. 2007; Katsuki et al. 2007). In addition, previous studies demonstrated that GLY induces oxidative damage and reduces the antioxidant defenses in vitro (Leipnitz et al. 2009) and in vivo (Seminotti et al. 2011), and compromises bioenergetics in brain of rats in vitro (Busanello et al. 2010). However, to the best of our knowledge, the in vivo effects of GLY on energy homeostasis have not been investigated.

Therefore, in the present work we evaluated the in vivo influence of GLY intracerebroventricular (ICV) administration on important parameters of energy metabolism, namely CO_2 production from glucose, the activities of citric acid cycle (CAC) enzymes citrate synthase (CS), isocitrate dehydrogenase (IDH), and aconitase (AC), of the respiratory chain complexes I–IV, of total creatine kinase (tCK) and its mitochondrial and cytosolic isoforms, and of Na^+, K^+-ATPase.

Experimental Procedures

Animals and Reagents

Thirty-day-old Wistar rats, obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, were used. 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). All reagents used were of analytical grade and purchased from Sigma Co. (St Louis, MO, USA).

Glycine (GLY) Intracerebroventricular (ICV) Administration

The rats were deeply anesthetized with equitesine (3.33 mL kg^{-1} intraperitoneally-i.p.), which is a mixture of 0.25 M chloral hydrate, 88 mM magnesium sulfate heptahydrate, 10 mg mL^{-1} sodium thiopental, 5.8 M propylene glycol, and 1.97 M ethanol, and thereafter placed in a stereotaxic apparatus. Two small holes were drilled in the skull and 2 μL of a 2.5 M GLY solution (5 μmol in a final volume of 2 μL) or NaCl (controls) at the same volume and concentration (each solution was prepared in water and pH was adjusted to 7.4 with NaOH) was slowly injected bilaterally over 3 min into each lateral ventricle via a needle connected by a polyethylene tube to a 10 μL Hamilton syringe. The needle was left in place for another 1 min before being gently removed. The coordinates for injection were as follows: 0.6 mm posterior to the bregma, 1.1 mm lateral to the midline, and 3.2 mm ventral from dura (Paxinos and Watson 1986). The correct position of the needle was tested by injecting 0.5 μL of methylene blue injection (4 % in saline solution) and carrying out histological analysis. The experimental protocol was approved by the Ethics Committee for animal research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, and followed the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85–23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

In some experiments, animals were pre-treated i.p. with *N*-acetylcysteine (NAC; 150 mg kg^{-1}) for 3 days, one injection per day (Farbiszewski et al. 2000), or creatine (CR; 50 mg kg^{-1}) for 3 days, two injections per day (Vasques et al. 2006). Control animals received the same volumes of saline solution (0.9 %). After pre-treatment, animals received ICV injection of GLY or NaCl. In the experiments designed to evaluate the role of NMDA glutamate receptors, the animals received a single injection of MK-801 (dizocilpine; 0.25 mg kg^{-1}, i.p.) (Ribeiro et al. 2006) 30 min before GLY injection.

Preparation of Cortical and Striatal Samples

The rats were sacrificed by decapitation without anesthesia 30 min or 24 h after ICV injection of either GLY or NaCl. The brain was rapidly excised on a Petridish placed on ice. The cerebral cortex and striatum were dissected, weighed, and homogenized in a specific buffer used for each technique. For CO_2 production, the cerebral tissues were homogenized (1:10, w/v) in Krebs-Ringer bicarbonate buffer, pH 7.4. For the determination of the activities of CS, IDH, AC, and the respiratory chain complexes, the cerebral cortex and the striatum were homogenized (1:20, w/v) in SET buffer, pH 7.4. The homogenates were centrifuged at 800×*g* for 10 min and the supernatants were kept at −70 °C until being used for enzyme activity determination. For tCK activity determination, the cerebral cortex and striatum were homogenized (1:10 w/v) in isosmotic saline solution. For preparation of mitochondrial

and cytosolic fractions, the homogenates were centrifuged at 1,000×*g* for 10 min at 4 °C and the pellet was discarded. The supernatant was then centrifuged at 27,000×*g* for 30 min at 4 °C. The cytosol (supernatant) was used for the determination of cytosolic creatine kinase (cCK) activity. The pellet containing the mitochondria was washed three times with saline solution and used as the mitochondrial fraction for the mitochondrial creatine kinase (mCK) enzymatic assay.

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

CO_2 Production

For CO_2 production, cortical and striatal homogenates containing 1.5–2 mg of protein were added to small flasks (11 cm^3), which were pre-incubated in a metabolic shaker at 37 °C for 10 min. After pre-incubation, [U-^{14}C] glucose (0.055 μCi) plus 5.0 mM of unlabeled glucose were added to the incubation medium in a final incubation volume of 500 μL. The flasks were gassed with a O_2:CO_2 (95:5) mixture and sealed with rubber stoppers and parafilm. Glass center wells containing a folded 65 mm/5 mm piece of filter paper were hung from the stoppers. After 60 min of incubation at 37 °C, 200 μL of 50 % trichloroacetic acid were added to the medium and 100 μL of benzethonium hydroxide were added to the center wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete $^{14}CO_2$ trapping and then opened. The filter papers were removed and added to vials containing scintillation fluid, and radioactivity was measured (Dutra-Filho et al. 1995). Results correspond to pmol glucose oxidized h^{-1} mg $protein^{-1}$.

Citric Acid Cycle (CAC) Enzyme Activities

Citrate synthase (CS) activity was measured according to Srere (1969), by determining DTNB reduction at $\lambda = 412$ nm, and calculated as nmol TNB min^{-1} mg $protein^{-1}$. Isocitrate dehydrogenase (IDH) activity was accessed by the method of Plaut (1969), by following NAD^+ reduction at wavelengths of excitation and emission of 340 and 466 nm, respectively, and calculated as nmol NADH min^{-1} mg $protein^{-1}$. The activity of aconitase (ACO) was measured according to Morrison (1954), following the reduction of $NADP^+$ at wavelengths of excitation and emission of 340 and 466 nm, respectively, and calculated as μmol NAPH min^{-1} mg $protein^{-1}$.

Respiratory Chain Complexes Activities

Mitochondrial respiratory chain enzyme activities (complexes I–III, II, II–III, and IV) were measured in homogenates with a protein concentration varying from 1.5 to 4.0 mg protein mL^{-1}. The activity of NADH: cytochrome *c* oxidoreductase (complex I–III) was assessed as described by Schapira et al. (1990). The activities of succinate: DCIP-oxidoreductase (complex II) and succinate: cytochrome *c* oxidoreductase (complex II–III) were determined according to the method of Fischer et al. (1985) and that of cytochrome *c* oxidase (complex IV) according to Rustin et al. (1994). The methods used to measure these activities were slightly modified, as described previously (da Silva et al. 2002). The activities of the respiratory chain complexes were calculated as nmol min^{-1} mg $protein^{-1}$.

Creatine Kinase (CK) Activity

CK activity was measured in the supernatants containing 0.4–1.2 μg of protein in a reaction mixture consisting of 60 mM Tris–HCl, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$, and 0.625 mM lauryl maltoside in a final volume of 100 μL. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of 0.3 μmol of ADP. The reaction was stopped after 10 min by the addition of 1 μmol of *p*-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962) with slight modifications as described previously (da Silva et al. 2004). The color was developed by the addition of 100 μL of 2 % α-naphthol and 100 μL of 0.05 % diacetyl in a final volume of 1 mL and read spectrophotometrically at 540 nm after 20 min. Results were calculated as μmol creatine min^{-1} mg $protein^{-1}$.

Na^+, K^+-ATPase Activity

The reaction mixture for the Na^+, K^+-ATPase determination consisted of 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, and purified synaptic membranes containing 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 (Tsakiris and Deliconstantinos 1984). Released inorganic

phosphate (Pi) was measured by the method of Chan et al. (1986). Enzyme-specific activity was calculated as nmol Pi released min^{-1} mg $protein^{-1}$.

Protein Determination

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

Statistical Analysis

Results are presented as 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 or one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the *F* value was significant. Only significant 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.

Results

Initially we investigated the effect of GLY ICV injection on CO_2 production from glucose in cerebral cortex and striatum from 30-day-old rats 30 min after GLY administration in order to evaluate whether this amino acid could affect the CAC functioning. It can be seen in Fig. 1 that CO_2 production was significantly decreased in striatum ($t_{(6)} = 4.101$, $P < 0.01$), but not in cerebral cortex. Moreover, we verified that the activities of the CAC enzymes CS ($t_{(6)} = 4.029$, $P < 0.01$) and IDH ($t_{(6)} = 2.48$, $P < 0.05$) were decreased in striatum, whereas the ACO activity was not modified (Fig. 2). None of the activities of CAC enzymes were altered in cerebral cortex (Fig. 2).

We also studied the effect of GLY administration on respiratory chain complex activities in an attempt to test whether GLY could reduce the activity of the electron transfer chain. Figure 3 shows that GLY injection inhibited complex IV activity in cerebral cortex ($t_{(9)} = 3.662$, $P < 0.01$) (Fig. 3d) and complex I–III activity in striatum ($t_{(8)} = 2.394$, $P < 0.05$) (Fig. 3b), without affecting complexes II (Fig. 3a) and II–III (Fig. 3c) in any of these brain structures 30 min after the injection of GLY. We also studied the effect of GLY on the activities of the respiratory chain complexes 24 h after the injection. GLY significantly decreased the activity of complex I–III in striatum ($t_{(8)} = 2.583$, $P < 0.05$) (Fig. 3b) and did not alter the various complex activities in cerebral cortex at this time point (Fig. 3a, c, d).

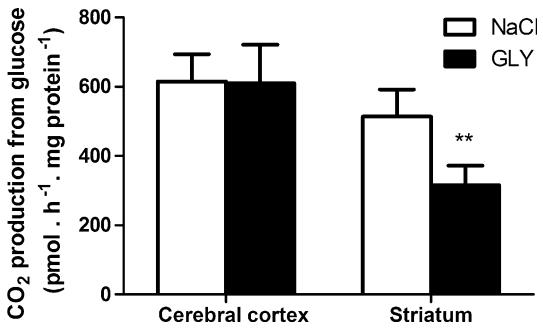

Fig. 1 Effect of intracerebroventricular (ICV) administration of glycine (GLY, 5 µmol) on CO_2 production from glucose in rat cerebral cortex and striatum. Data are expressed as mean ± SD for four to five independent experiments (animals) performed in triplicate and expressed as pmol CO_2 h^- g $tissue^{-1}$. $^{**}P < 0.01$, compared to rats that received ICV NaCl injection (Student's *t* test for unpaired samples)

The next step of our work evaluated the effects of GLY on tCK activity. We can observe in Fig. 4a that GLY markedly inhibited tCK activity in cerebral cortex ($t_{(6)} = 4.367$, $P < 0.01$) and striatum ($t_{(7)} = 2.638$, $P < 0.05$) 30 min after GLY injection. Furthermore, mCK activity was also significantly inhibited by GLY injection in both brain structures (cerebral cortex: $t_{(6)} = 3.381$, $P < 0.05$; striatum: $t_{(7)} = 6.075$, $P < 0.001$) (Fig. 4b), whereas the activity of cCK was not modified at this time point (Fig. 4c). We then examined the activities of tCK and its isoforms 24 h after GLY administration. We found a significant reduction of the activities of tCK (cerebral cortex: $t_{(7)} = 3.216$, $P < 0.05$; striatum: $t_{(8)} = 3.128$, $P < 0.05$) (Fig. 4a) and mCK (cerebral cortex: $t_{(7)} = 3.575$, $P < 0.01$; striatum: $t_{(6)} = 3.035$, $P < 0.05$) (Fig. 4b), but not of cCK (Fig. 4c).

The effect of GLY injection on synaptic Na^+, K^+-ATPase activity was assessed at two periods (30 min and 24 h) after GLY injection. Our results demonstrate that GLY did not modify Na^+, K^+-ATPase activity in cerebral cortex and striatum 30 min or 24 h after GLY injection (Table 1).

We then investigated the effects of NAC (antioxidant) and CR (energetic substrate and antioxidant) on GLY-induced decrease of the activities of the respiratory chain complexes and tCK in cerebral cortex and striatum 30 min after GLY ICV administration. NAC and CR totally prevented or attenuated the effects of GLY on the activities of tCK in these cerebral structures (cerebral cortex-NAC: $F_{(3,14)} = 6.364$, $P < 0.01$; CR: $F_{(3,19)} = 13.515$, $P < 0.001$) (striatum-NAC: $F_{(3,13)} = 4.363$, $P < 0.05$; CR: $F_{(3,13)} = 7.135$, $P < 0.01$) (Fig. 5a, b). Similar prevention of the inhibitory effect of GLY on complex IV activity was attained by these antioxidants in cerebral cortex (NAC: $F_{(3,14)} = 8.218$, $P < 0.01$; CR: $F_{(3,15)} = 4.580$, $P < 0.05$) (Fig. 7a, b). Furthermore, CR attenuated GLY-induced complex I–III inhibition in striatum (CR: $F_{(3,12)} = 4.30$, $P < 0.05$) (Fig. 6b), whereas NAC did not prevent this effect (Fig. 6a).

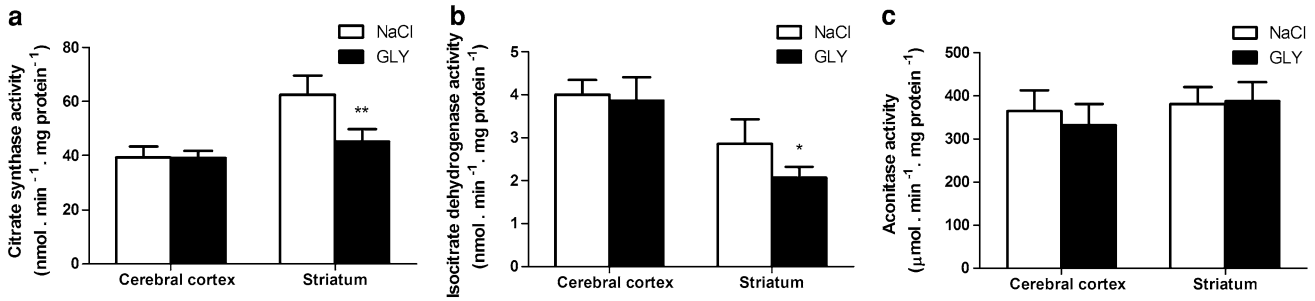

Fig. 2 Effect of intracerebroventricular (ICV) administration of glycine (GLY, 5 µmol) on citric acid cycle enzymes citrate synthase (CS) (**a**), isocitrate dehydrogenase (IDH) (**b**), and aconitase (ACO) (**c**) in cerebral cortex and striatum. Data are represented as mean ± SD for four to five independent experiments (animals) performed in triplicate and expressed as nmol TNB min^{-1} mg $protein^{-1}$ (CS), nmol NADH min^{-1} mg $protein^{-1}$ (IDH), and µmol NAPH min^{-1} mg $protein^{-1}$ (ACO). $^{*}P < 0.05$, $^{**}P < 0.01$, compared to rats that received ICV NaCl injection (Student's *t* test for unpaired samples)

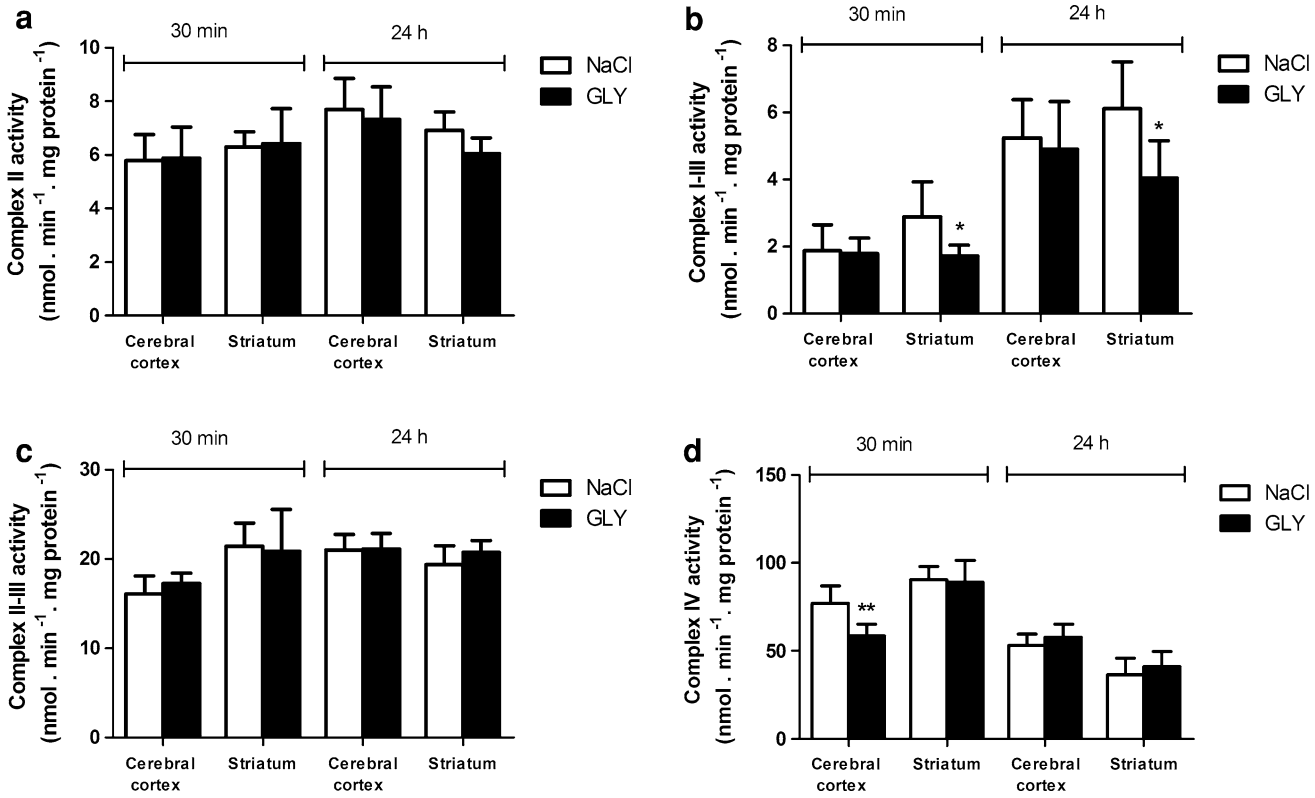

Fig. 3 Effect of intracerebroventricular (ICV) administration of glycine (GLY, 5 µmol) on the activities of the respiratory chain complexes I–IV in rat cerebral cortex and striatum 30 min or 24 h after GLY injection. Data are expressed as mean ± SD for five to six independent experiments (animals) performed in triplicate. The activity of complex II (**a**) is expressed as nmol DCIP reduced min^{-1} mg $protein^{-1}$ and complex I–III (**b**) as nmol cytochrome *c* reduced min^{-1} mg $protein^{-1}$. The activities of complexes II–III (**c**) and IV (**d**) are expressed, respectively, as nmol cytochrome *c* reduced min^{-1} mg $protein^{-1}$ and nmol cytochrome *c* oxidized min^{-1} mg $protein^{-1}$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to rats that received ICV NaCl injection (Student's *t* test for unpaired samples)

Finally, we tested the effect of the noncompetitive NMDA receptor antagonist MK-801 on the inhibitory effect of GLY on tCK and respiratory chain complex activities. MK-801 fully prevented the decrease of tCK activity in both brain structures (cerebral cortex: $F_{(3,14)} = 11.208$, $P < 0.001$) (striatum: $F_{(3,17)} = 10.012$, $P < 0.001$) (Fig. 5c) and of complex I–III in striatum ($F_{(3,15)} = 4.303$, $P < 0.05$) (Fig. 6c). However, GLY-induced decrease of the activity of complex IV was not modified by MK-801 in cerebral cortex (Fig. 7c).

Discussion

Patients affected by NKH have increased levels of GLY in all tissues, particularly in the brain, and present severe

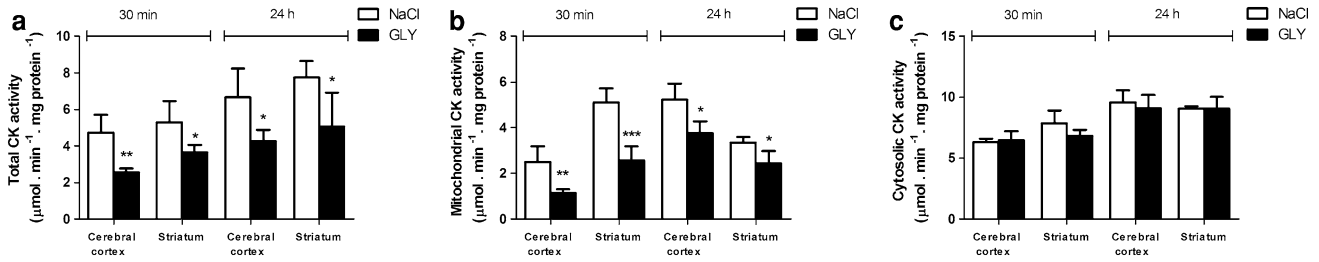

Fig. 4 Effect of intracerebroventricular (ICV) administration of glycine (GLY, 5 μmol) on total (**a**) mitochondrial (**b**) and cytosolic creatine kinase (CK) (**c**) activities in cerebral cortex and striatum 30 min or 24 h after GLY injection. Data are expressed as mean ± SD for four to six independent experiments (animals) performed in triplicate and are expressed as mmol creatine min^{-1} mg $protein^{-1}$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to rats that received ICV NaCl injection (Student's *t* test for unpaired samples)

Table 1 Effect of intracerebroventricular administration of glycine (GLY; 5 μmol) on Na^+, K^+-ATPase activity in cerebral cortex and striatum 30 min or 24 h after injection

	Na^+, K^+-ATPase activity			
	30 min		24 h	
	Control	GLY	Control	GLY
Cerebral cortex	702 ± 96.9	694 ± 21.4	786 ± 74.5	748 ± 66.0
Striatum	864 ± 67.6	786 ± 157	658 ± 47.8	698 ± 78.0

Values are mean ± standard deviation for four to five independent experiments (animals) and are expressed as nmol Pi released min^{-1} mg of $protein^{-1}$. No significant differences between groups were detected (one-way ANOVA)

neurological symptoms and brain atrophy. Although it has been suggested that overstimulation of the excitatory NMDA receptors plays an important role in the pathophysiology of NKH (Applegarth and Toone 2001; Hara et al. 1993), more research is necessary to elucidate other pathomechanisms possibly involved in the neurodegeneration found in the affected patients. In this particular, to the best of our knowledge, no in vivo studies investigating the effects of GLY on brain energy homeostasis have been performed. Therefore, in the present work we evaluated whether an acute ICV injection of GLY could compromise important parameters of energy metabolism in cerebral cortex and striatum of young rats.

GLY in vivo administration decreased CO_2 production from glucose in striatum, suggesting a disruption in the aerobic glycolytic pathway involving the CAC. Although we did not measure the activities of the enzymes of the glycolytic pathway, we demonstrated that the activities of the regulatory enzymes of the CAC, CS, and IDH were significantly inhibited by GLY in striatum that may possibly explain the reduction of CO_2 generation caused by this amino acid. We also tested the influence of GLY ICV administration on respiratory chain function by measuring the activities of complexes I–IV. GLY inhibited the activity of complex IV in cerebral cortex and of complex

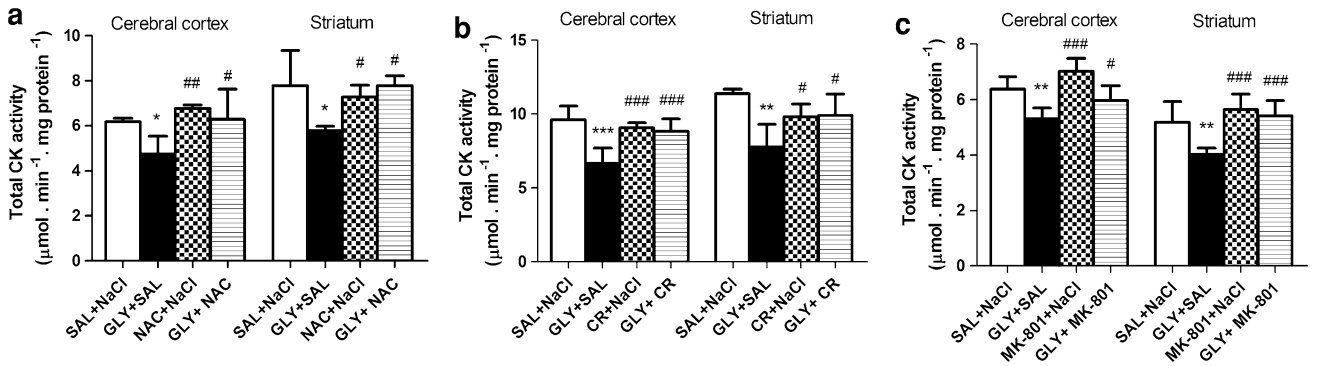

Fig. 5 Effect of *N*-acetylcysteine (NAC) (**a**), creatine (CR) (**b**), and MK-801 (**c**) on the total creatine kinase (CK) activity inhibition caused by glycine (GLY, 5 μmol) intracerebroventricular injection. Animals were intraperitoneally pre-treated with saline or NAC (3 days, one injection per day), CR (3 days, two injections per day), or MK-801 (30 min prior to GLY injection). Rats were sacrificed 30 min after GLY administration. Data are represented as mean ± SD for four to six independent experiments (animals) performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to rats pre-treated with saline and treated with NaCl; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to rats pre-treated with saline and treated with GLY (ANOVA followed by Duncan's multiple range test)

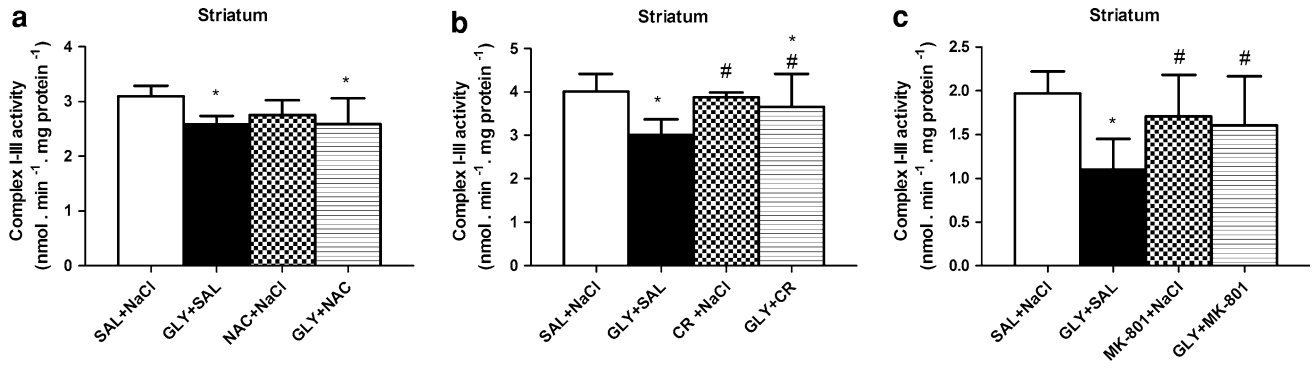

Fig. 6 Effect of *N*-acetylcysteine (NAC) (**a**), creatine (CR) (**b**), and MK-801 (**c**) on the complex I–III activity inhibition caused by glycine (GLY, 5 μmol) intracerebroventricular injection. Animals were intraperitoneally pre-treated with saline or NAC (3 days, one injection per day), CR (3 days, two injections per day), or MK-801 (30 min prior to GLY injection). Rats were sacrificed 30 min after GLY administration. Data are represented as mean ± SD for four to six independent experiments (animals) performed in triplicate. $^{*}P < 0.05$, compared to rats pre-treated with saline and treated with NaCl; $^{\#}P < 0.05$, compared to rats pre-treated with saline and treated with GLY (ANOVA followed by Duncan's multiple range test)

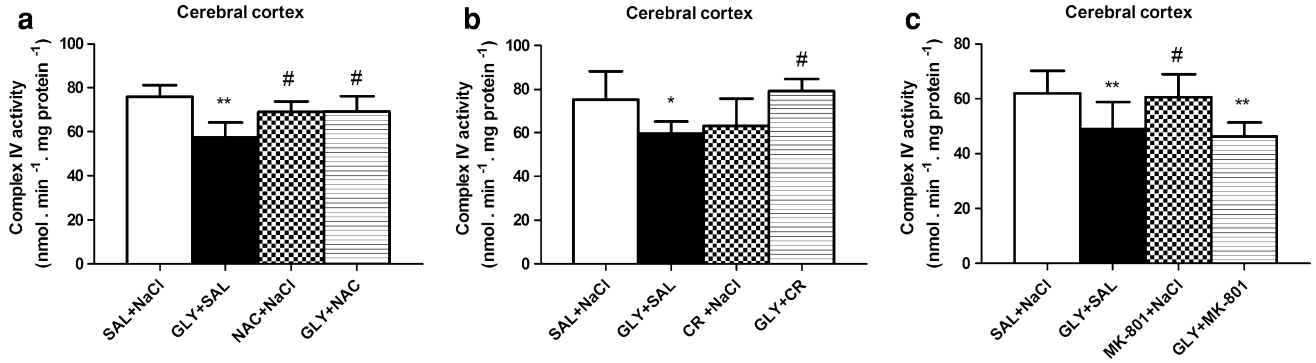

Fig. 7 Effect of *N*-acetylcysteine (NAC) (**a**), creatine (CR) (**b**), and MK-801 (**c**) on the complex IV activity inhibition caused by glycine (GLY, 5 μmol) intracerebroventricular injection. Animals were pre-treated intraperitoneally with saline or NAC (3 days, one injection per day), CR (3 days, two injections per day), or MK-801 (30 min prior to GLY injection). Rats were sacrificed 30 min after GLY injection. Data are represented as mean ± SD for four to six independent experiments (animals) performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to rats pre-treated with saline and treated with NaCl; $^{\#}P < 0.05$, compared to rats pre-treated with saline and treated with GLY (ANOVA followed by Duncan's multiple range test)

I–III in striatum at 30 min and of complex I–III in striatum at 24 h after its injection. The lack of effect of GLY on cytochrome *c* oxidase activity 24 h after its administration may be tentatively explained by mechanisms probably dependent on the presence of high concentrations of this amino acid in the brain structures. Alternatively, a compensatory biosynthesis of cytochrome *c* oxidase subunits following its inhibition may have occurred since this complex is critical to respiratory chain functioning (Rhein et al. 2009; Sauer et al. 2009; Arnold 2012). Considering that reduction of the electron transport chain flow (impaired oxidative phosphorylation) results in lower ATP synthesis, our present data suggest that energy production is compromised in vivo by GLY in cerebral cortex and striatum of young rats.

Next we verified that GLY markedly and selectively inhibited the mitochondrial isoform of CK 30 min and 24 h after GLY injection in both tissues, indicating a long-term inhibitory effect on this enzyme. Considering that CK plays an important role in cellular energy homeostasis, being mainly responsible for ATP buffering and transfer, it may be presumed that a disturbance of intracellular energy transfer is probably involved in the severe brain injury found in NKH.

We also verified that the antioxidants NAC and CR attenuated or fully prevented GLY-induced decrease of the activities of the complexes of respiratory chain and CK in cerebral cortex and striatum. Since NAC itself is a scavenger of several reactive species and a precursor of GSH (Halliwell and Gutteridge 2007), the most important brain antioxidant, it is proposed that GLY impairs the respiratory chain and inhibits CK via increased production of reactive species, corroborating with previous reports demonstrating that GLY induces oxidative stress in rat brain (Leipnitz

et al. 2009; Seminotti et al. 2011). It should be also emphasized here that CK molecule contains essential cysteine thiol groups for it is catalytic function that is highly vulnerable to oxidation and that GSH is a naturally occuring thiol-reducing antioxidant (Halliwell and Gutteridge 2007).

The same line of thinking may be assumed for CR since this compound is capable to reduce the generation of reactive species by acting as a free radical scavenger (Sestili et al. 2006) or through its stimulatory effects on mitochondrial respiration (Kay et al. 2000), allowing efficient recycling of ADP inside mitochondria by CK and leading to tight coupling of mitochondrial respiration (Meyer et al. 2006). It may be suggested that these mechanisms were involved in the prevention of GLY-induced inhibitory effects observed for CR in the present investigation.

Another interesting finding of the present work was that the noncompetitive NMDA receptor antagonist MK-801 prevented the decrease of the activities of complex I–III in striatum and CK in both brain structures caused by GLY. This prevention was probably related to role of GLY as a co-agonist of NMDA glutamate receptors. It is feasible that overstimulation of NMDA receptors by GLY leads to enhanced generation of reactive species that may attack CK and respiratory chain complexes resulting in their partial inactivation. Therefore, our present data highlight a role for NMDA receptor in GLY inhibitory effects and corroborate with previous findings demonstrating that MK-801 prevented the lipid peroxidation induced by GLY in vivo (Seminotti et al. 2011). This is also in line with the observations that excitotoxicity may lead to bioenergetics failure through increased production of reactive species and consequent oxidation and inactivation of several mitochondrial transport chain complexes (Zhang et al. 1990) and CK (Stachowiak et al. 1998).

We cannot at present explain the differential effects of MK-801 and NAC on GLY-induced inhibition of complexes I–III and IV of the respiratory chain complexes in striatum and cerebral cortex of rats, respectively. However, it should be noted that the expression of the subunits of NMDA receptor and of the respiratory chain complexes changes in the various brain regions (Clay and Ragan 1988; Rustin et al. 1994). It is possible that this differential expression may confer distinct reactivity of NMDA receptor and electron transport chain complexes to MK-801 and NAC. In this regard, previous reports demonstrated that MK-801-induced alterations of bioenergetic parameters, evaluated by the concentrations of glycolytic and Krebs cycle intermediates, differ in cerebral cortex, as compared to striatum and other brain structures (Brenner et al. 2005; Eyjolfsson et al. 2011). Furthermore, expression of different component subunits of cytochrome *c* oxidase in a brain region-specific manner has been previously shown in some pathological conditions, such as inflammation, hypoxia, or exposition to toxins (Sundar Boyalla et al. 2011; Roemgens et al. 2011; Arnold 2012). Therefore, we cannot exclude that the mechanisms underlying the neuroprotective action of MK-801 and NAC depend on the isoforms and subunits of the respiratory chain complexes and of NMDA receptor of each brain region (Sundar Boyalla et al. 2011; Roemgens et al. 2011; Arnold 2012).

Finally, we found that GLY did not alter the activity of Na^+, K^+-ATPase, suggesting that this amino acid does not compromise the membrane potential generation and the cellular volume control performed by Na^+, K^+-ATPase in vivo.

In conclusion, our study demonstrate that in vivo administration of GLY inhibits the CAC through the enzymes CS and IDH, the electron transport chain, and also CK in cerebral cortex and striatum, indicating that the accumulation of this toxic amino acid causes disruption of mitochondrial energy production, buffering, and transfer. Therefore, it is proposed that this pathomechanism contributes, at least in part, to the encephalopathy of NKH patients. Considering that NAC, CR, and MK-801 acted as neuroprotective agents in our in vivo model of NKH, this raises the possibility that the administration of these compounds may represent a potential adjuvant therapeutic strategy in NKH.

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Capítulo II

Glycine administration alters MAPK signaling pathway and causes neuronal damage in cerebral cortex and striatum of rats: putative mechanisms involved in the neurological dysfunction in nonketotic hyperglycinemia

Alana Pimentel Moura, Belisa dos Santos Parmeggiani, Juciano Gasparotto, Mateus Grings, Gabriela Miranda, Bianca Seminotti, José Cláudio Fonseca Moreira, Daniel Pens Gelain, Moacir Wajner, Guilhian Leipnitz.

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Alana Pimentel Moura[1], Belisa Parmeggiani[1], Juciano Gasparotto[1], Mateus Grings[1], Gabriela Miranda[1], Bianca Seminotti[1], José Cláudio Fonseca Moreira[1], Daniel Pens Gelain[1], Moacir Wajner[1,2], Guilhian Leipnitz[1].

[1]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

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

* Corresponding Author: Guilhian Leipnitz

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul. Rua Ramiro Barcelos N° 2600 – Attached, CEP: 90035-003, Porto Alegre, RS – Brasil. Phone: +55 51 3308-5560, fax: +55 51 3308-5535, e-mail: guilhian@ufrgs.br

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ABSTRACT

High glycine (GLY) levels have been shown to induce neurotoxic effects in the central nervous system of patients with nonketotic hyperglycinemia (NKH). Since the exact mechanisms involved in the pathophysiology of brain injury observed in this disorder are not totally established, we evaluated the effect of a single intracerebroventricular administration of GLY on the immunocontent of synaptophysin, and on the phosphorylation of Tau protein and the mitogen-activated protein kinases (MAPK) p38, ERK1/2 and JNK in cerebral cortex and striatum of rats. We also performed histopathological analysis to assess glial fibrillary acidic protein (GFAP), a marker of glial reactivity, and to evaluate the compaction and structure of myelin by fluoromyelin staining. The parameters were analyzed 30 min or 24 h after GLY administration. Our results showed that GLY decreased the phosphorylation of Tau in cerebral cortex and striatum 30 min and 24 h after its administration. On the other hand, synaptophysin levels were decreased by GLY in striatum at 30 min, and in cerebral cortex at 24 h after its injection. GLY also decreased the phosphorylation of p38, ERK1/2 and JNK 30 min after its administration in both brain structures evaluated. GLY-induced decrease of p38 phosphorylation in striatum was attenuated by the pre-treatment with the NMDA receptor antagonist MK-801. In contrast, GFAP and fluoromyelin staining were not altered 30 min after GLY infusion. Based on these findings, it may be presumed that MAPK decreased phosphorylation associated to alterations of markers of neuronal injury induced by GLY may contribute to the neurological dysfunction observed in NKH.

Keywords: glycine, MAPK, synaptophysin, Tau protein, rat brain.

INTRODUCTION

Nonketotic hyperglycinemia (NKH) is an autosomal recessive inborn error of metabolism caused by the deficient activity of glycine (GLY) cleavage system (GCS), which is the main catabolic pathway for GLY. This deficiency leads to the accumulation of GLY in cerebrospinal fluid (CSF), plasma and tissues of affected patients, with a high CSF/plasma ratio. GCS is an intramitochondrial enzyme complex that is formed by the P-protein, which decarboxylates GLY to release carbon dioxide and transfers the aminomethyl group to a lipoate on the H-protein, and the T-protein that releases ammonia and forms methylenetetrahydrofolate, after which the reduced lipoate is reoxidized by L-protein.

The classical NKH is the most severe and frequent phenotype of this disorder and it is reported that 70-75 % of patients with this phenotype have a mutation in *GLDC* gene that encodes the P-protein [1-3]. The symptoms of classical NKH appear neonatally with lethargy, myoclonic jerks, muscular hypotonia and apnea that frequently lead to coma and death. Surviving individuals have profound neurological impairment and intractable seizures [4,3,5]. Cerebral MRI findings include progressive brain atrophy, hypoplasia of the corpus callosum, gliosis and vacuolating myelinopathy [6-8].

The pathogenesis of the neurological dysfunction observed in NKH has been attributed to the accumulation of GLY in brain of affected patients. In this context, it was showed that an increased CSF/plasma GLY ratio correlates with the severity of the disorder [2]. Furthermore, previous reports suggested that GLY induces excitotoxic damage, since this molecule is a co-agonist of NMDA glutamate receptor [9-13]. This is in line with a study that showed that the NMDA receptor antagonist MK-801 prevents bioenergetics dysfunction caused by GLY *ex vivo* in brain of rats [14]. It was further demonstrated that GLY alters redox homeostasis and provokes glial reactivity in brain of rats [15-18]. Nevertheless, the exact pathomechanisms underlying the brain abnormalities in this disorder are not fully established.

Regarding excitotoxicity, it is well known that this event leads to a cascade of deleterious intracellular effects that include calcium overload with consequent dysregulation of several signaling pathways, such as the mitogen-activated protein kinases (MAPK), oxidative stress and energy metabolism impairment that may lead to neuronal damage [19]. Since GLY is suggested to cause excitotoxicity, in the present work we evaluated the *ex vivo* influence of GLY intracerebroventricular (ICV) administration on the phosphorylation of Tau protein and the MAPK p38, ERK and JNK, as well as and immunocontent of synaptophysin in

cerebral cortex and striatum of young rats. We also performed histopathological analysis to assess glial reactivity and myelin structure after GLY injection.

Material and Methods

Animals and reagents

Thirty-day-old Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, were used. 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). All reagents used were of analytical grade and purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The experiments were approved by the local Animal Ethics Commission (Universidade Federal do Rio Grande do Sul) under the number 23787, and the National Animal Rights Regulations (Law 11.794/2008). The guidelines of National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication n^{o}. 80-23, revised 1996), and Directive 2010/63/EU were followed. All efforts were made to minimize the number of animals used and their suffering.

Glycine (GLY) administration

The rats were deeply anesthetized with equitesine (3.33 mL.kg^{-1}, intraperitoneally-ip), which is a mixture of 0.25 M chloral hydrate, 88 mM magnesium sulfate heptahydrate, 10 mg.mL^{-1} sodium thiopental, 5.8 M propyleneglycol, and 2.0 M ethanol, and thereafter placed in a stereotaxic apparatus. Two small holes were drilled in the skull and 2 µL of a 2.5 M GLY solution (5 µmol in a final volume of 2 µL) or NaCl (control) at the same volume and concentration (each solution was prepared in water and pH was adjusted to 7.4 with NaOH) were slowly injected bilaterally over 3 min into each lateral ventricle via a needle connected by a polyethylene tube to a 10 µL Hamilton syringe. The needle was left in place for another 1 min before being gently removed. The coordinates for injection were as follows: 0.6 mm posterior to the bregma, 1.1 mm lateral to the midline, and 3.2 mm ventral from dura [20]. The correct position of the needle was tested by injecting 0.5 µL of methylene blue injection (4 % in saline solution) and carrying out histological analysis. In

the experiment designed to evaluate the role of the NMDA glutamate receptor on GLY effects, the animals received a single injection of MK-801 (dizocilpine; 0.25 $mg.kg^{-1}$, ip) [21] 30 min before GLY injection.

Preparation of samples and Western Blot Analysis

The rats were euthanized by decapitation 30 min or 24 h after GLY injection without anesthesia and had their brain rapidly excised on a Petri dish placed on ice. Cerebral cortex or striatum was homogenized in RIPA buffer containing protease and phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM aprotinin and 1 % protease inhibitor cocktail) and centrifuged at 10,000 *g* for 10 min at 4 °C. Supernatant protein concentrations were determined by the method of Bradford [22] , then denaturated in 4x Laemli buffer (250 mM Tris, 8 % SDS, 40 % glicerol and 0.002 % bromophenol blue, pH 6.7) and 10 % 2-mercaptoethanol. These samples were then heated at 98 ºC for 5 min and equal amounts of protein (30 μg/well) were fractionated by SDS-PAGE and electro-blotted onto nitrocellulose membranes. Protein loading and electro-blotting efficiency were verified through Ponceau S staining. Afterwards the membrane was blocked in 5 % albumin prepared with Tween-Tris-buffered saline (TTBS; 100 mM Tris-HCl, pH 7.6, containing 70 mM NaCl and 0.1 % Tween-20), and incubated overnight at 4 °C with the primary antibodies diluted at 1:1.000 in TTBS (anti-p38, anti-phospho-p38, anti-ERK1/2, anti-phospho-ERK1/2, anti-Tau, anti-phospho-Tau and anti-synaptophysin were purchased from Cell Signaling Technology; anti-JNK and anti-phospho-JNK from R&D systems) and washed with TTBS. The membrane was incubated with anti-IgG from mouse or rabbit (according the species that originated the primary antibody) linked to a peroxidase for 2 h at room temperature (1:10.000 dilution range) and washed with TTBS again. The immunoreactivity was detected by enhanced chemiluminescence using Millipore Immobilon™ Western chemiluminescent HRP substrate in a CCD camera (*GE* ImageQuant LAS 4000). Densitometric analysis of the membranes was performed with ImageJ software. Blots were developed to be linear in the range used for densitometry.

Immunohistochemical studies

After GLY administration, animals were anesthetized with an injection (ip) of a mixture of ketamine (80 $mg.kg^{-1}$) and xylazine (10 $mg.kg^{-1}$) until complete unresponsiveness to nociceptive stimuli and then transcardially perfused with 0.9 % saline and 10 % paraformaldehyde (PAF) prepared in 0.1 M phosphate

buffer, pH 7.4, to fix the brain. Fixed brains were removed, post-fixed by immersion in PAF during 24 h and then sectioned on a vibrating microtome to obtain 30-50 μm thick consecutive coronal series. Immunohistochemistry was performed in cerebral cortex and striatum. For each animal and staining procedure, 3 to 6 equivalent sections were immunostained. Free-floating sections were washed with PBS, submitted to antigen retrieval by boiling in 10 mM sodium citrate, pH 6.0, during 10 min, and washed twice for 10 min. The slices were then permeabilized with PBS plus 0.1-0.3% Triton X-100 (PBST) for 20 min and treated with blocking buffer (PBS containing 0.3 % Triton X-100 and 5 % bovine serum albumin) for 1 h. Afterwards, slices were incubated with the antibodies anti-GFAP (Sigma-Aldrich Co., 1:300) or the fluoromyelin probe (Sigma-Aldrich Co.). All dilutions were carried out in PBST. After a 4 °C overnight incubation, sections were rinsed in PBS and incubated at room temperature for 2 h with a secondary antibody (1:500) conjugated to fluorescent probes (Molecular Probes). Sections were then washed, mounted using fluoroshield (Sigma-Aldrich Co.) and imaged in a FV300 Olympus confocal microscope provided with 488 and 546 nm lasers. Primary or secondary antibodies were omitted in negative controls [23].

Data normalization and statistical analysis

Sample protein content was quantified for data normalization according to Bradford method [22]. Data were analyzed using the Student's *t* test for unpaired samples or one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the F value was significant with GraphPad 5.0 software. Only significant values are shown in the text. Differences between groups were rated significant at $P < 0.05$. All data presented here are the result of three or more independent experiments.

RESULTS

GLY administration decreases synaptophysin content in rat cerebral cortex and striatum

We first investigated the effects of GLY ICV administration on the levels of synaptophysin, a membrane protein of presynaptic vesicles, in rat cerebral cortex and striatum 30 min and 24 h after GLY administration. GLY significantly decreased synaptophysin in cerebral cortex at 24 h [$t_{(6)}$=-2.503, $P<0.05$]

(Fig. 2), and in striatum 30 min after the injection [$t_{(6)}$= 2.5, P<0.05] (Fig. 1), indicating that GLY causes synaptotoxicity in the brain.

GLY decreases Tau protein phosphorylation in rat cerebral cortex and striatum

The influence of GLY injection on the phosphorylation of Tau, a protein involved in the regulation of cell microtubule dynamics and stability, was also examined in rat brain. It can be observed in Figures 3 and 4 that GLY decreased Tau phosphorylation in cerebral cortex 30 min [$t_{(6)}$=3.340, P<0.05] and 24 h [$t_{(6)}$=5.152, P<0.01] after its administration. In addition, GLY decreased Tau phosphorylation also in striatum in both periods tested [30 min: $t_{(6)}$=3.755; P<0.01; 24 h: $t_{(6)}$=3.628; P<0.05] (Fig. 3 and 4). These data show that high levels of GLY impair the neuronal microtubule function.

GLY administration decreases MAPK phosphorylation in rat cerebral cortex and striatum

We next examined the effects of GLY injection on the protein immunocontent of the MAPK p38, ERK1/2 and JNK in rat brain since Tau is a substrate of these kinases. Our results demonstrate that GLY decreased the phosphorylation of p38, ERK1/2 and JNK in cerebral cortex [p38: $t_{(6)}$=3.768; P<0.01; ERK1/2: $t_{(5)}$=2.968; P<0.05; JNK: $t_{(6)}$=9.086; P<0.001] (Fig. 5) and striatum [p38: $t_{(5)}$=5.576; P<0.01; ERK1/2: $t_{(6)}$=13.37; P<0.001; JNK: $t_{(5)}$=4.903; P<0.01] (Fig. 6) of young rats 30 min after GLY injection.

NMDA receptor antagonist MK-801 prevents GLY-induced decrease of the phosphorylation of p38 in rat striatum

We then evaluated the influence of the pre-treatment with the NMDA antagonist MK-801 on GLY-induced decrease of p38 phosphorylation once it has been reported that GLY exerts excitotoxicity via NMDA receptor [9-13] and that the activities of MAPK may be influenced by this receptor [24,25]. We verified that MK-801 prevented the decreased p38 phosphorylation caused by GLY in rat striatum 30 min after its administration [striatum p38 $F_{(2,8)}$=6.191; P<0.03] (Fig. 7), suggesting that this MAPK is altered by GLY via NMDA receptor activation.

GLY administration does not alter GFAP and myelin structure in rat cerebral cortex and striatum

The effect of GLY administration on GFAP and fluoromyelin staining in rat cerebral cortex and striatum was further investigated. It can be observed that GLY did not alter these parameters in the brain regions evaluated (Fig. 8 and 9), implying that this amino acid does not cause glial reactivity and does impair myelin structure in brain of young rats.

Discussion

Patients affected by NKH usually present in the neonatal period lethargy, hypotonia and seizures, whose pathophysiology is not yet established. However, previous data demonstrated that high levels of GLY causes excitotoxicity via NMDA receptor overstimulation, induces oxidative stress and bioenergetics dysfunction in rat brain [17,14,2,26,27]. Considering these GLY neurotoxic effects described in the literature, in the present study we investigated the *ex vivo* effects of GLY administration on MAPK signaling pathways in brain of rats once the activities of these kinases may be altered by NMDA receptor activation and/or reactive species. We also examined whether the toxic effects of GLY could cause neuronal damage in rat brain.

In the first part of our work we analyzed the effects of a single GLY ICV administration on the markers of neuronal damage synaptophysin levels and Tau phosphorylation. Our results showed that GLY decreased the immunocontent of synaptophysin in rat cerebral cortex and striatum. It is well known that synaptophysin is an abundant integral membrane protein of pre-synaptic vesicles essential for the formation of synapse, regulation of neurotransmitter release and synaptic plasticity [28-30], thus having a crucial role in cognitive function. In this context, previous studies showed that mice lacking synaptophysin exhibit behavioral alterations and learning deficits [31] and that the loss of this protein in the hippocampus of patients with Alzheimer's disease correlates with the cognitive decline of these individuals [32]. Moreover, it was also demonstrated that synaptophysin can be downregulated in response to stress conditions, leading to impairment of synaptic integrity [33,34]. Thus, it may be presumed that GLY induces synaptotoxicity that possibly leads to the neuronal damage.

GLY also decreased Tau phosphorylation in rat brain. Since this protein is involved in the outgrowth of neural processes, axonal transport, development of neuronal polarity, and maintenance of normal neuron morphology [35,36], these data reinforce the view that GLY causes neuronal damage. Moreover, our findings corroborate with Zambrano et al. [37] that showed decrease of Tau protein phosphorylation in rat hippocampus cells exposed to hydrogen peroxide. It should be considered here that previous findings from our group demonstrated that GLY increases reactive species production *in vitro* and *in vivo* in rat brain [15,17,18].

Considering that Tau protein is a substrate for the MAPK and that the activity of these kinases may be modulated by NMDA receptor overstimulation, we next assessed the effect of GLY administration on the phosphorylation of p38, ERK and JNK. GLY decreased the phosphorylation of the three MAPK, which is in accordance with our results showing a decrease in the phosphorylation of Tau protein. Nevertheless, we cannot rule out that GLY-induced Tau phosphorylation decrease can also occur due to the activation of phosphatases, besides the deactivation of MAPK. Furthermore, these data are in line with previous reports showing dysregulation of MAPK signaling pathways in different models of neurological disorders with involvement of excitotoxicity, oxidative stress and bioenergetics dysfunction, including schizophrenia [38,39], depression [40-42], mental retardation [43] and Alzheimer`s disease [44].

We then tested the effect of the NMDA receptor antagonist MK-801 on GLY-induced decrease phosphorylation of p38 since a large body of evidence have demonstrated that the sustained activation of NMDA receptor leads to a cascade of intracellular events that include calcium influx and alterations in the phosphorylation status of the MAPK [45,46]. It should be also emphasized here that the p38 pathway has important roles in transducing stress-related signals involved in cell survival, apoptosis, and inflammatory cytokine production [47-50] and that this MAPK was showed to mediate the pathogenesis of glutamate excitotoxicity [50,51]. We found that MK-801 prevented the decrease of p38 phosphorylation induced by GLY, which corroborates the data reported by Wang et al. [45] that reported that an increased calcium influx induces ERK phosphorylation decrease in hippocampus of mice submitted to chronic hypoxia, which was prevented by the NMDA receptor antagonist memantine [45]. It is also noteworthy that there are evidences suggesting that NMDA receptor activation may be associated with a decrease in the phosphorylation of cytoskeletal proteins, such as Tau [52,53,36]. So, it can be assumed that GLY, as an NMDA receptor co-

agonist, induces MAPK decreased phosphorylation that leads to Tau protein reduced phosphorylation, as previously reported in other pathological situations [36,45].

Brain abnormalities observed in patients affected by NKH consist of progressive cortical brain atrophy with white matter leukodystrophy, gliosis and vacuolating myelinopathy [6-8]. In this particular, it was already shown that GLY administration induces glial reactivity in brain of neonatal rats [18]. Considering this, we assessed the effects of GLY on GFAP levels and myelin compaction and structure and verified that these markers were not altered by the amino acid. We cannot at present explain why we did not observe glial reactivity induced by GLY, as previously demonstrated in neonatal rats, but it may be speculated that the brain of newborn rats is more susceptible to GLY neurotoxic effects than young rats [18]. Moreover, in the present study we analyzed the effects of GLY on the central nervous system of young rats 30 min after its injection, while the data in the literature reported that GLY-induced gliosis occurs 5 days after GLY administration [18].

In conclusion, this is the first report showing that GLY alters MAPK signaling pathways, which are classically involved in neuronal stress [19,54,55], and causes neuronal damage reflected by decreased synaptophysin levels and Tau protein phosphorylation. Our data also provide evidence that the stimulation of NMDA receptor by GLY is the mechanism involved in the dysregulation of MAPK signaling pathways and possibly to decreased phosphorylation of Tau protein. Finally, our results reinforce the view that the use of NMDA receptor antagonists is beneficial for patients affected by NKH.

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Legends to Figures

Fig.1 Effect of intracerebral administration of glycine (GLY; 5 µmol) on the immunocontent of synaptophysin in cerebral cortex (a) and striatum (b) of young rats. Rats were euthanized 30 min after GLY injection. Representative immunoblots are shown as mean ± SD for four independent experiments (animals) normalized by β-actin immunocontent. *P < 0.05, compared to control (Student's *t* test for unpaired samples).

Fig.2 Effect of intracerebral administration of glycine (GLY; 5 µmol) on the immunocontent of synaptophysin in cerebral cortex (a) and striatum (b) of young rats. Rats were euthanized 24 h after GLY injection. Representative immunoblots are shown as mean ± SD for four independent experiments (animals) normalized by β-actin immunocontent. *P < 0.05, compared to control (Student's *t* test for unpaired samples).

Fig.3 Effect of intracerebral administration of glycine (GLY; 5 µmol) on the immunocontent of Tau protein in cerebral cortex (a) and striatum (b) of young rats. Rats were euthanized 30 min after GLY injection. Representative immunoblots are shown as mean ± SD for four independent experiments (animals). *P < 0.05, **P < 0.01, compared to control (Student's *t* test for unpaired samples).

Fig.4 Effect of intracerebral administration of glycine (GLY; 5 µmol) on the immunocontent of Tau protein in cerebral cortex (a) and striatum (b) of young rats. Rats were euthanized 24 h after GLY injection. Representative immunoblots are shown as mean ± SD for four independent experiments (animals). *P < 0.05, compared to control (Student's *t* test for unpaired samples).

Fig.5 Effect of intracerebral administration of glycine (GLY; 5 µmol) on the immunocontent of the mitogen-activated protein kinases (MAPK) p38 (a), ERK (b) and JNK (c) in cerebral cortex of young rats. Rats were euthanized 30 min after GLY injection. Representative immunoblots are shown as mean ± SD for three to four independent experiments (animals). *P < 0.05, **P < 0.01, ***P < 0.001, compared to control (Student's *t* test for unpaired samples).

Fig.6 Effect of intracerebral administration of glycine (GLY; 5 μmol) on the immunocontent of the mitogen-activated protein kinases (MAPK) p38 (a), ERK (b) and JNK (c) in striatum of young rats. Rats were euthanized 30 min after GLY injection. Representative immunoblots are shown as mean ± SD for three to four independent experiments (animals). **P < 0.01, ***P < 0.001, compared to control (Student's *t* test for unpaired samples).

Fig.7 Effect of MK-801 (0.25 $mg.kg^{-1}$) on glycine (GLY; 5 μmol)-induced p38 phosphorylation decrease in striatum of young rats. Rats were euthanized 30 min after GLY injection. Representative immunoblots are shown as mean ± SD for three independent experiments (animals). *P < 0.05, compared to control (Duncan multiple range test).

Fig.8 Effect of intracerebral administration of glycine (GLY; 5 μmol) on glial fibrillary acidic protein (GFAP) immunofluorescence staining in cerebral cortex (a) and striatum (b) of young rats. Rats were euthanized 30 min after GLY injection. Representative images were obtained from three independent experiments (animals) per group. GFAP is shown with magnification of 40 X (scale bar of 200 μm).

Fig.9 Effect of intracerebral administration of glycine (GLY; 5 μmol) on fluoromyelin immunofluorescence staining in striatum of young rats. Rats were euthanized 30 min after GLY injection. Representative images were obtained from three independent experiments (animals) per group. Fluoromyelin is shown with magnification of 40 X (scale bar of 200 μm).

FIGURES

Figure 1.

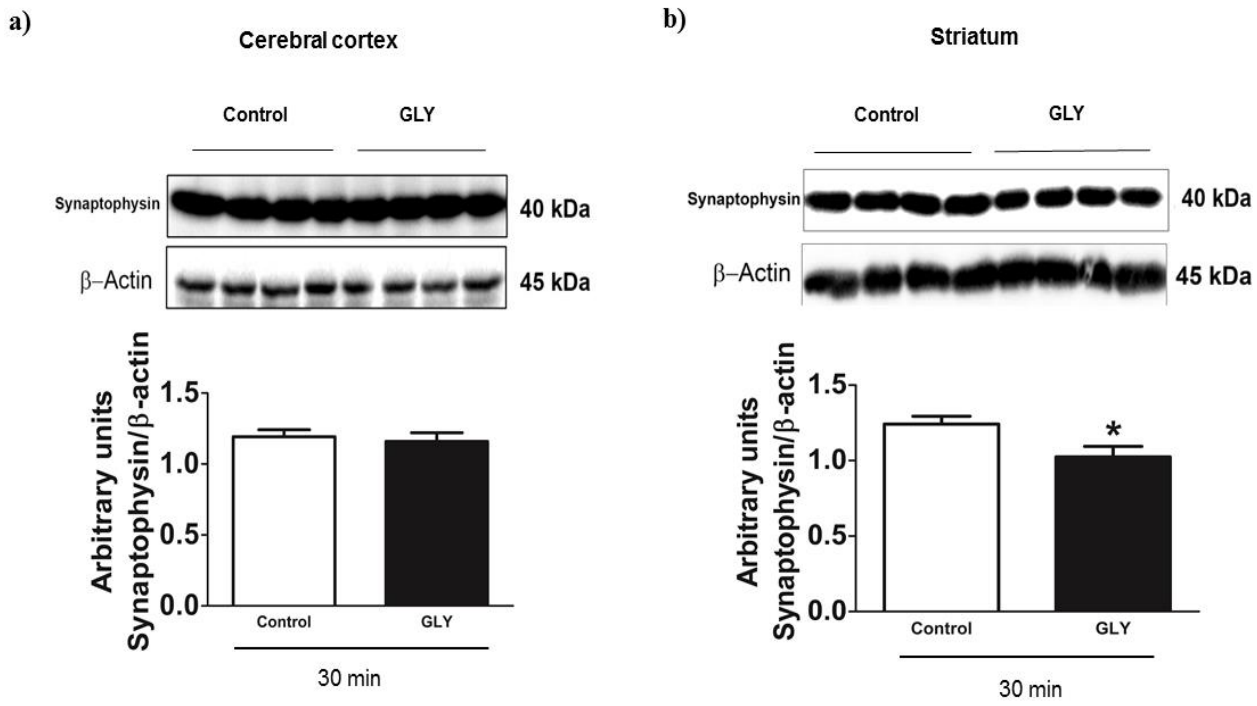


Figure 2.

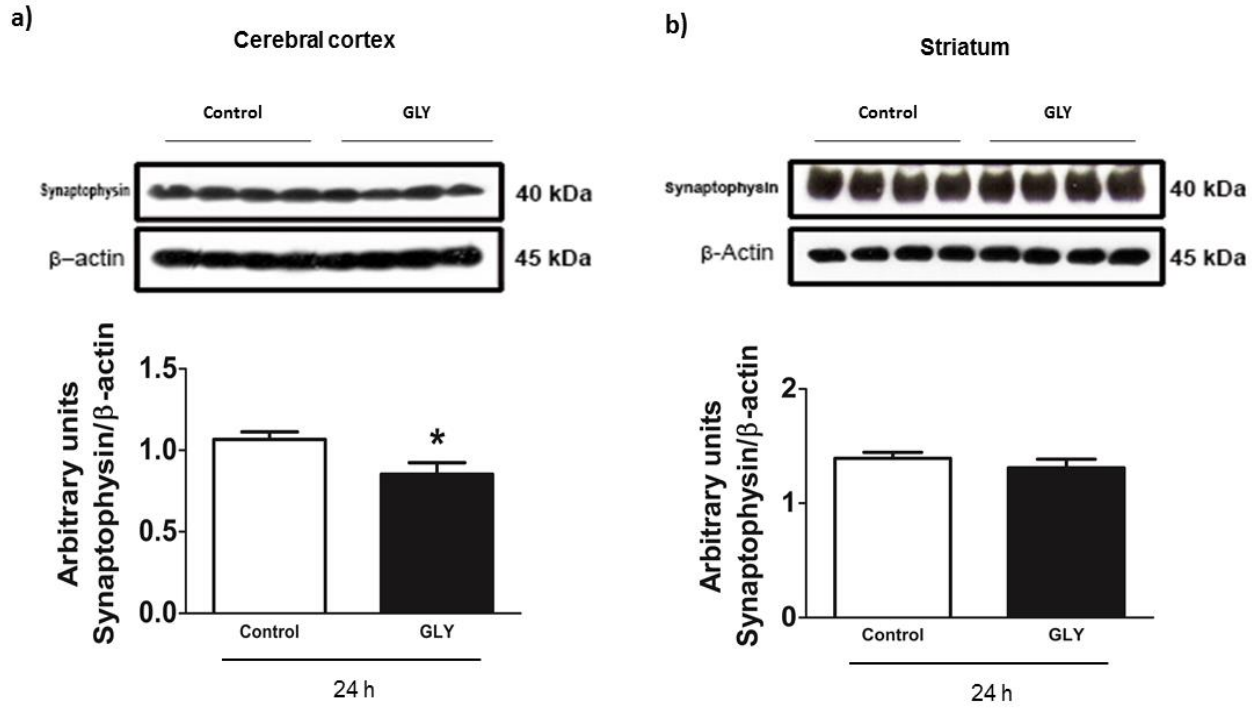

a)
Cerebral cortex
Control
GLY
Synaptophysin
β–actin
40 kDa
45 kDa
Arbitrary units
Synaptophysin/β-actin
1.5
1.0
0.5
0.0
*
Control
GLY
24 h
b)
Striatum
Control
GLY
Synaptophysin
β-Actin
40 kDa
45 kDa
Arbitrary units
Synaptophysin/β-actin
2
1
0
Control
GLY
24 h

Figure 3.

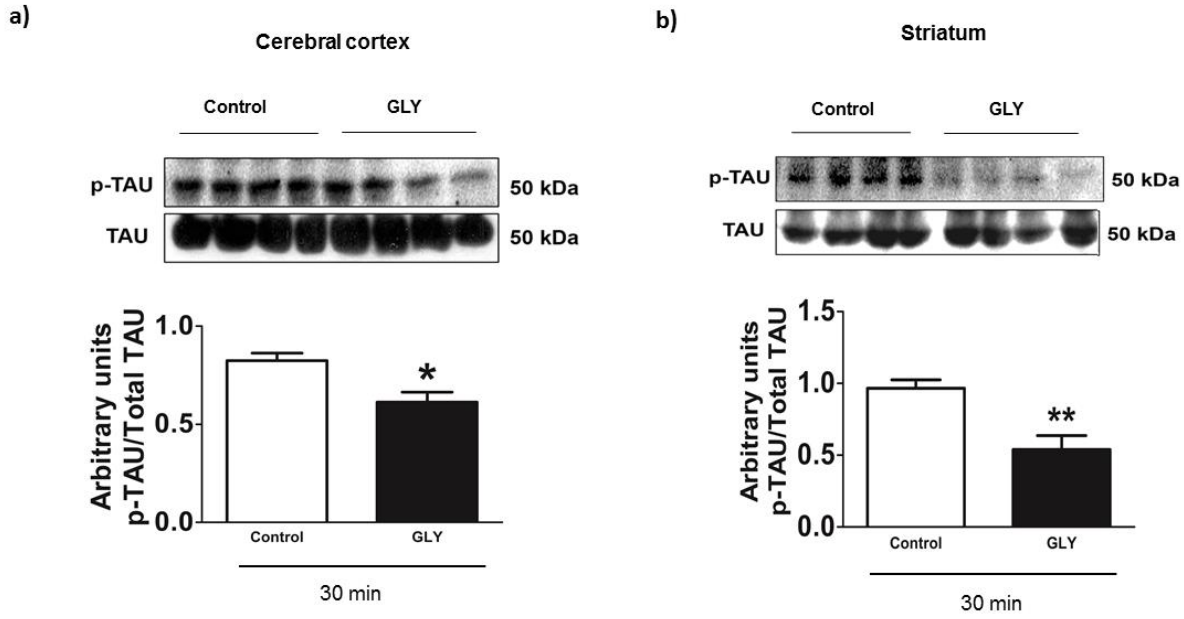

a)
Cerebral cortex
Control
GLY
p-TAU
TAU
50 kDa
50 kDa
Arbitrary units
p-TAU/Total TAU
1.0
0.5
0.0
*
Control
GLY
30 min
b)
Striatum
Control
GLY
p-TAU
TAU
50 kDa
50 kDa
Arbitrary units
p-TAU/Total TAU
1.5
1.0
0.5
0.0
**
Control
GLY
30 min

Figure 4.

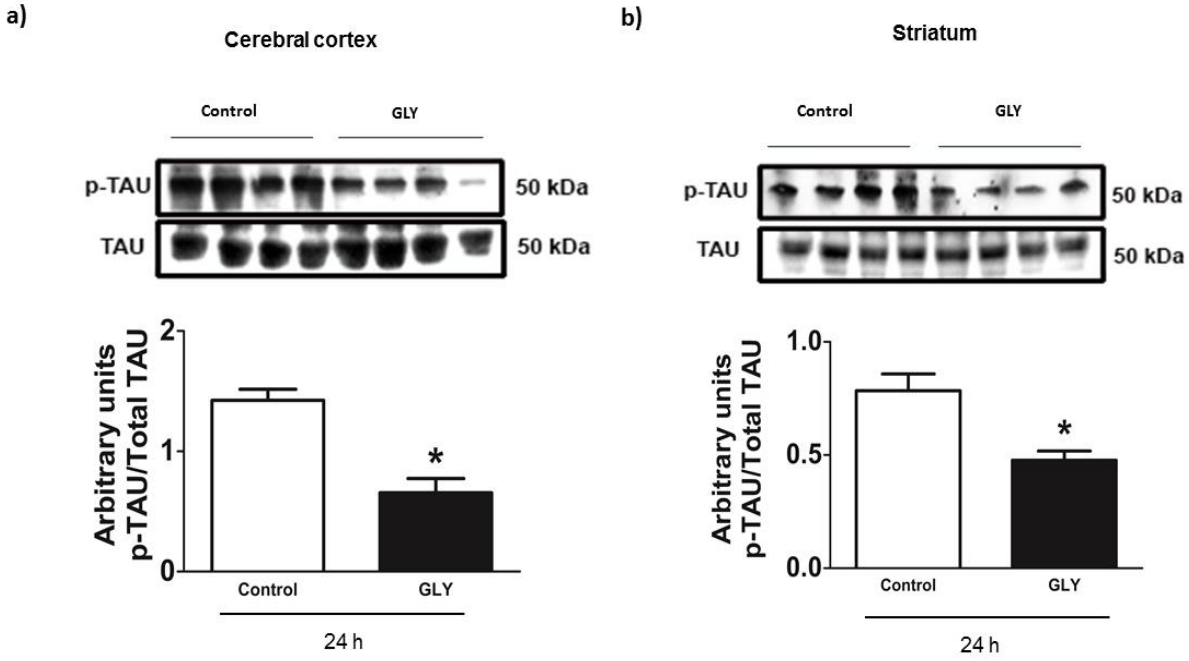

a)
Cerebral cortex
Control
GLY
p-TAU
50 kDa
TAU
50 kDa
Arbitrary units
p-TAU/Total TAU
2
1
0
*
Control
GLY
24 h
b)
Striatum
Control
GLY
p-TAU
50 kDa
TAU
50 kDa
Arbitrary units
p-TAU/Total TAU
1.0
0.5
0.0
*
Control
GLY
24 h

Figure 5.

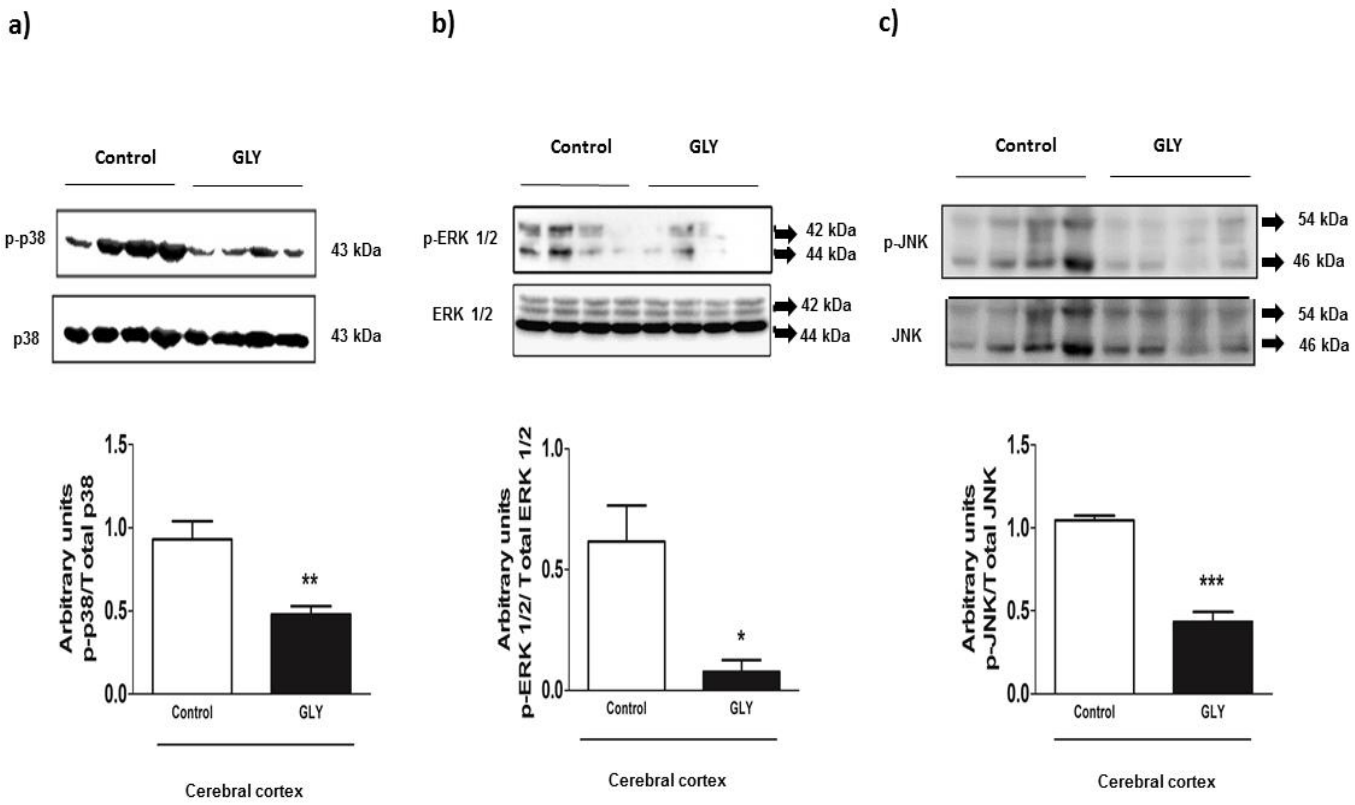

a)
b)
c)
Control
GLY
p-p38
43 kDa
p38
43 kDa
p-ERK 1/2
42 kDa
44 kDa
ERK 1/2
42 kDa
44 kDa
p-JNK
54 kDa
46 kDa
JNK
54 kDa
46 kDa
Arbitrary units p-p38/Total p38
Arbitrary units p-ERK 1/2/ Total ERK 1/2
Arbitrary units p-JNK/Total JNK
**
*
***
Cerebral cortex

Figure 6.

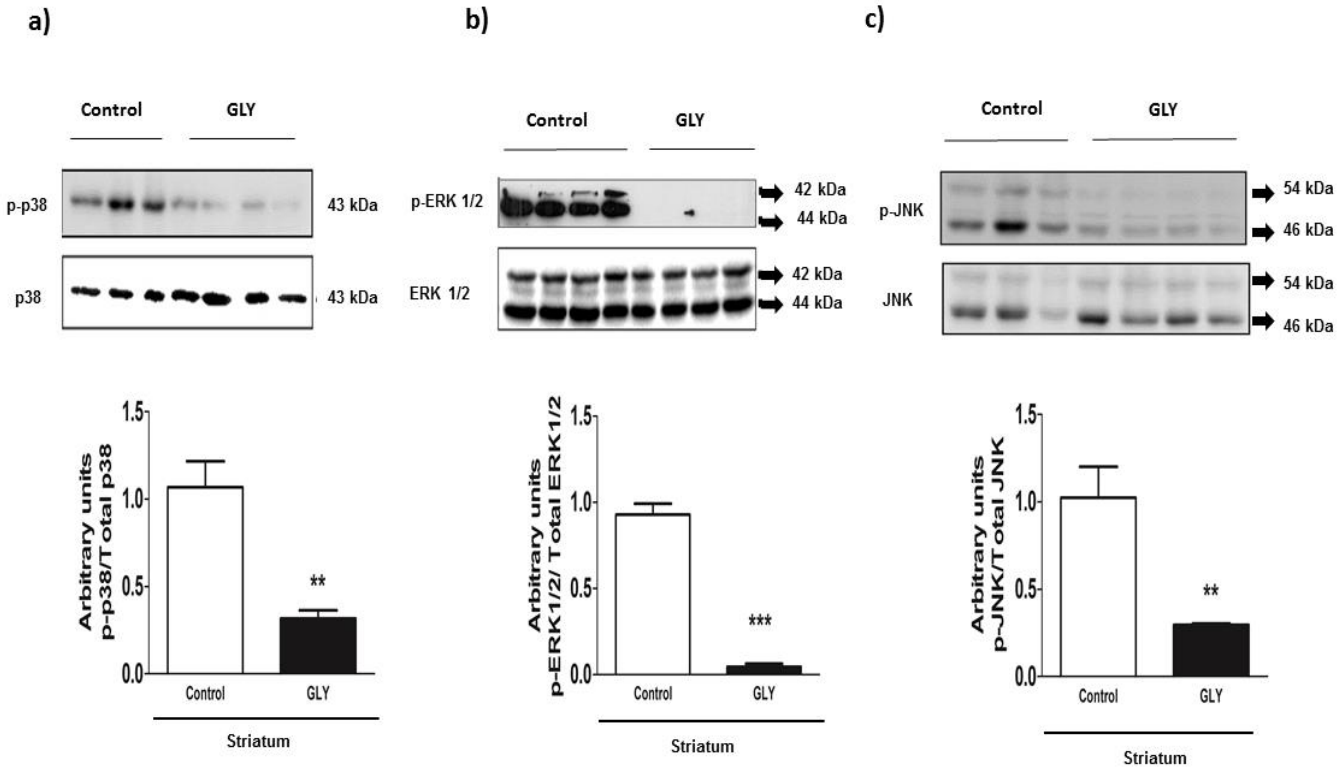

a)
b)
c)
Control
GLY
p-p38
43 kDa
p38
43 kDa
p-ERK 1/2
42 kDa
44 kDa
ERK 1/2
42 kDa
44 kDa
p-JNK
54 kDa
46 kDa
JNK
54 kDa
46 kDa
Arbitrary units p-p38/Total p38
Arbitrary units p-ERK1/2/ Total ERK1/2
Arbitrary units p-JNK/Total JNK
1.5
1.0
0.5
0.0
**
***
Striatum

Figure 7.

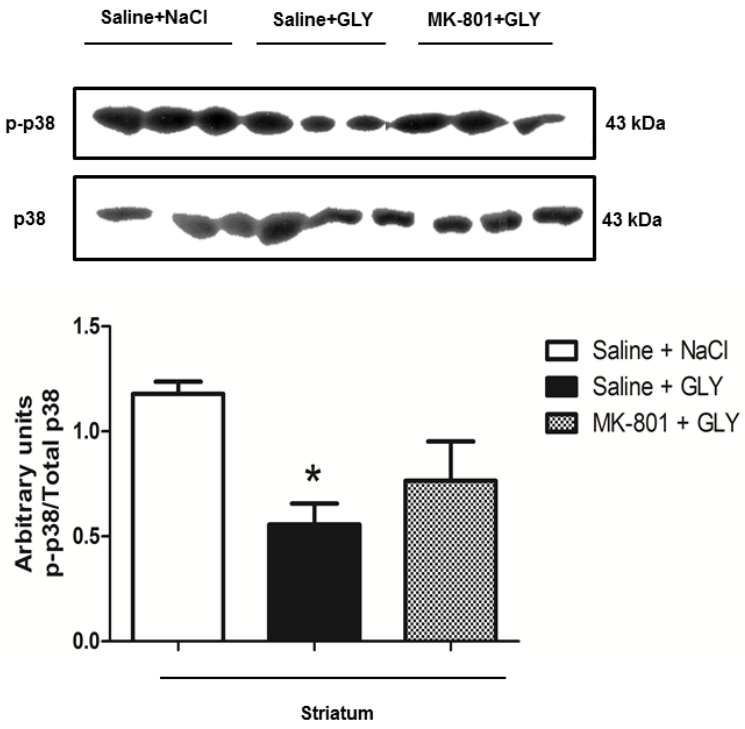

Saline+NaCl
Saline+GLY
MK-801+GLY
p-p38
43 kDa
p38
43 kDa
Arbitrary units
p-p38/Total p38
1.5
1.0
0.5
0.0
*
Saline + NaCl
Saline + GLY
MK-801 + GLY
Striatum

Figure 8.

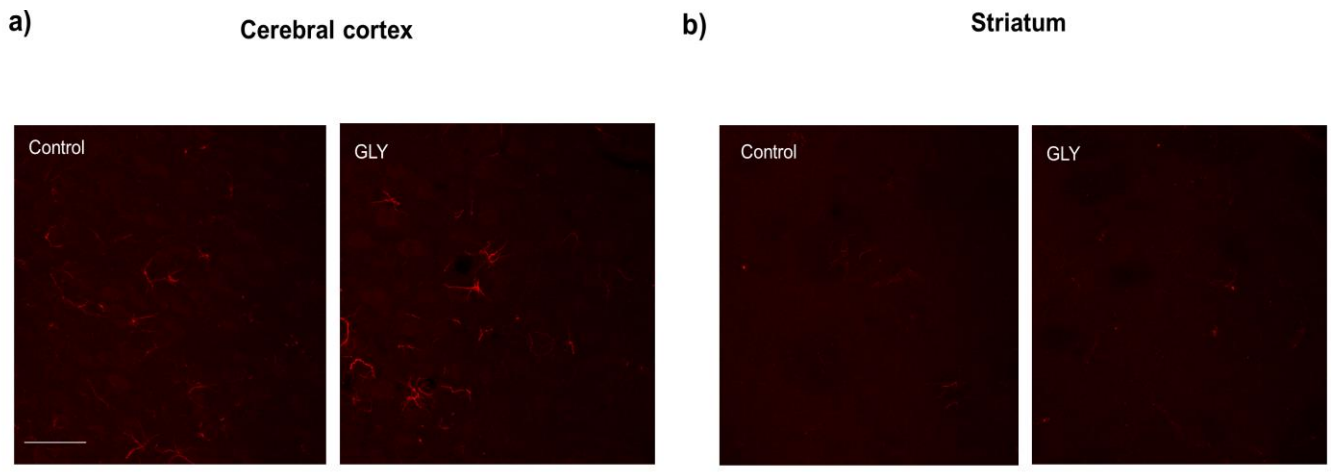
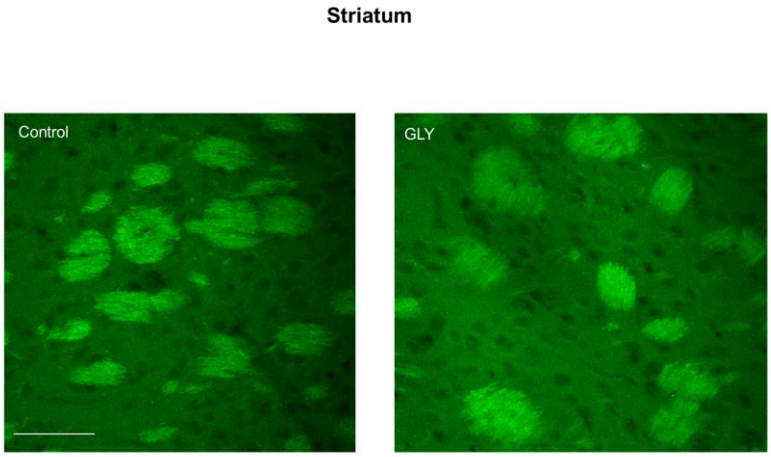


Figure 9.



Capítulo III

Intracerebral glycine administration impairs energy and redox homeostasis and induces glial reactivity in cerebral cortex of newborn rats

Alana Pimentel Moura, Belisa Parmeggiani, Mateus Grings, Leonardo de Moura Alvorcem, Rafael Mello Boldrini, Anna Paula Bumbel, Marcela Moreira Motta, Bianca Seminotti, Moacir Wajner, Guilhian Leipnitz

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Intracerebral Glycine Administration Impairs Energy and Redox Homeostasis and Induces Glial Reactivity in Cerebral Cortex of Newborn Rats

Alana Pimentel Moura[1] · Belisa Parmeggiani[1] · Mateus Grings[1] · Leonardo de Moura Alvorcem[1] · Rafael Mello Boldrini[1] · Anna Paula Bumbel[1] · Marcela Moreira Motta[1] · Bianca Seminotti[1] · Moacir Wajner[1,2] · Guilhian Leipnitz[1]

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Abstract Accumulation of glycine (GLY) is the biochemical hallmark of glycine encephalopathy (GE), an aminoacidopathy characterized by severe neurological dysfunction that may lead to early death. In the present study, we evaluated the effect of a single intracerebroventricular administration of GLY on bioenergetics, redox homeostasis, and histopathology in brain of neonatal rats. Our results demonstrated that GLY decreased the activities of the respiratory chain complex IV and creatine kinase, induced reactive species generation, and diminished glutathione (GSH) levels 1, 5, and 10 days after GLY injection in cerebral cortex of 1-day-old rats. GLY also increased malondialdehyde (MDA) levels 5 days after GLY infusion in this brain region. Furthermore, GLY differentially modulated the activities of superoxide dismutase, catalase, and glutathione peroxidase depending on the period tested after GLY administration. In contrast, bioenergetics and redox parameters were not altered in brain of 5-day-old rats. Regarding the histopathological analysis, GLY increased S100β staining in cerebral cortex and striatum, and GFAP in corpus callosum of 1-day-old rats 5 days after injection. Finally, we verified that melatonin prevented the decrease of complex IV and CK activities and GSH concentrations, and the increase of MDA levels and S100β staining caused by GLY. Based on our findings, it may be presumed that impairment of redox and energy homeostasis and glial reactivity induced by GLY may contribute to the neurological dysfunction observed in GE.

Keywords Glycine · Melatonin · Bioenergetic dysfunction · Oxidative stress · Glial reactivity · Rat brain

Introduction

Glycine encephalopathy (GE), also called non-ketotic hyperglycinemia, is an autosomal recessive disease caused by deficient activity of glycine (GLY) cleavage system (GCS), an intramitochondrial enzyme complex composed by four proteins: P-protein (a pyridoxal phosphate-dependent glycine decarboxylase), H-protein (a lipoic acid containing hydrogen-carrier protein), T-protein (tetrahydrofolate-requiring amino methyltransferase), and L-protein (lipoamide dehydrogenase). The prevalence of GE is estimated at 1:60,000 [1, 2], and it has been reported that up to 80 % of the affected patients have a defect in the P-protein. GCS defect results in the accumulation of GLY in all tissues of patients, particularly in the central nervous system (CNS), reaching concentrations as high as 7.3 mM [1, 3].

Most patients with GE present the classical or neonatal form of this disorder, whose symptoms appear in the first days of life and include lethargy, hypotonia, and seizures, frequently leading to coma and early death. Other less frequent forms of GE with late onset are characterized by behavioral problems, cognitive deficit, and developmental delay. Cerebral MRI findings include progressive brain atrophy, hypoplasia of the corpus callosum, and variable degrees of gliosis [4, 5]. Treatment is not effective, but generally consists at decreasing

✉ Guilhian Leipnitz
guilhian@ufrgs.br

[1] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos No. 2600, 90035-003 Porto Alegre, RS, Brazil

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

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GLY levels and avoiding the effects of GLY at neurotransmitter receptors [6–8].

Although the pathophysiology of brain damage observed in GE is not established, it has been associated to the high tissue levels of GLY. In the CNS, GLY may cause excitotoxicity, since this amino acid is a co-agonist of NMDA glutamate receptor [9–13]. Furthermore, previous in vitro and in vivo studies demonstrated that GLY induces oxidative damage and compromises bioenergetics in brain of young rats [14–16]. It should be noted that GLY has been suggested to exert neurotoxic effects already in utero [7, 17].

Since to the best of our knowledge the in vivo effects of GLY in brain of neonatal rats have not yet been investigated, we evaluated the in vivo influence of GLY intracerebroventricular (ICV) administration (a chemical model of GE) on important parameters of energy metabolism and redox status, namely the activities of the respiratory chain complexes I to IV and of creatine kinase (CK), malondialdehyde (MDA) levels, GSH concentrations, 2′,7′-dichlorofluorescin (DCFH) oxidation, as well as the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in cerebral cortex of neonatal rats. We also investigated whether GLY could cause brain damage by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and histopathological analysis for gliosis and neuronal death in cerebral cortex, striatum, and corpus callosum. In addition, we evaluated the effects of MEL, a neuroprotective agent, on the alterations exerted by GLY in newborn rat brain.

Material and Methods

Animals and Reagents

We used 1- and 5-day-old Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. The animals were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 hours) 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). All reagents used were of analytical grade and purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

The experiments were approved by the local Animal Ethics Commission (*Comissão de Ética no Uso de Animais*-Universidade Federal do Rio Grande do Sul) under the number 23787, and the National Animal Rights Regulations (Law 11.794/2008). The guidelines of National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996), and Directive 2010/63/EU were followed. All efforts were made to minimize the number of animals used and their suffering.

Glycine and Melatonin Administration

Each littermate of 1- or 5-day-old rats was injected into the fourth ventricle, as previously described [18, 19], with a dose of 0.2 μmol/g of GLY (treated group) or phosphate-buffered saline (PBS; control group). After the injection, the rat pups were returned to their respective dams. The correct position of the needle (size 3 mm) was tested by injecting 1 μL of methylene blue (4 % in saline solution) followed by histological analysis. One-day-old rats were euthanized 1, 5, or 10 days after GLY administration, whereas 5-day-old rats were killed 1 day after GLY injection. In other experiments, 1-day-old rats also received one diary intraperitoneal injection of 20 μmol/g MEL (prepared in dimethyl sulfoxide (DMSO)) during five consecutive days [20]. The first injection of MEL was performed 1 h before the administration of PBS or GLY. All rat pups were injected once a day at 10:00 a.m. to ensure that there were no differences in circadian rhythm between experimental groups. The rats were euthanized 1 h after the last injection of MEL. It was verified that MEL per se did not alter the biochemical parameters evaluated in rat cerebral cortex.

Preparation of Samples

The rats were euthanized by decapitation without anesthesia and had their brain rapidly excised on a Petri dish placed on ice. The cerebral cortex was dissected, weighed, and homogenized in a specific buffer used for each technique. For the determination of the activities of the respiratory chain complexes, the cerebral cortex was homogenized (1:20, *w*/*v*) in SET buffer (250 mM sucrose, 2.0 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM Trizma base), pH 7.4. The homogenates were centrifuged at 800×*g* for 10 min, and the supernatants were kept at −70 °C until being used for enzyme activity determination. For CK activity measurement, the cerebral cortex was homogenized (1:10, *w*/*v*) in isosmotic saline solution. For the evaluation of the redox homeostasis parameters, the brain structure was homogenized in 10 volumes (1:10, *w*/*v*) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 750×*g* for 10 min at 4 °C to discard nuclei and cell debris [21] and the supernatants utilized for the determination of the parameters. Tissue slices (25 mg) were also isolated for DCFH oxidation measurement.

Respiratory Chain Complex Activities

Mitochondrial respiratory chain enzyme activities (complexes I–III, II, II–III, and IV) were measured in the supernatants with a protein concentration varying from 1.5 to 4.0 mg protein/mL. The activity of NADH/cytochrome *c* oxidoreductase

(complexes I–III) was assessed as described by Schapira [22]. The activities of succinate/DCIP-oxidoreductase (complex II) and succinate/cytochrome *c* oxidoreductase (complex II–III) were determined according to the method of Fischer [23] and that of cytochrome *c* oxidase (complex IV) according to Rustin [24]. The methods described to measure these activities were slightly modified, as described in details in a previous report [25]. The activities of the respiratory chain complexes were calculated as nanomoles per minute milligram of protein.

Creatine Kinase Activity

The activity of CK was assessed in the supernatants containing 0.4–1.2 μg of protein in a reaction mixture consisting of 60 mM Tris–HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$, and 0.625 mM lauryl maltoside in a final volume of 100 μL. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of 0.3 μmol of ADP. The reaction was stopped after 10 min by the addition of 1 μmol of *p*-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes [26] with slight modifications as described previously [27]. The color was developed by the addition of 100 μL of 2 % α-naphtol and 100 μL of 0.05 % diacetyl in a final volume of 1 mL and read spectrophotometrically at 540 nm after 20 min. Results were calculated as micromoles creatine per minute per milligram of protein.

Determination of Malondialdehyde Levels

MDA levels were measured according to the method described by Yagi [28] with some modifications. One hundred microliters of cortical supernatants containing 0.3 mg of protein were treated with 200 μL of 10 % trichloroacetic acid and 300 μL of 0.67 % thiobarbituric acid in 7.1 % sodium sulfate and incubated for 2 h in a boiling water bath. The mixture was then cooled on running tap water and the resulting pink-stained pigment was extracted with 400 μL of butanol. Fluorescence of the pigment was read at 515 and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was carried out using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. MDA levels were calculated as nanomoles MDA per milligram of protein.

2′,7′-Dichlorofluorescin Oxidation

Reactive species (RS) production was assessed by determining DCFH oxidation [29]. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl, and incubated with tissue slices during 30 min at 37 °C. DCFH-DA is hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of RS. The DCF fluorescence intensity correlates to the amount of RS formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. Calibration curve was performed with standard DCF (0.1–1 μM), and data were calculated as picomoles DCF formed per gram of tissue.

Glutathione Concentrations

GSH concentrations were measured according to Browne and Armstrong [30] with some modifications. One hundred and eighty-five microliters of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and 15 μL of *o*-phthaldialdehyde (1 mg/mL) were added to 30 μL of sample (0.3–0.5 mg of protein) previously deproteinized with metaphosphoric acid. This mixture was incubated at room temperature in a dark room for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.001–1 mM), and the concentrations were calculated as nanomoles GSH per milligram of protein.

Superoxide Dismutase Activity

SOD activity was determined according to Marklund [31]. This assay was based on the capacity of pyrogallol to autooxidize, a process highly dependent on superoxide, which is a substrate for SOD. Thus, the autoxidation of pyragollol is inhibited by SOD, whose activity can be indirectly measured in a spectrophotometer at 420 nm. The reaction medium contained 50 mM Tris buffer/1 mM EDTA, pH 8.2, 80 U/mL CAT, 0.38 mM pyrogallol, and approximately 1 μg of protein. A calibration curve was performed with purified SOD as standard to calculate the activity of SOD in the samples. The results were reported as units per milligram of protein.

Catalase Activity

CAT activity was determined according to Aebi [32] by monitoring the absorbance decrease at 240 nm due to the decomposition of hydrogen peroxide in a reaction medium containing 20 mM hydrogen peroxide, 0.1 % Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and approximately 1 μg of protein. The specific activity was calculated as units per milligram of protein.

Glutathione Peroxidase Activity

GPx activity was measured according to the method of Wendel [33] by monitoring the disappearance of NADPH at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM EDTA, pH 7.7, 2 mM GSH, 0.1 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH, and approximately 3 μg of

protein. The specific activity was calculated as units per milligram of protein.

MTT Reduction

Cell viability was determined in cerebral cortex slices by measuring the reduction of MTT to a dark violet formazan product [34]. First, cortical slices were washed twice with 500 μL Hank's balanced salt solution (HBSS) and transferred to a 48-well plate in which each well contained 300 μL HBSS. The reaction was started with the addition of 0.5 mg mL^{-1} MTT, and after 45 min of incubation at 37 °C, the medium was removed and the slices were dissolved in dimethyl sulfoxide. The rate of MTT reduction was measured spectrophotometrically at a wavelength of 570 nm and a reference wavelength of 630 nm. Results were compared with control samples to which 100 % viability was attributed.

Immunohistochemical Studies

Animals were anesthetized with an intraperitoneal (i.p.) injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) until complete unresponsiveness to nociceptive stimuli and then transcardially perfused with 0.9 % saline and 10 % paraformaldehyde (PAF) prepared in 0.1 M phosphate buffer, pH 7.4, to fix the brain. Fixed brains were removed, post-fixed by immersion in PAF during 24 h, and then sectioned on a vibrating microtome to obtain 30–50-μm-thick consecutive coronal series. Immunohistochemistry was performed in cerebral cortex, striatum, and corpus callosum. For each animal and staining procedure, four to eight equivalent sections were immunostained. Free-floating sections were washed with PBS, submitted to antigen retrieval by boiling in 10 mM sodium citrate, pH 6.0, during 10 min, and washed twice for 10 min. The slices were then permeabilized with PBS plus 0.1–0.3 % Triton X-100 (PBST) for 20 min and treated with blocking buffer (PBS+0.3 % Triton X-100+5 % bovine serum albumin) for 60 min. Afterwards, slices were incubated with the antibodies anti-NeuN (Millipore, 1:300), anti-GFAP (Sigma-Aldrich, 1:300), and anti-S100β (Sigma-Aldrich, 1:400). All dilutions were carried out in PBST. After a 4 °C overnight incubation, sections were rinsed in PBS and incubated at room temperature for 120 min with secondary antibodies (1:500) conjugated to fluorescent probes (Molecular Probes). Sections were then washed, mounted in fluoroshield (Sigma), and imaged in a FV300 Olympus confocal microscope provided with 488- and 546-nm lasers. Primary or secondary antibodies were omitted in negative controls [18].

Protein Determination

Protein content was measured by the method of Lowry [35] using bovine serum albumin as standard.

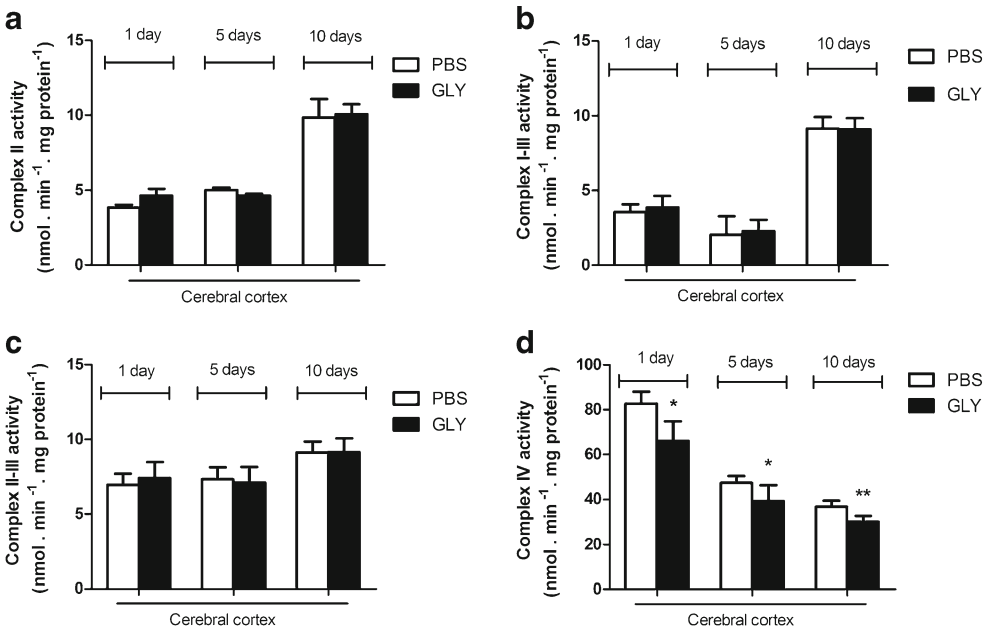

Fig. 1 Effect of intracerebral administration of glycine (*GLY*; 0.2 μmol g^{-1}) on the activities of the respiratory chain complexes I to IV in cerebral cortex of 1-day-old rats. Neonatal rats were euthanized 1, 5, or 10 days after GLY injection. The activity of complex II (**a**) is expressed as nanomoles DCIP reduced per minute per milligram of protein and complexes I–III (**b**) as nanomoles cytochrome *c* reduced per minute per milligram of protein. The activities of complexes II–III **(c)** and IV (**d**) are expressed, respectively, as nanomoles cytochrome *c* reduced per minute per milligram of protein and nanomoles cytochrome *c* oxidized per minute per milligram of protein. Data are represented as mean±SD for four to six independent experiments (animals) performed in triplicate. **P*<0.05; ***P*<0.01, compared with control (Student's *t* test for unpaired samples)

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 or one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the *F* value was significant. Only significant 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.

Results

We first observed that GLY ICV administration at the dose of 0.2 μmol/g into the brain of 1-day-old rats did not cause behavioral alterations. On the other hand, the rats receiving higher doses (0.25 μmol/g and higher) died in less than 2 min after GLY injection.

GLY Administration Decreases Complex IV and CK Activities in Cerebral Cortex of Newborn Rats

We evaluated the effects of GLY injection (0.2 μmol/g) on cellular energy metabolism in cerebral cortex of 1-day-old rats by determining the activities of the respiratory chain complexes and CK. Figure 1 demonstrates that GLY inhibited the activity of complex IV 1 day ($t_{(6)}=2.595$; $P<0.05$), 5 days ($t_{(8)}=2.33$; $P<0.05$), or 10 days ($t_{(7)}=3.755$; $P<0.01$) after its administration (Fig. 1d), whereas the activities of the complexes I–III, II, and II–III were not significantly altered at any period tested (Fig. 1a–c). GLY was also able to inhibit the activity of CK 1 day ($t_{(9)}=3.780$; $P<0.01$), 5 days ($t_{(8)}=2.765$; $P<0.05$), or 10 days ($t_{(8)}=2.537$; $P<0.05$) after its injection (Fig. 2).

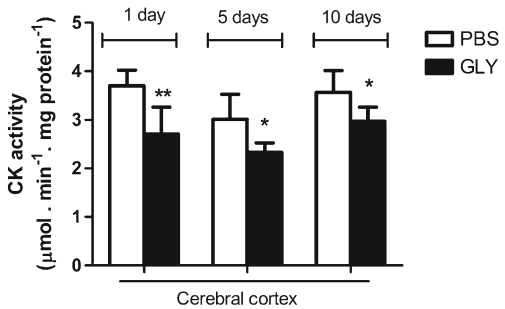

Fig. 2 Effect of intracerebral administration of glycine (*GLY*; 0.2 μmol g^{-1}) on creatine kinase (*CK*) activity in cerebral cortex of 1-day-old rats. Neonatal rats were euthanized 1, 5, or 10 days after GLY injection. Data are represented as mean±SD for four to six independent experiments (animals) performed in triplicate and are expressed as micromoles per milligram of protein. *$P<0.05$; **$P<0.01$, compared with control (Student's *t* test for unpaired samples)

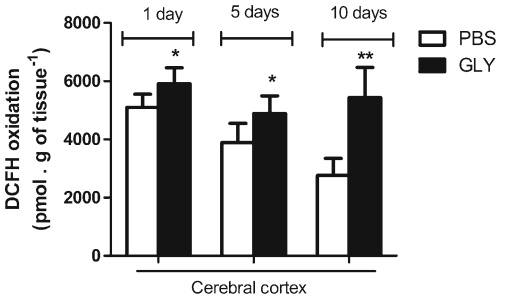

Fig. 3 Effect of intracerebral administration of glycine (*GLY*; 0.2 μmol g^{-1}) on 2′,7′-dichlorofluorescin oxidation (*DCFH*) in cerebral cortex of 1-day-old rats. Neonatal rats were euthanized 1, 5, or 10 days after GLY injection. Data are represented as mean±SD for four to six independent experiments (animals) performed in triplicate and expressed as picomoles per gram of tissue. *$P<0.05$; **$P<0.01$, compared with control (Student's *t* test for unpaired samples)

GLY Administration Increases Reactive Species Generation and Induces Lipid Peroxidation in Cerebral Cortex of Newborn Rats

The next set of experiments was performed in order to examine whether GLY disturbs redox homeostasis in neonatal rat cerebral cortex. Our results show that GLY significantly increased DCFH oxidation 1 day ($t_{(8)}=-2.432$; $P<0.05$), 5 days ($t_{(8)}=-2.483$; $P<0.05$), or 10 days ($t_{(7)}=-4.411$; $P<0.01$) after its administration (Fig. 3). It was also verified that GLY increased MDA levels in brain of 1-day-old rats at five days after injection ($t_{(9)}=-3.547$; $P<0.01$) (Fig. 4).

GLY Administration Decreases GSH Concentrations and Modulates Antioxidant Enzyme Activities in Cerebral Cortex of Newborn Rats

Regarding the antioxidant defenses, GLY decreased GSH concentrations 1 day ($t_{(7)}=2.736$; $P<0.05$), 5 days ($t_{(9)}=2.297$; $P<0.05$), or 10 days ($t_{(9)}=3.591$; $P<0.01$) after its infusion (Fig. 5). GLY also significantly reduced SOD activity at

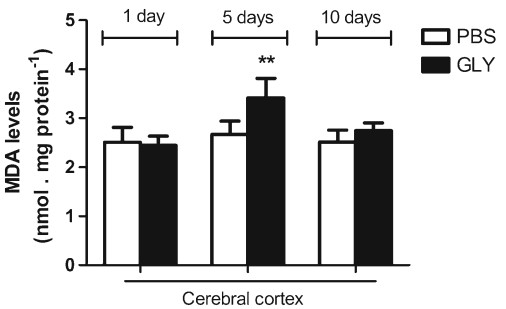

Fig. 4 Effect of intracerebral administration of glycine (*GLY*; 0.2 μmol g^{-1}) on malondialdehyde (*MDA*) levels in cerebral cortex of 1-day-old rats. Neonatal rats were euthanized 1, 5, or 10 days after GLY injection. Data are represented as mean±SD for four to six independent experiments (animals) performed in triplicate and are expressed as nanomoles per milligram of protein. **$P<0.01$, compared with control (Student's *t* test for unpaired samples)

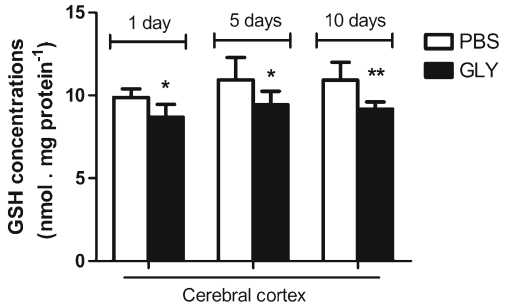

Fig. 5 Effect of intracerebral administration of glycine (*GLY*; 0.2 μmol g^{-1}) on glutathione (*GSH*) concentrations in cerebral cortex of 1-day-old rats. Neonatal rats were euthanized 1, 5 or 10 days after GLY injection. Data are represented as mean±SD for four to six independent experiments (animals) performed in triplicate and are expressed as nanomoles milligram of protein. *P<0.05; **P<0.01, compared with control (Student's t test for unpaired samples)

10 days ($t_{(9)}$=4.693; P<0.01) (Fig. 6a) and CAT activity at 5 days ($t_{(9)}$=2.613; P<0.05) (Fig. 6b) after injection. On the other hand, GPx activity was increased at 1 day ($t_{(8)}$=−3.132; P<0.05) but decreased at 5 days ($t_{(8)}$=7.153; P<0.001), and 10 days ($t_{(7)}$=2.734; P<0.05) (Fig. 6c) after GLY infusion.

GLY Administration Does Not Alter Energy and Redox Homeostasis in Cerebral Cortex of 5-Day-Old Rats

Next, we investigated the effects of GLY on energy and redox homeostasis in older (5-day-old) rats. Table 1 shows that the amino acid did not change the activity of CK, MDA levels, DCFH oxidation, and GSH concentrations 1 or 5 days after GLY injection in cerebral cortex of 5-day-old rats. GLY also did not alter the activities of the respiratory chain complexes, SOD and CAT one day after its injection (Table 1). These data imply that newborn rat brain is more vulnerable to GLY toxic effects.

GLY Administration Increases S100β in Cerebral Cortex and Striatum and GFAP in Corpus Callosum of Newborn Rats

We then evaluated the influence of GLY administration on S100β, GFAP, and NeuN markers in cerebral cortex, striatum, and corpus callosum 5 days after its administration in order to investigate whether GLY could cause brain injury. Figure 7 demonstrates that GLY significantly increased the number of cells stained with S100β in cerebral cortex ($t_{(4)}$=−3.441; P<0.05) and striatum ($t_{(4)}$=−2.937; P<0.05) 5 days after its administration (Fig. 7). We also found that GLY increased GFAP staining in corpus callosum ($t_{(4)}$=0.758; P<0.05) without significant alterations in cerebral cortex and striatum (Fig. 8). In contrast, NeuN marker was not changed in any brain structure evaluated (data not shown). Aiming to evaluate whether GLY could cause cell death at longer periods after its administration, we examined the effects of GLY on NeuN staining and MTT reduction 15 days after the injection into the cerebral cortex of 1-day-old rats. It can be observed that, even at a longer period after the administration, GLY did not significantly alter MTT reduction (Table 2) and NeuN staining (data not shown).

MEL Treatment Prevents GLY-Induced Neurotoxic Effects in Cerebral Cortex of Newborn Rats

We finally assessed the influence of MEL, a potent free radical scavenger, on the neurotoxicity exerted by GLY administration in cerebral cortex of 1-day-old rats at 5 days after its injection. MEL per se did not alter the parameters of energy and redox homeostasis (Fig. 9). MEL treatment totally prevented GLY-induced inhibition of the activities of complex IV ($F_{(3,\ 16)}$=3.760; P<0.05) (Fig. 9a) and CK ($F_{(3,\ 12)}$=10.152; P<0.01) (Fig. 9b). The antioxidant also prevented the decrease of GSH concentrations ($F_{(3,\ 13)}$=3.601; P<0.05) (Fig. 9c) and the increase of MDA levels ($F_{(3,\ 13)}$=3.722; P<0.05) (Fig. 9d) caused by GLY. In addition, we found that MEL attenuated GLY-induced increase of S100β staining in cerebral cortex ($F_{(2,\ 8)}$=32.424; P<0.01) (Fig. 10).

Discussion

Neonatal GE is a debilitating disorder characterized by severe neurological dysfunction [1, 7]. Although the onset of symptoms usually occurs in the first days of life, there is evidence

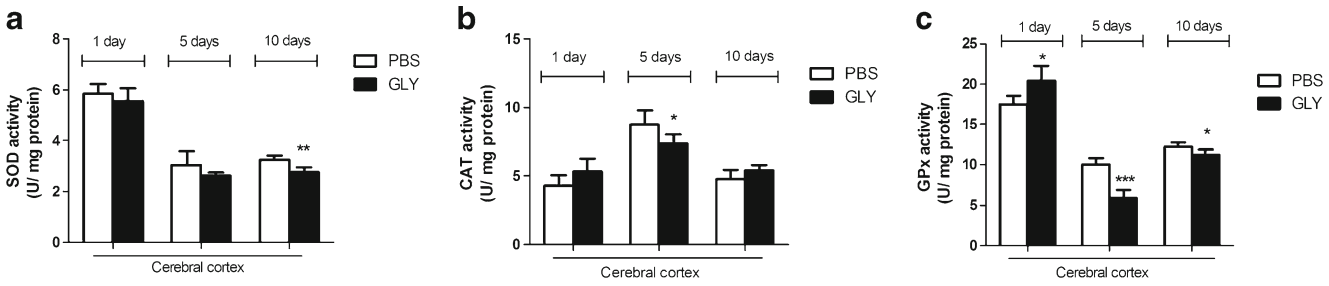

Fig. 6 Effect of intracerebral administration of glycine (*GLY*; 0.2 μmol g^{-1}) on the activities of superoxide dismutase (*SOD*) (**a**), catalase (*CAT*) (**b**), and glutathione peroxidase (*GPx*) (**c**) in cerebral cortex of 1-day-old rats. Neonatal rats were euthanized 1, 5, or 10 days after the GLY injection. Data are represented as mean±SD for four to six independent experiments (animals) performed in triplicate and are expressed as units per milligram of protein. *P<0.05; **P<0.01; ***P<0.001, compared with control (Student's t test for unpaired samples)

Table 1 Effect of intracerebral administration of glycine (GLY; 0.2 μmol g^{-1}) on creatine kinase (CK) and respiratory chain complex IV activities, malondialdehyde (MDA) levels, 2′,7′-dichlorofluorescin (DCFH) oxidation, glutathione (GSH) concentrations and the activities of the enzymes superoxide dismutase (SOD) and catalase (CAT) in cerebral cortex of 5-day-old rats

	1 day after administration (A)		5 days after administration (B)	
	PBS	GLY	PBS	GLY
CK activity	2.67±0.10	2.22±0.46	6.00±0.34	5.33±0.79
Complex IV activity	22.4±4.70	17.4±3.69	–	–
MDA levels	1.47±0.22	1.74±0.19	1.47±0.22	1.74±0.19
GSH concentrations	5.18±0.51	5.26±0.42	5.18±0.51	5.26±0.42
DCFH oxidation	2991±711.9	3216±1009	1799±626.9	1845±644.9
SOD activity	3.69±0.39	3.52±0.22	–	–
CAT activity	3.79±0.41	3.70±0.19	–	–

Five-day-old rats were euthanized 1 day (A) or 5 days (B) after GLY injection. Data are represented as mean±SD for four to six independent experiments (animals) performed in triplicate and expressed as micromoles per milligram of protein (CK activity), nanomoles cytochrome *c* oxidized per min per milligram of protein (complex IV activity), nanomoles milligram of protein (MDA and GSH levels), picomoles per gram of tissue (DCFH oxidation), and units per milligram of protein (SOD and CAT activities). No significant differences between groups were detected (Student's *t* test for unpaired samples)

pointing for brain injury already in utero [5, 7, 36], implying that high levels of GLY may exert damage during the fetal period. Furthermore, treatment for GE is not effective [37] so that the search for new adjuvant therapies is needed to improve patients' quality of life. In order to clarify these issues, we investigated the effects of GLY administration on energy metabolism, redox homeostasis and histopathology in brain of 1- and 5-day-old rats. The analysis was carried out in rats with these ages because we aimed to mimic the neonatal form of GE, which is characterized by GLY accumulation in the brain of patients in utero and early in life [7, 17].

GLY inhibited the activities of the respiratory chain complex IV and CK in cerebral cortex of newborn rats. Since complex IV plays a major role in the electron transport chain flow and consequently in ATP synthesis and CK is essential for ATP buffering and transfer, it may be presumed that production and transfer of energy are compromised in vivo by GLY in newborn rat brain. This is in accordance with previous findings showing that GLY impairs energy metabolism in brain of young rats [37]. We also verified that the free radical scavenger MEL prevented the GLY-induced decrease of complex IV and CK activities, implying that both enzymes are vulnerable to reactive species generated by GLY [38]. In this

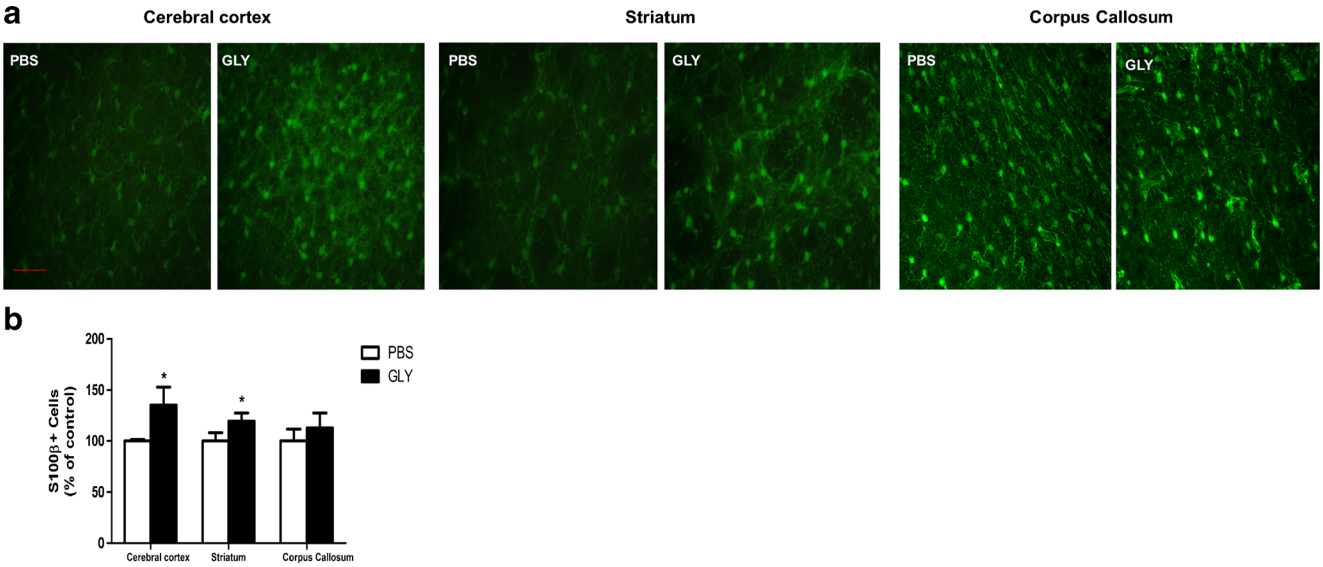

Fig. 7 Effect of intracerebral administration of glycine (*GLY*; 0.2 μmol g^{-1}) on S100β immunofluorescence staining in cerebral cortex, striatum, and corpus callosum of 1-day-old rats. Neonatal rats were euthanized five days after GLY injection. Representative images of S100β immunofluorescence staining in rat cerebral cortex, striatum, and corpus callosum slices. Calibration bar indicates 200 μm (**a**). Quantification of S100β staining in rat cerebral cortex, striatum, and corpus callosum slices (**b**). Data are represented as mean±SD for three independent experiments (animals) and are expressed as percentage of controls. *$P<0.05$, compared with control (Student's *t* test for unpaired samples)

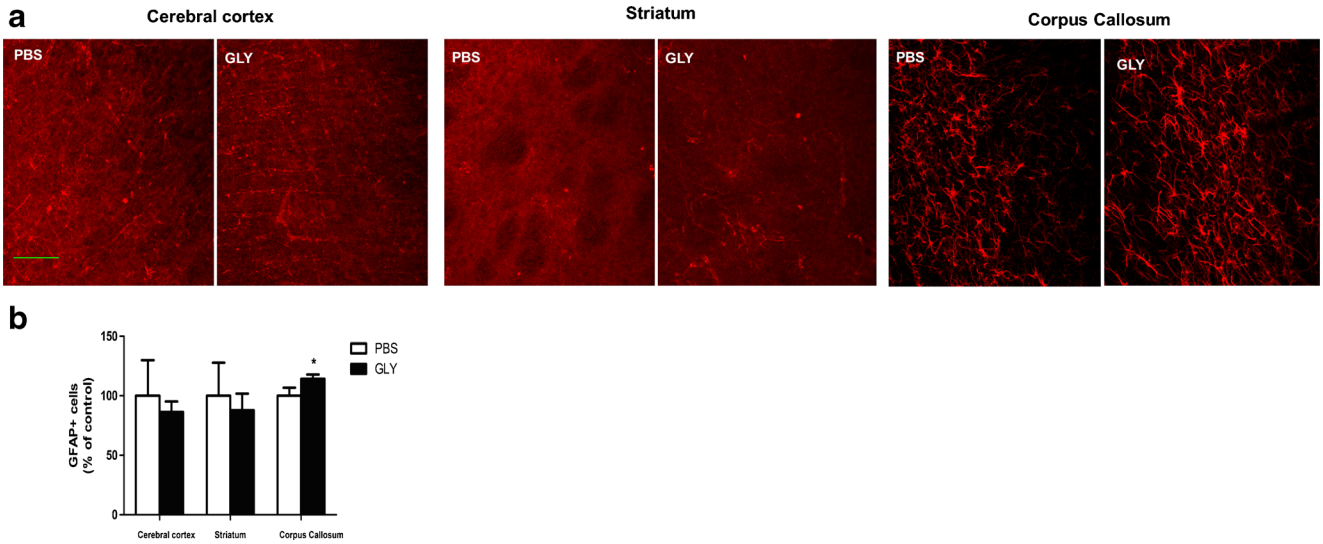

Fig. 8 Effect of intracerebral administration of glycine (*GLY*; 0.2 μmol g^{-1}) on glial fibrillary acidic protein (*GFAP*) immunofluorescence staining in cerebral cortex, striatum, and corpus callosum of 1-day-old rats. Representative images of GFAP immunofluorescence staining in rat cerebral cortex, striatum, and corpus callosum slices. Calibration bar indicates 200 μm (**a**). Quantification of GFAP staining in rat cerebral cortex, striatum, and corpus callosum slices (**b**). Data are represented as mean±SD for three independent experiments (animals) and are expressed as percentage of controls. *$P<0.05$, compared with control (Student's *t* test for unpaired samples)

context, it was suggested that GLY increases the generation of peroxyl and hydroxyl radicals [14, 39] and that MEL is an efficient scavenger of these free radicals [40–43]. However, we cannot rule out that the protective effects of MEL on the respiratory chain may also be a result of direct interactions that stabilize respiratory chain components in the inner mitochondrial membrane or via the induction of gene expression of complex IV subunits [44–46].

We also observed that GLY increased reactive species generation and induced lipid peroxidation in neonatal brain. These results are in line with previous in vitro and in vivo studies showing that GLY disturbs redox homeostasis in CNS of young rats [14, 16, 39]. Although we did not investigate the reasons by which GLY induced lipid peroxidation 5 days, but not 10 days after GLY administration, it is widely known that after an oxidative insult cells are able to activate signaling pathways that lead to increased expression of antioxidant enzymes in order to reestablish redox homeostasis [47–50]. Treatment with MEL prevented the lipid oxidative damage exerted by GLY, reinforcing that MEL is able to scavenge the reactive species generated by GLY, thus protecting against the GLY-induced pro oxidant insults. It should be noted here that hydroxyl and peroxyl radicals, which were suggested to be generated by GLY in vitro, are thermodynamically capable of initiating lipid peroxidation [38].

In addition, brain antioxidant defenses were also altered by GLY administration. We found that GLY decreased the concentrations of the major brain antioxidant GSH, a marker of non-enzymatic antioxidant capacity of tissues [38]. The decrease of GSH concentrations caused by GLY was fully prevented by MEL, probably due to the scavenging properties of MEL [44, 51]. Furthermore, the fact that MEL is able to maintain a normal GSH homeostasis is possibly related to the prevention exerted by MEL on GLY-induced decrease of CK activity, since this enzyme possesses critical sulfhydryl groups highly vulnerable to oxidative attack [38], which may be protected by GSH.

Regarding the enzymatic antioxidant defenses, we verified that GLY reduced SOD and CAT activities, whereas GPx activity was increased 1 day (short-term effect) and decreased 5 or 10 days (long-term effect) after GLY administration. We cannot at present explain the reasons why the activities of antioxidant enzymes are differentially altered or modulated by GLY. Nevertheless, it is feasible that the short-term increase of GPx activity induced by GLY could be related to an induction of the expression of this antioxidant enzyme at the gene level that might have occurred as a compensatory mechanism in response to increased formation of hydrogen peroxide and/or lipid peroxide [38, 52–54]. On the other hand, the decrease of the activities of SOD and CAT, as well as of GPx at longer periods may be due to inhibition caused by

Table 2 Effect of intracerebral administration of glycine (GLY; 0.2 μmol g^{-1}) on cell viability determined by MTT reduction assay in cerebral cortex slices from 1-day-old rats

	PBS	GLY
MTT reduction	100±22.3	96.3±23.0

One-day-old rats were euthanized 15 days after GLY injection. Data are represented as mean±SD for five independent experiments (animals) performed in triplicate and expressed as percentage of control (100 % viability). No significant differences between groups were detected (Student's *t* test for unpaired samples)

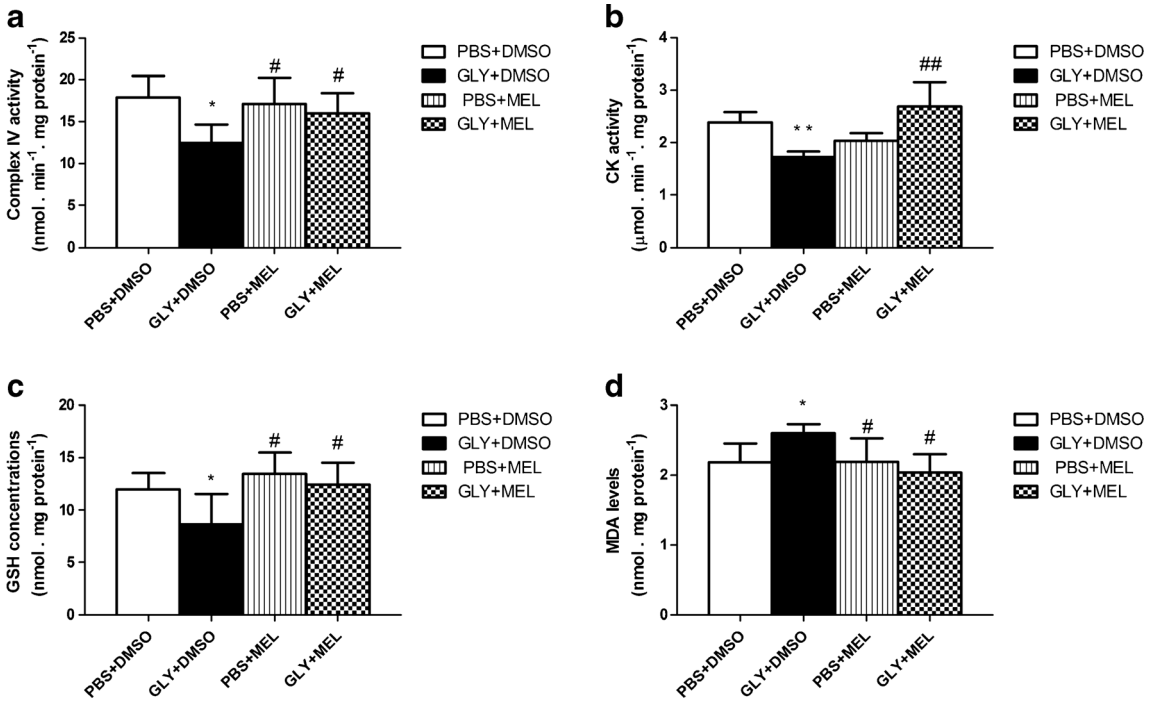

Fig. 9 Effect of melatonin (*MEL*; 20 μmol g^{-1}) on glycine (*GLY*)-induced decrease of the activities of respiratory chain complex IV (**a**) and creatine kinase (*CK*) (**b**) and of glutathione (*GSH*) concentrations (**c**) and increase of MDA levels (**d**) in cerebral cortex of 1-day-old rats. Neonatal rats were euthanized 5 days after GLY injection. Data are represented as mean±SD for four to six independent experiments (animals). **P*<0.05; ***P*<0.01, compared with control; $^{\#}P$<0.05; $^{\#\#}P$<0.01, compared with GLY (Duncan multiple range test)

excessive production of reactive species elicited by GLY causing a site-specific amino acid modification in these enzyme structures [53]. Interestingly, investigators studying the effects of metabolites that accumulate in other inborn errors of

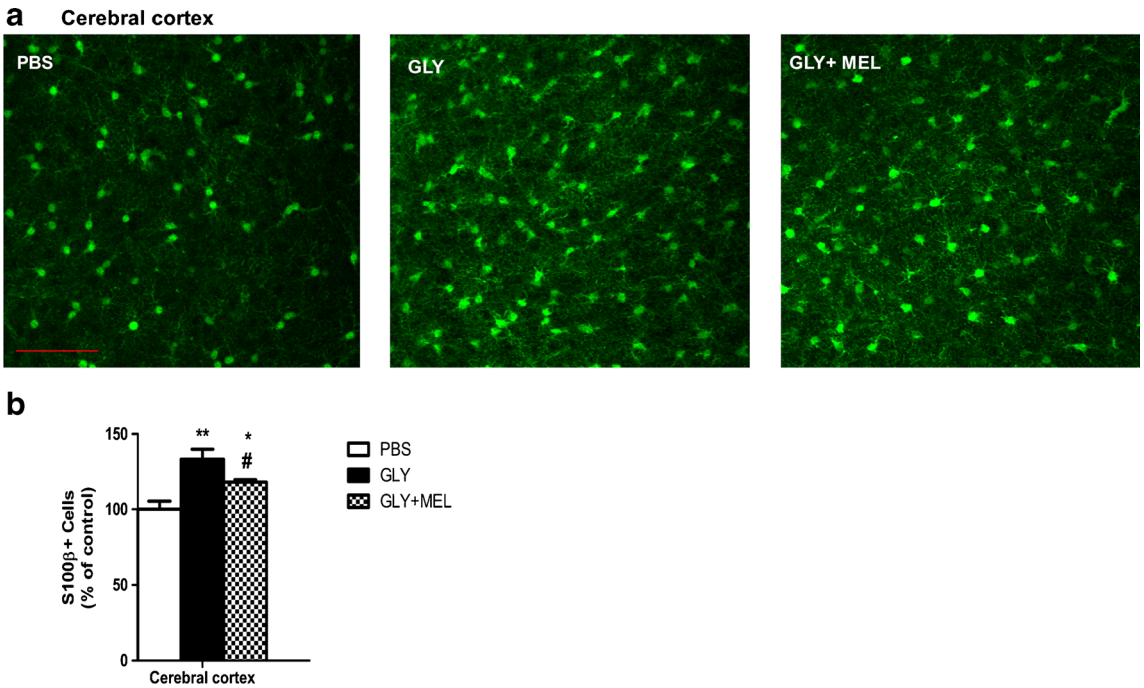

Fig. 10 Effect of melatonin (*MEL*; 20 μmol g^{-1}) on glycine (*GLY*)-induced increase of S100β immunofluorescence staining in cerebral cortex of 1-day-old rats. Neonatal rats were euthanized five days after GLY injection. Representative images of S100β immunofluorescence staining in rat cerebral cortex slices. Calibration bar indicates 200 μm (**a**). Quantification of S100β staining in rat cerebral cortex slices (**b**). Data are represented as mean±SD for three independent experiments (animals) and are expressed as percentage of controls. **P*<0.05; ***P*<0.01, compared with control; $^{\#}P$<0.05, compared with GLY (Duncan multiple range test)

metabolism also observed differential effects on the activities of antioxidant enzymes [55–58].

Otherwise, GLY did not alter the parameters of energy and redox homeostasis in older rats, indicating that the brain of newborn rats is more vulnerable to GLY neurotoxic effects. In this context, it is known that the neonatal brain is particularly sensitive to free radical attack once it has an immature mitochondrial free radical scavenging system with low levels of enzymatic and non-enzymatic antioxidants [59, 60]. Furthermore, this assumption is in accordance with previous data suggesting that elevation of GLY levels causing brain abnormalities already occurs at prenatal and neonatal stages [17, 61, 62].

Neuropathological findings in patients affected by GE consist of progressive cortical brain atrophy with leukodystrophy of the white matter, abnormalities in corpus callosum, and gliosis [1, 63]. Aiming to evaluate whether GLY could cause brain injury, we assessed the effects of GLY administration on GFAP, S100β, and NeuN immunohistochemical staining, as well as on MTT reduction. GLY increased the number of cells stained with S100β in cerebral cortex and striatum, and with GFAP in corpus callosum, suggesting that this amino acid induces glial reactivity, a condition characterized by proliferation and activation of astrocytes and accompanied by high expression of GFAP and S100β proteins that occurs in response to brain insults [64–67]. Thus, it may be presumed that GLY causes brain damage by inducing glial reactivity. This is in line with data of the literature showing that this process is involved in the pathogenesis of other neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, and multiple sclerosis [60–66]. We also found that MEL attenuated GLY-induced increase of S100β staining in rat cerebral cortex, indicating that reactive species are implicated in GLY-induced glial reactivity. In contrast, we did not find alterations in NeuN staining and MTT reduction, implying that GLY does not induce cell death. It may be speculated that compensatory mechanisms involving cell adaptive responses have occurred in response to GLY oxidative insults, albeit they could not counteract the glial reactivity already in progress. On the other hand, we cannot rule out that higher concentrations of GLY may cause cell death. It should be emphasized here that we were not able to investigate the effects of doses higher than 0.2 μmol/g because the rats died shortly after the administration.

In conclusion, this is the first report showing that GLY, the amino acid accumulating in GE, induces bioenergetic dysfunction, oxidative stress, and glial reactivity in neonatal brain. These findings reinforce the hypothesis that high GLY levels induce neurotoxic effects in utero and/or early in life [7, 17, 68, 69]. It was also shown that MEL exerted beneficial effects against GLY neurotoxicity preventing oxidative stress, bioenergetic dysfunction, and glial reactivity caused by this compound, so that the administration of MEL could be considered as an adjuvant therapy to other pharmacological agents for patients affected by GE.

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Capítulo IV

Evidence that glycine induces lipid peroxidation and decreases glutathione concentrations in rat cerebellum

Alana Pimentel Moura, Mateus Grings, Gustavo Flora Marcowich, Anna Paula Bumbel, Belisa Parmeggiani, Leonardo de Moura Alvorcem, Moacir Wajner, Guilhian Leipnitz

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Evidence that glycine induces lipid peroxidation and decreases glutathione concentrations in rat cerebellum

Alana Pimentel Moura · Mateus Grings · Gustavo Flora Marcowich · Anna Paula Bumbel · Belisa Parmeggiani · Leonardo de Moura Alvorcem · Moacir Wajner · Guilhian Leipnitz

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Abstract Patients with non-ketotic hyperglycinemia (NKH) present severe neurological symptoms and brain abnormalities involving cerebellum. Although the pathomechanisms underlying the cerebellum damage have not been studied, high tissue levels of glycine (GLY), the biochemical hallmark of this disorder have been suggested to contribute to the neuropathology of this disease. We investigated the in vitro effects of GLY on important parameters of oxidative stress and energy metabolism in cerebellum of 30-day-old rats. Our results show that GLY increased 2′,7′-dichlorofluorescin oxidation, suggesting that reactive species production are augmented by GLY in the cerebellum. However, hydrogen peroxide generation was not altered by GLY. GLY also increased thiobarbituric acid-reactive substances (TBA-RS) levels and reduced the glutathione (GSH) content, indicating that this amino acid provokes lipid oxidative damage and compromises the non-enzymatic antioxidant defenses, respectively, in cerebellum. The antioxidants melatonin and trolox (the hydrosoluble analog of vitamin E) prevented the GLY-induced increase of TBA-RS and decrease of GSH in cerebellum, indicating the involvement of hydroxyl and peroxyl radicals in these effects. The NMDA receptor antagonist MK-801 also attenuated GLY-induced decrease of GSH, suggesting that this effect is mediated through NMDA receptor. In contrast, GLY did not alter the protein carbonyl formation and total and protein-bound sulfhydryl group content, as well as catalase and superoxide dismutase activities. Furthermore, GLY did not alter the activities of the respiratory chain complexes and creatine kinase. Our present data indicate that oxidative stress elicited by GLY in vitro may be a potential pathomechanism involved in the cerebellar dysfunction observed in NKH.

Keywords Non-ketotic hyperglycinemia · Glycine · Cerebellum · Oxidative stress · Energy metabolism

Introduction

Non-ketotic hyperglycinemia (NKH), also called glycine (GLY) encephalopathy, is an inborn error of metabolism caused by the deficient activity of GLY cleavage system (GCS), a mitochondrial multienzyme complex present in the brain, liver, kidney and placenta [1]. GCS contains the components P protein (a GLY decarboxylase), T protein (a tetrahydrofolate-dependent protein), H protein (a lipoic acid containing hydrogen-carrier protein) and L protein (lipoamide dehydrogenase). Defects in the P, H and T proteins have been identified in NKH, leading to the accumulation of glycine (GLY) in body fluids and tissues of patients, particularly in the central nervous system (CNS) [2]. In this context, brain GLY concentrations may be up to 7.0 mM with an average of 4.0–4.8 mM, but vary within different areas [3], with higher levels in the cerebellar cortex, spinal cord and basal ganglia, compared to the cerebral cortex [4].

A. P. Moura · M. Grings · G. F. Marcowich · A. P. Bumbel · B. Parmeggiani · L. de Moura Alvorcem · M. Wajner · G. Leipnitz (✉)
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul, Rua Ramiro Barcelos N° 2600 – Attached, Porto Alegre, RS CEP 90035-003, Brazil
e-mail: guilhian@ufrgs.br

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

Depending on the age of the onset and severity, five NKH variants can be identified: neonatal, infantile, mild-episodic, late-onset and transient [1, 5]. The most frequent and severe presentation of NKH is the classic or neonatal form, which is usually caused by defects in the P protein of GCS. Patients generally have spasticity, apnea, lethargy, pronounced psychomotor development delay, as well as seizures and coma that often lead to early death. Cerebral findings include progressive brain atrophy, vacuolation in cerebellum, decreased or absent myelination in the white matter and hypoplasia of the corpus callosum [6–8]. Cerebellar hypogenesis and atrophy, and variable degrees of gliosis are also found [9–12]. The other NKH forms are characterized by heterogeneous phenotypes and variable neurological features that often include behavioral problems, cognitive deficits and other developmental delays. NKH with symptom onset between 1 month and 2 years of age is described as the infantile form, whereas the late-onset NKH is characterized by symptom onset after 2 years of age [1].

Although the pathogenesis of the brain lesions found in NKH is still not established, it is reported that the higher the CSF/plasma GLY ratio, the more severe is the disease [1, 13]. In this context, GLY may act both as an excitatory and an inhibitory neurotransmitter, depending on the brain region. Elevated levels of GLY in cerebral cortex induce excitotoxicity through overstimulation of N-methyl-D-aspartate (NMDA) glutamate receptor, leading to seizures [2, 14–17]. On the other hand, GLY-mediated inhibitory neurotransmission in the spinal cord and brain stem may cause apnea and hypotonia [1, 7]. Furthermore, previous in vitro and in vivo studies in cerebral cortex and striatum of rats demonstrated that GLY induces oxidative stress [18, 19] and compromises bioenergetics [20, 21]. Taken together, these findings indicate that neurotransmission impairment, oxidative stress and bioenergetic dysfunction are involved in brain damage found in NKH affected patients.

However, to the best of our knowledge, the mechanisms underlying the cerebellar damage in NKH have not yet been investigated. Therefore, we evaluated the in vitro influence of GLY on redox homeostasis and bioenergetics in the cerebellum of rats. We determined 2′,7′-dichlorofluorescin (DCFH) oxidation and hydrogen peroxide production (reactive species generation), thiobarbituric acid-reactive substances (TBA-RS) (lipid peroxidation), reduced glutathione (GSH) concentrations (non-enzymatic antioxidant defenses), carbonyl formation and total and protein-bound sulfhydryl group content (protein oxidative damage), as well as catalase (CAT) and superoxide dismutase (SOD) activities (enzymatic antioxidant defenses). The activities of mitochondrial complexes I to IV (electron flow through respiratory chain) and creatine kinase (CK) (intracellular energy transfer) were also evaluated.

Experimental procedures

Animal and reagents

We used 62 30-day-old Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, UFRGS. We used male rats for the evaluation of oxidative stress parameters and rats of both sexes for energy metabolism parameters. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. The "Principles of Laboratory Animal Care" (NIH publication n̊. 80–23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, number 23,787. All efforts were made to minimize the number of animals used and their suffering. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA. GLY was dissolved on the day of the experiments in the buffer used for each assay to final concentrations in the incubation medium ranging from 1.0 to 5.0 mM, and the pH adjusted to 7.4.

Preparation of cerebellum samples and incubation

Animals were killed by decapitation without anesthesia and the cerebellum was dissected, weighed and homogenized in 10 volumes (w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 750 *g* for 10 min at 4 °C to discard nuclei and cell debris [22]. The pellet was discarded and the supernatant was separated and pre-incubated for 1 h at 37 °C with GLY. Controls did not contain this metabolite in the incubation medium. Immediately after incubation, aliquots were taken to determine TBA-RS, GSH concentrations, carbonyl formation, total sulfhydryl group content and CAT and SOD activities. For the measurement of protein-bound sulfhydryl groups, cerebellum protein resuspensions were prepared according to Kowaltowski et al. [23] with slight modifications. The supernatants were treated with 200 μL of 6.5 % trichloroacetic acid and centrifuged at 15,000 g during 2 min in order to precipitate the proteins. The pellet was then washed with 6.5 % trichloroacetic acid and resuspended 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 and 0.01 % bovine serum albumin to obtain the protein resuspension. DCFH oxidation was measured in cerebellum slices (25 mg), which were also exposed to GLY for 1 h at 37 °C before the measurement of the parameter. Hydrogen peroxide production was examined in mitochondrial fractions prepared according to Rosenthal

et al. [24], which were exposed to GLY during 30 min. In some experiments, antioxidants or the NMDA receptor antagonist MK-801 (dizocilpine; 0.01 mM) were co-incubated with supernatants at the following final concentrations: 1 mM reduced glutathione (GSH), 1 mM melatonin (MEL), and 0.0075 mM Trolox (TRO, soluble α-tocopherol). We used these classical antioxidants due to their scavenging properties toward different reactive oxygen and nitrogen species, because GSH is an effective thiol group protective agent. The doses of antioxidants used in the present study were selected according to the literature and to previous experiments demonstrating that these doses are capable of preventing oxidative damage and do not alter *per se* the biochemical parameters analyzed [18, 25, 26]. For the determination of the activities of the respiratory chain complexes I–III, II, II–III and IV, the cerebellum was homogenized (1:20,w/v) in SETH buffer (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 UI.mL^{-1} heparin), pH 7.4. The homogenates were centrifuged at 800 *g* for 10 min and the supernatants were kept at −70 °C until being used for enzyme activity determination. For the measurement of CK activity, the cerebellum was homogenized (1:10 w/v) in isosmotic saline solution. The homogenates were pre-incubated for 30 min at 37 °C with GLY. Controls in all experiments did not contain the amino acid in the incubation medium. We always performed the experiments using blanks in order to detect artifacts and validate our methodology.

2′,7′-Dichlorofluorescin (DCFH) oxidation

The production of reactive species was determined according to the method of Lebel et al. [27] with slight modifications. Cerebellum slices were exposed to GLY for 1 h at 37 °C. Afterward, the tissue slices were incubated with 0.005 mM 2′,7′-dichlorofluorescein diacetate (DCF-DA) prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl, during 30 min at 37 °C. DCF-DA crosses the cell membrane and is deacetylated by esterases to DCFH in the intracellular medium. This product is oxidized by reactive species into the highly fluorescent compound dichlorofluorescein (DCF). Fluorescence was measured using wavelengths of 480 nm (excitation) and 535 nm (emission). The calibration curve was performed with standard DCF (0.001–0.01 mM).The production of reactive species was calculated as pmol DCF.g of tissue^{-1} and expressed as percentage of control.

Mitochondrial hydrogen peroxide (H_2O_2) release

Mitochondrial preparations (0.2 mg protein.mL^{-1}) supported by 5 mM glutamate and 5 mM malate as substrates were incubated in standard reaction medium in the presence of 0.01 mM Amplex red, 1 U.mL^{-1} horseradish peroxidase and 5 mM GLY. The fluorescence was monitored during 1800 s on a Hitachi F-4500 spectrofluorometer operated at excitation and emission wavelengths of 563 and 587 nm, respectively. Antimycin A (0.1 μg.mL^{-1}) was added at the end of the measurements [28]. Data were expressed as fluorescence arbitrary units (FAU).

Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS were determined according to Esterbauer and Cheeseman [29]. Three hundred microliters of 10 % trichloroacetic acid were added to 150 μL of cerebellum supernatants and centrifuged at 3000 *g* for 10 min. Then, 300 μL of the supernatants (containing approximately 0.3 mg of protein) was transferred to a tube and incubated with 300 μL of 0.7 % thiobarbituric acid in 7.1 % sodium sulfate in a boiling water bath for 25 min. The resulting pink-stained product was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane (2.5–25 nmol.L^{-1}). TBA-RS values were calculated as nmol.mg protein^{-1} and expressed as percentage of control.

Reduced glutathione (GSH) concentrations

GSH concentrations were measured according to Browne and Armstrong [30]. One volume of metaphosphoric acid was added to 100 μL of the pre-treated samples, which were centrifuged for 10 min at 7000 *g*. Then, 185 μL of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and 15 μL of o-phthaldialdehyde (1 mg.mL^{-1}) were added to 30 μL of supernatants (0.3–0.5 mg of protein). This mixture was incubated at room temperature in a dark room for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.001–0.5 mM). The concentrations were calculated as nmol.mg protein^{-1} and expressed as percentage of control.

Protein carbonyl content

Protein carbonyl formation was examined spectrophotometrically according to Reznick and Packer [31]. Two hundred microliters of the pre-incubated cerebellum supernatants (containing approximately 0.3 mg of protein) were treated with 400 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) prepared in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for 1 h. Six hundred microliters of 20 % trichloroacetic acid were added to the samples to precipitate the proteins and the tubes centrifuged for 5 min at 9000 *g*. The pellet was then washed with 1 mL ethanol:ethyl acetate (1:1, v/v) and dissolved in 550 μL of 6 M guanidine prepared in 2.5 N HCl at 37 °C for 5 min. The

difference between the DNPH-treated and HCl-treated samples was used to calculate the carbonyl formation determined at 365 nm. The results were calculated as nmol of carbonyl groups.mg of protein^{-1} and expressed as percentage of control, using the extinction coefficient of 22,000 $\times$ 10^6 nmol.mL^{-1} for aliphatic hydrazones.

Total and protein-bound sulfhydryl group content

This assay was performed according to Aksenov and Markesbery [32] in total cerebellum supernatants (total sulfhydryl group content) and in cerebellum protein resuspension. It is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by thiol groups, generating a yellow-stained compound TNB whose absorption is measured spectrophotometrically at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were calculated as nmol TNB.mg protein^{-1} and expressed as percentage of control.

Superoxide dismutase (SOD) activity

SOD activity was assayed according to Marklund [33]. The method evaluates the autoxidation of pyrogallol in the presence of superoxide anion ($O_2^{\bullet -}$), which is a substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. The reaction medium contained 50 mM Tris buffer/1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U.mL^{-1} CAT, 0.38 mM pyrogallol and approximately 1 μg of protein. A calibration curve was performed with purified SOD as standard to calculate the activity of SOD present in the samples. One unit (U) of the enzyme is defined as the amount required for inhibiting pyrogallol autoxidation by 50 % per min. The results are reported as U.mg protein^{-1} and expressed as percentage of control.

Catalase (CAT) activity

CAT activity was determined according to Aebi [34] by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1 % Triton X-100, 10 mM potassium phosphate buffer, pH 7.0 and approximately 1 μg of protein. One unit (U) of the enzyme is defined as 1 μmol of H_2O_2 consumed per minute. The specific activity was calculated as U.mg protein^{-1} and expressed as percentage of control.

Respiratory chain complexes activities

Mitochondrial respiratory chain enzyme activities (complexes I–III, II, II–III and IV) were measured in homogenates with a protein concentration varying from 1.5 to 4.0 mg protein.mL^{-1}. The activity of NADH:cytochrome *c* oxidoreductase (complex I–III) was assessed as described by Schapira et al. [35]. The activities of succinate: DCIP-oxidoreductase (complex II) and succinate:cytochrome *c* oxidoreductase (complex II–III) were determined according to the method of Fischer et al. [36] and that of cytochrome *c* oxidase (complex IV) according to Rustin et al. [37]. The methods used to measure these activities were slightly modified, as previously described [38]. The activities of the respiratory chain complexes were calculated as nmol.min^{-1}.mg protein^{-1} and expressed as percentage of control.

Creatine kinase (CK) activity

CK activity was measured in a reaction mixture consisting of 60 mM Tris–HCl, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$, 0.625 mM lauryl maltoside and supernatants containing 0.4–1.2 μg of protein in a final volume of 100 μL. The reaction was started by the addition of 0.3 μmol of ADP and stopped after 10 min by the addition of 1 μmol of p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes [39] with slight modifications [40]. The color was obtained by the addition of 100 μL of 2 % α-naphthol and 100 μL of 0.05 % diacetyl in a final volume of 1 mL and read spectrophotometrically at 540 nm after 20 min. Results were calculated as μmol creatine.min^{-1}.mg protein^{-1} and expressed as percentage of control.

Protein determination

Protein concentration was measured by the method of Lowry et al. [41] using bovine serum albumin as standard.

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 one-way analysis of variance (ANOVA) followed by the post hoc Duncan multiple range test, when F was significant. Only significant F values are shown in the text. Differences between groups were considered 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.

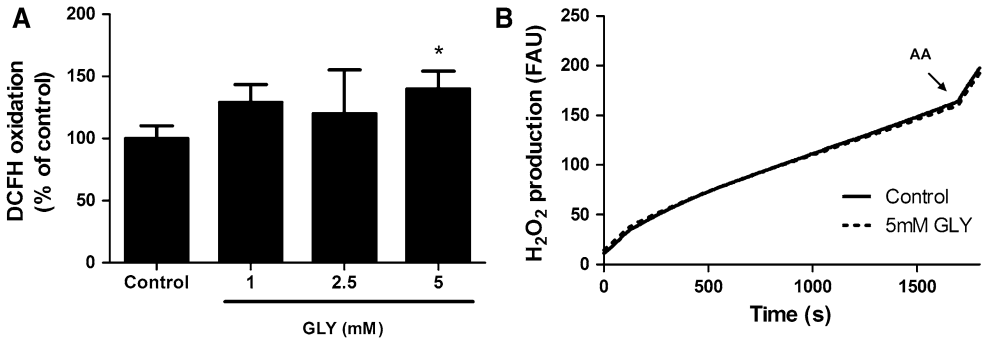

Fig. 1 Effect of glycine (GLY) on 2′,7′-dichlorofluorescin (DCFH) oxidation (**a**) and hydrogen peroxide production (**b**) in rat cerebellum. Cerebellum slices were incubated in the presence of GLY (1.0–5.0 mM) and DCFH oxidation was measured afterward. Values are mean ± standard deviation for four to five independent experiments (animals) and are expressed as percentage of controls (DCFH oxidation [pmol.g of tissue^{-1}]: Control: 275 ± 27.7; 1 mM GLY: 353 ± 42.94; 2.5 mM GLY: 323 ± 69.07; 5 mM GLY: 382 ± 31.68). *$P < 0.05$, compared to controls (Duncan multiple range test) (**a**). For the experiments measuring hydrogen peroxide production, cerebellum mitochondrial preparations (0.5 mg protein.mL^{-1}), 2.5 mM glutamate plus 2.5 mM malate as substrates and 5 mM GLY were added to the incubation medium in the beginning of the assay. Antimycin A (AA) (0.1 μg.mL^{-1}) was added at the end of assays, as indicated in the figure. Traces are representative of four independent experiments and were expressed as fluorescence arbitrary units (FAU) (**b**). For a and b, the control group did not contain GLY in the incubation medium

Results

GLY increases reactive species production in rat cerebellum

In the first set of experiments, we investigated the in vitro effect of GLY on reactive species production in rat cerebellum. Figure 1a shows that GLY increased DCFH oxidation in cerebellum slices [$F_{(3,16)} = 3.239$, $P < 0.05$] (1 mM GLY: 129 %; 2.5 mM GLY: 119 %; 5 mM GLY: 139 %), indicating that elevated levels of this amino acid increase the formation of reactive species. In contrast, H_2O_2 production was not altered by GLY in mitochondrial preparations of cerebellum during thirty min of incubation in the presence of glutamate and malate as substrates (Fig. 1b).

GLY induces lipid peroxidation in rat cerebellum

We evaluated the in vitro effect of GLY on TBA-RS levels and found that the amino acid significantly increased this parameter [$F_{(3,20)} = 3.779$, $P < 0.05$] (1 mM GLY: 100 %; 2.5 mM GLY: 114 %; 5 mM GLY: 125 %) in a dose-dependent manner [$\beta = 0.67$, $P < 0.005$] (Fig. 2a), indicating that GLY induces lipid peroxidation in rat cerebellum. We then investigated the role of free radical scavengers on GLY-induced lipid oxidative damage. Our results show that MEL and TRO [$F_{(4,15)} = 5.129$; $P < 0.01$], but not GSH, were able to fully prevent the GLY-induced increase of TBA-RS levels (5 mM GLY: 138 %; 5 mM GLY + 1 mM MEL: 112 %; 5.0 mM GLY + 0.0075 mM TRO: 113 %; 5.0 mM GLY + 1 mM GSH: 136 %) (Fig. 2b).

GLY reduces GSH concentrations in rat cerebellum

The effect of GLY on GSH concentrations in cerebellar supernatants from rats was also measured. We observed that GLY significantly decreased GSH content [$F_{(3,16)} = 6.578$; $P < 0.01$] (1 mM GLY: 84 %; 2.5 mM GLY: 82 %; 5 mM GLY: 79 %) (Fig. 3a) and that the free radical scavenger MEL, but not TRO, attenuated this effect [$F_{(3,16)} = 5.417$; $P < 0.01$] (5 mM GLY: 66 %; 5 mM GLY + 1 mM MEL: 79 %; 5 mM GLY + 0.0075 mM TRO: 63 %) (Fig. 3b). We further verified that MK-801, a non-competitive NMDA receptor antagonist, attenuated this effect [$F_{(2,11)} = 4.07$; $P < 0.05$] (5 mM GLY: 70 %; 5 mM GLY + 0.01 mM MK-801: 93 %) (Fig. 3c).

GLY does not induce protein oxidative damage or alter enzymatic antioxidant defenses in rat cerebellum

Next, we observed that GLY did not significantly alter the carbonyl formation, total sulfhydryl group content and protein-bound sulfhydryl groups, indicating that this compound does not provoke protein damage (Table 1). Moreover, GLY does not modify the activities of the antioxidant enzymes CAT and SOD in vitro (Table 1).

GLY does not change the activities of the respiratory chain complexes and CK in rat cerebellum

Finally, we determined the effect of GLY on the activities of complexes I–III, II, II–III and IV of the respiratory chain and CK and verified that these parameters were not altered by the amino acid (Table 2).

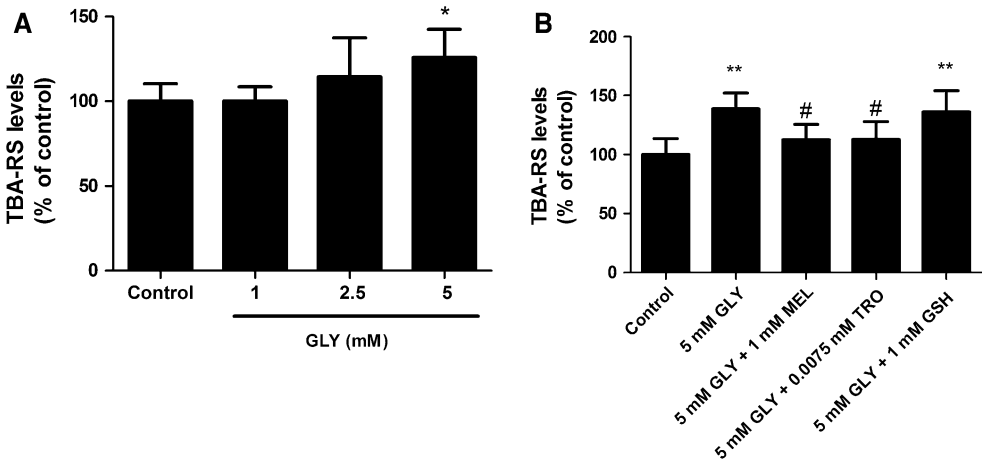

Fig. 2 Effect of glycine (GLY) on thiobarbituric acid-reactive substances (TBA-RS) in rat cerebellum (**a**). In some experiments, cerebellum supernatants were incubated in the presence of GLY (5 mM) and melatonin (MEL, 1 mM), trolox (TRO, 0.0075 mM) or glutathione (GSH, 1 mM) (**b**). Values are mean ± standard deviation for four to six independent experiments (animals) and are expressed as percentage of controls (TBA-RS [nmol.mg protein^{-1}]: (**a**) Control: 0.12 ± 0.01; 1 mM GLY: 0.12 ± 0.01; 2.5 mM GLY: 0.14 ± 0.03; 5 mM GLY: 0.15 ± 0.01; (**b**) Control: 1.52 ± 0.20; 5 mM GLY: 2.09 ± 0.23; 5 mM GLY + MEL: 1.56 ± 0.21; 5 mM GLY + -TRO: 1.55 ± 0.16; 5 mM GLY + GSH: 2.05 ± 0.23). Controls did not contain the tested compounds in the incubation medium. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to controls, $^{\#}P < 0.05$, compared to 5 mM GLY (Duncan multiple range test)

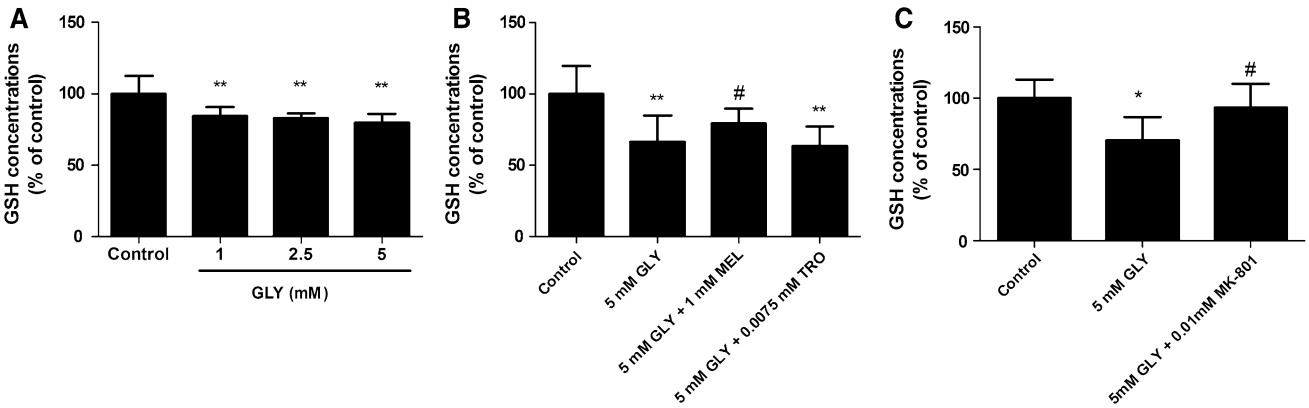

Fig. 3 Effect of glycine (GLY) on glutathione (GSH) concentrations in rat cerebellum (**a**). In some experiments, cerebellum supernatants were incubated in the presence of GLY (5 mM) and the antioxidants melatonin (MEL, 1 mM) or trolox (TRO, 0.0075 mM) (**b**). Cerebellum slices were incubated in the presence of the non-competitive NMDA receptor antagonist MK-801 (0.01 mM) (**c**). Values are mean ± standard deviation for five independent experiments (animals) and are expressed as percentage of controls (GSH concentrations [nmol.mg protein^{-1}]: **a** Control: 12.2 ± 1.54; 1 mM GLY: 10.2 ± 0.61; 2.5 mM GLY: 10.0 ± 1.28; 5 mM GLY: 9.77 ± 1.68; **b** Control: 18.1 ± 3.51; 5 mM GLY: 11.5 ± 1.71; 5 mM GLY + 1 mM MEL: 13.4 ± 1.70; 5 mM GLY + 0.0075 mM TRO: 11.3 ± 2.05; **c** Control: 3.97 ± 0.50; 5 mM GLY: 2.73 ± 0.46; 5 mM GLY + 0.01 mM MK-801: 3.68 ± 0.65). Controls did not contain the tested compounds in the incubation medium. $^{**}P < 0.01$ compared to controls, $^{\#}P < 0.05$, compared to 5 mM GLY (Duncan multiple range test)

Discussion

NKH affected patients present high GLY levels in CNS and atrophy and degeneration of cerebellum [2, 10, 42], whose pathophysiology has not been studied yet. In the present work, we evaluated whether GLY (up to 5.0 mM) could alter parameters of oxidative stress and energy metabolism in vitro in rat cerebellum.

We first verified that GLY increased the oxidation of DCFH, suggesting that GLY augments the generation of reactive species in rat cerebellum. However, hydrogen peroxide is not involved in GLY-induced increase of DCFH oxidation, since the production of this reactive species was not modified by GLY in cerebellum mitochondria. GLY also induced lipid peroxidation, as reflected by the increase of TBA-RS levels, corroborating with

Table 1 Effect of glycine (GLY) on carbonyl formation, total and protein-bound sulfhydryl group content and the activities of the enzymes catalase (CAT) and superoxide dismutase (SOD) in rat cerebellum

Protein oxidative damage				
	Control	1 mM	2.5 mM	5 mM
Carbonyl formation	100 ± 19.5	94.0 ± 27.4	104 ± 23.6	96.0 ± 18.1
Total sulfhydryl content	100 ± 4.14	93.0 ± 7.41	102 ± 2.73	95.0 ± 5.50
Protein-bound sulfhydryl group content	100 ± 6.11	98.0 ± 7.0	110 ± 12.08	106.0 ± 15.0
Antioxidant enzyme activities				
	Control	1 mM	2.5 mM	5 mM
Catalase (CAT)	100 ± 9.12	89.0 ± 14.2	93.0 ± 13.2	94.0 ± 13.7
Superoxide dismutase (SOD)	100 ± 8.05	100 ± 2.53	101 ± 2.59	100 ± 4.29

Values are mean ± standard deviation for four to six independent experiments (animals) and are expressed as percentage of controls (Carbonyl formation [nmol.mg protein^{-1}]: Control: 2.03 ± 0.40; 1 mM GLY: 1.88 ± 0.50; 2.5 mM GLY: 2.09 ± 0.51; 5 mM GLY: 1.97 ± 0.61; Total sulfhydryl group content [nmol.mg protein^{-1}]: Control: 32.2 ± 2.52; 1 mM GLY: 32.2 ± 2.51; 2.5 mM GLY: 35.2 ± 1.67; 5 mM GLY: 33.0 ± 2.29; Protein-bound sulfhydryl groups [nmol.mg protein^{-1}]: Control: 33.3 ± 2.03; 1 mM GLY: 32.9 ± 3.83; 2.5 mM GLY: 36.5 ± 2.62; 5 mM GLY: 35.7 ± 6.87; CAT activity [U.mg protein^{-1}]: Control: 4.10 ± 0.37; 1 mM GLY: 4.10 ± 0.37; 2.5 mM GLY: 3.87 ± 0.51; 5 mM GLY: 3.87 ± 0.13; SOD activity [U.mg protein^{-1}]: Control: 6.40 ± 0.52; 1 mM GLY: 6.39 ± 0.49; 2.5 mM GLY: 6.40 ± 0.46; 5 mM GLY: 6.39 ± 0.45). Controls did not contain the tested compounds in the incubation medium. No significant differences between groups were detected (One-way ANOVA)

Table 2 Effect of glycine (GLY) on the activities of respiratory chain complexes and creatine kinase (CK) in rat cerebellum

	Control	1.0 mM	2.5 mM	5.0 mM
CI-III	100 ± 27.5	103 ± 55.6	115 ± 55.6	109 ± 40.5
CII	100 ± 7.70	102 ± 30.7	101 ± 27.0	101 ± 12.5
CII-III	100 ± 14.2	104 ± 20.8	99.0 ± 13.8	100 ± 14.2
CIV	100 ± 9.50	97.0 ± 18.5	89.0 ± 14.3	88.0 ± 16.5
CK	100 ± 15.0	103 ± 6.80	93.0 ± 14.8	87.0 ± 4.80

Values are mean ± standard deviation for four to six independent (animals) experiments per group and are expressed as percentage of controls (activities of complexes I–IV: CI-III [nmol cytochrome c reduced.min^{-1}.mg protein^{-1}]: Control: 4.77 ± 1.31; 1.0 mM GLY: 4.45 ± 1.05; 2.5 mM GLY: 5.25 ± 1.75; 5.0 mM GLY: 4.95 ± 1.56; CII [nmol DCIP reduced.min^{-1}.mg protein^{-1}]: Control: 5.82 ± 0.39; 1.0 mM GLY: 5.92 ± 1.58; 2.5 mM GLY: 5.87 ± 1.42; 5.0 mM GLY: 5.96 ± 1.10; CII-III [nmol cytochrome c reduced.min^{-1}.mg protein^{-1}]: Control: 8.47 ± 1.19; 1.0 mM GLY: 8.73 ± 1.29; 2.5 mM GLY: 8.36 ± 1.19; 5.0 mM GLY: 8.40 ± 1.16; CIV [nmol cytochrome c oxidized.min^{-1}.mg protein^{-1}]: Control: 81.8 ± 7.80; 1.0 mM GLY: 79.6 ± 19.1; 2.5 mM GLY: 73.6 ± 17.6; 5.0 mM GLY: 68.8 ± 10.5; CK [nmol creatine.min^{-1}.mg protein^{-1}]: Control: 1.57 ± 0.24; 1.0 mM GLY: 1.62 ± 0.26; 2.5 mM GLY: 1.48 ± 0.36; 5.0 mM GLY: 1.36 ± 0.23). No significant differences between groups were detected (One-way ANOVA)

in vitro and in vivo recent studies showing that GLY increases TBA-RS levels in cerebral cortex and striatum of rats [18, 19]. This effect indicates that GLY induces the generation of malondialdehyde, an end product of membrane fatty acid peroxidation [43]. We also observed that this effect was totally prevented by MEL and TRO, suggesting that hydroxyl and peroxyl radicals, which are scavenged by these antioxidants, are the main free radicals involved in GLY effects in cerebellum. These findings reinforce the data of Leipnitz et al. [18] that showed that the same free radical scavengers (MEL and TRO) prevent the lipoperoxidation caused by GLY in rat cerebral cortex. It is noteworthy that several studies report that MEL and its derivatives, besides acting as efficient scavengers of hydroxyl and peroxyl radicals and peroxynitrite, are capable of preventing lipid peroxidation by maintaining cell membranes in a state of optimal fluidity [44–47]. MEL also reduces electron leakage and reactive species generation from electron transport chain by stimulating the activities of complexes I and IV [44]. Regarding to TRO, the hydrosoluble analog of vitamin E, it is well known that this compound is a potent peroxyl radical scavenger, therefore acting as a chain-breaking antioxidant that prevents the propagation of free radicals in membranes [48].When peroxyl radicals are generated, these react 1000 times faster with vitamin E than with polyunsaturated fatty acids [48]. Therefore, it is presumed that GLY provokes a pro-oxidant effect on polyunsaturated fatty acids from cerebellum mediated by reactive species generation that may lead to cell membrane damage.

The concentrations of GSH, the major brain antioxidant, were decreased by GLY. This effect was attenuated by MEL, reinforcing the involvement of the hydroxyl radical in GLY pro-oxidant effects. These data are also in line with previous data in cerebral cortex demonstrating that MEL prevents the GSH decrease caused by GLY [18]. However, it should be noted that the reduction of GSH concentrations

is probably due to reactive species generation elicited by GLY and also to a direct effect of GLY, since it was suggested that this amino acid is capable to directly react with sulfhydryl groups [18]. MK-801 also prevented the decrease of GSH concentrations induced by GLY, implying that this effect is probably mediated by NMDA receptor. So, considering that the measurement of endogenous GSH levels is used to evaluate the non-enzymatic antioxidant capacity of a tissue to prevent the oxidative damage to biomolecules, it is concluded that the rat cerebellar non-enzymatic antioxidant defenses were compromised by GLY [43].

In contrast, GLY did not alter the carbonyl and total and protein-bound sulfhydryl group content, indicating that GLY does not provoke protein damage in rat cerebellum. Moreover, GLY does not alter the activities of SOD and CAT in vitro, implying that this enzymatic antioxidant system is not compromised.

With respect to the parameters of energy metabolism, GLY did not change the activities of the respiratory chain complexes and CK. These findings are different from those previously observed [20] probably due to the brain region studied in each report (cerebellum or cerebral cortex). In this context, it should be noted that the expression of the subunits of the respiratory chain complexes changes in the various brain regions [37, 49, 50]. So, it is possible that this differential expression of subunits may confer distinct tissue reactivity to GLY exposure. Regarding to the CK activity, the differences may be explained by the fact that the cerebellum contains the highest level of CKB (the most abundant CK isoform in brain) mRNA, leading to elevated CKB levels, compared with the other brain regions analyzed, which confer differential vulnerability of this enzyme [51, 52]. Taken together the present data and previous studies [20], it seems that the cerebellum is less vulnerable to GLY toxic effects on cell energy homeostasis than cerebral cortex.

Considering that GLY increases the production of reactive species, induces lipid peroxidation and decreases GSH concentrations in rat cerebellum, our present data indicate that GLY, the amino acid accumulating in NKH, induces oxidative stress in this brain region. It is noteworthy that the CNS is a tissue particularly vulnerable to increased reactive species due to its low antioxidant defenses compared with other tissues [43] and high content of polyunsaturated fatty acids. It should be also emphasized that GCS is highly expressed in cerebellum compared to other brain regions [53], implying that a defect in this system could lead to a marked accumulation of GLY in this brain structure. Furthermore, our findings suggest that at least some of GLY pro-oxidant effects in cerebellum are mediated by NMDA receptor, which is in line with previous reports that showed that GLY acts an excitatory neurotransmitter in cerebellum [1, 5, 54].

In conclusion, we demonstrate here for the first time that GLY induces lipid peroxidation and impairs non-enzymatic antioxidant defenses in rat cerebellum. Therefore, it is presumed that oxidative stress may be a relevant pathomechanism underlying the cerebellum damage observed in NKH affected patients.

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PARTE III

Discussão e Conclusões

III.1. DISCUSSÃO

A HNC é uma aminoacidopatia causada por um defeito no SCG, cuja principal característica bioquímica é o aumento dos níveis de Gli no plasma e nos tecidos dos indivíduos afetados, especialmente no SNC. A forma neonatal ou clássica da doença tem uma sintomatologia bastante grave que compromete o SNC de forma devastadora e que inclui convulsões, hipotonia, dificuldades respiratórias, letargia e encefalopatia que pode evoluir para um quadro de coma e até mesmo causar a morte do paciente. Dentre os achados neuropatológicos da HNC podem ser citados atrofia progressiva cortical e cerebelar, hipoplasia do corpo caloso, alterações na mielinização e degeneração da substância branca com diferentes graus de gliose (Shuman et al., 1978; Raghavendra et al., 2007; Bekiesiniska-Figatowska et al., 2001; Hennermann et al., 2012; Tsuyusaki et al., 2012). Alguns relatos na literatura mostram que tais anormalidades cerebrais estão presentes já no período fetal (Shuman et al., 1978; Brun et al., 1979; Hennermann et al., 2012; Pai et al., 2015).

A patogênese do dano cerebral apresentado pelos pacientes acometidos pela HNC ainda não está totalmente esclarecida, embora estudos já tenham sugerido que a elevação dos níveis de Gli no cérebro está intimamente ligada com a gravidade da doença (Hamosh e Johnston, 2001). O primeiro efeito proposto na literatura para a Gli foi indução de excitotoxicidade, visto que esse aminoácido atua como um co-agonista do receptor glutamatérgico do tipo NMDA e, portanto, o seu acúmulo pode superestimular esses receptores. Na última década, estudos realizados em cérebro de ratos demonstraram que a Gli também pode causar disfunção bioenergética e induzir estresse oxidativo *in vitro* (Leipnitz et al., 2009; Busanello et al., 2010). Também foi verificado que a injeção intraestriatal (IE) de Gli em ratos jovens induz lipoperoxidação e dano proteico, e altera as

defesas antioxidantes no estriado desses animais (Seminotti et al., 2011), corroborando com os achados *in vitro*. Contudo, no cenário atual de pesquisa ainda não foram realizados estudos investigando o efeito *ex vivo* da injeção ICV de Gli sobre o metabolismo energético cerebral em ratos jovens, bem como o seu efeito sobre o imunoconteúdo de proteínas que atuam em vias de sinalização que podem estar alteradas em situações de estresse oxidativo e dano neuronal. Também não há relatos sobre os efeitos da Gli no cérebro de ratos neonatos, tampouco foram realizados estudos sobre as ações da Gli em cerebelo, outra estrutura cerebral afetada na HNC.

O primeiro capítulo da presente tese abordou os efeitos de uma única injeção ICV de Gli (dose de 5 μmol) sobre importantes parâmetros do metabolismo energético em cérebro de ratos de 30 dias de vida. Foi verificado que a injeção de Gli causa uma série de alterações na homeostase energética cerebral tanto em córtex cerebral quanto em estriado 30 min e 24 h após a administração desse aminoácido. A Gli diminuiu a produção de CO_2 a partir de glicose pelo CAC em estriado 30 min após a injeção, porém não alterou esse parâmetro em córtex cerebral no mesmo tempo estudado. A atividade de duas enzimas cruciais para o funcionamento do CAC, a citrato sintase (CS) e a isocitrato desidrogenase (IDH), foi diminuída no tempo de 30 min também em estriado. Esses resultados demonstram que a injeção de Gli causa um prejuízo na atividade do CAC, o que provavelmente causa um comprometimento na produção dos equivalentes reduzidos necessários para a síntese de ATP pela mitocôndria.

O passo seguinte do capítulo I foi a investigação do funcionamento dos complexos da CTE em córtex cerebral e estriado 30 min ou 24 h após administração ICV de Gli. Foi observado que a Gli diminuiu a atividade do complexo I-III em estriado nos dois tempos estudados, e também diminuiu a atividade do complexo IV em córtex cerebral no tempo de

30 min. Considerando que a redução do fluxo de elétrons da CTE provavelmente resulta na diminuição na síntese de ATP, os nossos resultados sugerem que a produção de energia é comprometida pela Gli em ambas regiões cerebrais. Por outro lado, o fato de terem sido observados efeitos diferenciados da Gli sobre os complexos da CTE no córtex cerebral e estriado pode estar relacionado com a variabilidade na composição de subunidades dos complexos da CTE em diferentes partes do cérebro (Clay e Ragan, 1988; Rustin et al., 1994; Roemgens et al., 2011; Sundar Boyalla et al., 2011; Arnold, 2012).

A injeção ICV de Gli também diminuiu a atividade da creatina cinase total (tCK) tanto em córtex cerebral quanto em estriado em ambos os tempos estudados. Com o objetivo de elucidar qual isoforma da CK foi inibida pela Gli, separamos as frações citosólica e mitocondrial e medimos a atividade enzimática. Verificamos que a mCK da CK foi inibida nos dois tecidos e tempos estudados, enquanto que a cCK não foi comprometida. Nossos resultados corroboram com achados *in vitro* da literatura que mostraram que a Gli inibe a tCK e a mCK em córtex cerebral de ratos jovens (Busanello et al., 2010). Portanto, é presumido que a Gli prejudica a transferência e a produção de energia em diferentes compartimentos celulares (mitocôndria e citosol).

Por outro lado, a enzima Na^+,K^+-ATPase não foi alterada pela administração da Gli em nenhuma estrutura cerebral ou tempo avaliado, indicando que a Gli não altera a geração do potencial de membrana e o controle de volume celular regulados por essa enzima. Esses resultados estão em aparente controvérsia com os dados *in vitro* já publicados mostrando inibição da atividade da Na^+,K^+-ATPase pela Gli (Busanello et al., 2010). Contudo, isso pode ser explicado pelo fato de que nos experimentos *in vitro* foi realizada uma incubação da Gli com membranas plasmáticas purificadas de homogeneizados de cérebro de ratos, o

que proporciona uma exposição direta da enzima ao aminoácido, já que a membrana celular não está íntegra.

Para uma melhor investigação dos possíveis mecanismos pelos quais a Gli causa disfunção bioenergética, decidimos estudar a influência do antioxidante NAC, do substrato energético e antioxidante creatina (CR) e do antagonista do receptor NMDA dizocilpina (MK-801) sobre os efeitos exercidos pela administração de Gli. A NAC e a CR foram capazes de prevenir o comprometimento induzido pela Gli nas atividades da CK e do complexo IV em córtex cerebral e estriado de ratos. Quanto ao complexo I-III, somente a CR atenuou a diminuição da atividade desse complexo causado pela Gli em estriado. Já o MK-801 preveniu o efeito da Gli sobre a CK (córtex e estriado) e o complexo I-III (estriado), porém parece não atuar sobre a inibição do complexo IV causada pela Gli. A prevenção mediada pelos antioxidantes da maior parte dos efeitos exercidos pela Gli indica que a toxicidade desse aminoácido é mediada, pelo menos em parte, pela geração de espécies reativas. O quadro de estresse oxidativo pode, por sua vez, comprometer o funcionamento dos complexos da CTE e de enzimas como a CK que contém resíduos de aminoácidos vulneráveis ao ataque oxidativo. Pode ser presumido que tanto a NAC quanto a CR sejam capazes de sequestrar radicais livres gerados pela Gli, enquanto que a CR também pode promover a reciclagem de ATP a ADP dentro da mitocôndria, melhorando o acoplamento da respiração mitocondrial.

Com relação aos efeitos do MK-801, o fato de esse composto ter prevenido os efeitos de inibição causados pela Gli sobre a atividade da CK e do complexo I-III indica que a Gli induz excitotoxicidade via receptores NMDA. Deve ser salientado aqui que já é bem sabido que a excitotoxicidade causa um aumento no influxo celular de cálcio e consequentemente um acréscimo na produção de espécies reativas (Halliwell e Gutteridge,

2007), o que está de acordo com o fato de que antioxidantes (NAC e CR) também previnem os efeitos tóxicos da Gli. O fato de que esses compostos (MK-801, CR e NAC) tiveram alguns efeitos preventivos diferenciados sobre os parâmetros alterados pela Gli em córtex cerebral e estriado pode ser explicado pela diferente distribuição das subunidades dos complexos da CTE e também dos receptores NMDA (isoformas diferentes e variação na quantidade de proteína) nas regiões cerebrais. Nesse particular, tem sido demonstrado em outros estudos que tais diferenças podem conferir uma variabilidade na resposta do cérebro a diferentes compostos protetores (Boyalla et al., 2011; Roemgens et al., 2011; Arnold, 2012).

Portanto, no capítulo I foi evidenciado que a injeção ICV de Gli em ratos jovens compromete a formação, transferência e tamponamento de energia, prejudicando dessa forma a homeostase energética cerebral. Além isso, considerando que os efeitos da Gli foram prevenidos por antioxidantes e pelo antagonista do receptor NMDA MK-801, presume-se que a Gli induz estresse oxidativo provavelmente mediado por excitotoxicidade via receptor NMDA.

No capítulo II foi investigado o efeito da injeção ICV da Gli (dose de 5 µmol) sobre o imunoconteúdo proteico da sinaptofisina e fosforilação da Tau em córtex cerebral e estriado de ratos jovens. A administração de Gli diminuiu a fosforilação da Tau em córtex cerebral e estriado 30 min e 24 h após a injeção. Já o conteúdo de sinaptofisina foi diminuído em córtex cerebral 24 h após a injeção de Gli, ao passo que no tempo de 30 min o conteúdo dessa proteína foi alterado apenas em estriado. Nesse contexto, visto que já foi mostrado que a Gli aumenta a produção de espécies reativas *in vitro* e *ex vivo* (Leipnitz et al., 2009; Seminotti et al., 2011), nossos achados corroboram os resultados de Zambrano e colaboradores (2004) que mostraram que uma alta concentração de peróxido de hidrogênio

(500 μM) induz redução da fosforilação da Tau em células de hipocampo de ratos. A Tau é uma proteína regulatória da dinâmica e estabilidade dos microtúbulos e, portanto, está envolvida no transporte axonal, desenvolvimento da polaridade neuronal, manutenção da morfologia neuronal e crescimento neuronal. Já a sinaptofisina é uma proteína marcadora de sinapses, mais especificamente do botão pré-sináptico (Wang e Liu et al., 2008; Morris et al., 2011; Zhang et al., 2014). Portanto, nossos resultados indicam que a Gli causa dano neuronal por alterar a fosforilação da Tau e o imunocontéudo de sinaptofisina.

Com o objetivo de descobrir se modificações em vias de sinalização poderiam estar envolvidas no dano neuronal causado pela Gli, examinamos o efeito da injeção de Gli sobre a fosforilação das MAPK. É importante ser comentado aqui que vários estudos já evidenciaram que as MAPK são alteradas em situações patológicas com envolvimento de excitotoxicidade, estresse oxidativo e disfunção bioenergética (Kurokawa et al., 2011; Fan et al., 2012; Subramaniam et al., 2004; Wang et al., 2015). Verificamos que a Gli diminuiu a fosforilação da p38, ERK e JNK 30 min após a sua administração em córtex cerebral e estriado. Também observamos que o MK-801 atenuou a diminuição da fosforilação da p38 em estriado. Wang e colaboradores (2015) evidenciaram que a indução de hipóxia em ratos causa um aumento no influxo de cálcio e a diminuição da fosforilação da ERK no hipocampo desses animais. O mesmo estudo também mostrou que a memantina, um antagonista do receptor NMDA, preveniu o aumento no influxo de cálcio e a diminuição na fosforilação da ERK (Wang et al., 2015). Assim, pode ser presumido que a Gli, através de sua ação como co-agonista do receptor NMDA, aumenta as concentrações intracelulares de cálcio, o que leva à diminuição da fosforilação das MAPK. Ainda considerando que a Tau é

um substrato das MAPK, é concebível que a diminuição da fosforilação das MAPK seja o evento responsável pela diminuição da fosforilação da Tau.

Entretanto, a administração de Gli não alterou a marcação de GFAP e fluoromielina em cérebro de ratos jovens. Esses resultados indicam que a Gli não induz reatividade glial e não causa alterações na mielinização em animais com 30 dias de vida nas condições estudadas.

Para dar continuidade ao estudo da patogênese da HNC, no capítulo III estudamos o efeito da injeção ICV de Gli (0,2 $\mu mol.g^{-1}$) sobre a homeostase energética e redox em ratos neonatos (1 ou 5 dias de idade) eutanasiados 1, 5 ou 10 dias após a administração com o objetivo de mimetizar, ao menos parcialmente, a forma clássica da HNC, cujas anormalidades cerebrais tem início no período fetal ou nas primeiras semanas de vida do paciente (Van Hove et al., 2000; Applegarth e Toone, 2001; Hamosh e Johnston, 2001). O primeiro parâmetro analisado foi o funcionamento da cadeia respiratória mitocondrial através da medida da atividade dos complexos I-III, II, II-III e IV no córtex cerebral de ratos neonatos. A Gli diminuiu a atividade do complexo IV em córtex cerebral de ratos de 1 dia de vida 1, 5 e 10 dias após a infusão, enquanto que os outros complexos não foram alterados. Além disso, a Gli inibiu a atividade da enzima CK também em ratos de 1 dia de idade nos mesmos tempos testados para os complexos da CTE. Essa combinação de resultados envolvendo parâmetros do metabolismo energético corroboram com os dados mostrados no capítulo I do presente trabalho e mostram que a Gli compromete a produção e a transferência intracelular de energia. Verificamos ainda que o tratamento intraperitoneal (IP) com o antioxidante melatonina (MEL) preveniu os efeitos inibitórios da Gli sobre as atividades da CK e do complexo IV, mostrando um possível envolvimento dos radicais hidroxil e peroxil nesses efeitos, já que a MEL é um eficiente sequestrador dessas espécies

reativas. É provável também que a MEL previna a inibição da Gli sobre a atividade do complexo IV através da promoção da estabilização dos complexos proteicos da CTE ou ainda induzindo a expressão dos genes das subunidades do complexo IV, como descrito em outros trabalhos da literatura (Martin et al., 2000; Reyes-Toso et al., 2006; Garcia et al., 2014).

Quanto aos parâmetros de estresse oxidativo, observamos inicialmente que a Gli aumentou a produção de espécies reativas (oxidação de DCFH) em animais de 1 dia de idade 1, 5 e 10 dias após a injeção. A Gli também aumentou a lipoperoxidação no período de 5 dias, e diminuiu a concentração de GSH 1, 5 e 10 dias após a administração, indicando que altas concentrações de Gli no primeiro dia pós-natal induzem lipoperoxidação e comprometem as defesas antioxidantes não enzimáticas, o que está de acordo com resultados encontrados *in vitro* (Leipnitz et al., 2009) e reforça que a Gli leva a um desequilíbrio da homeostase redox no SNC em ratos. O tratamento com a MEL preveniu o dano oxidativo lipídico e a diminuição de GSH causados pela Gli.

Quando foram investigadas as atividades das enzimas antioxidantes, constatamos que a injeção de Gli exerceu um papel modulador sobre essas atividades, diminuindo a atividade da SOD no período de 10 dias, e da CAT no tempo de 5 dias após a injeção. Já a atividade da GPx foi aumentada pela Gli 1 dia, mas diminuída 5 e 10 dias após a administração do aminoácido. Tais achados mostram efeitos diferenciais da Gli sobre as atividades das defesas antioxidantes enzimáticas, principalmente sobre a GPx. O aumento da atividade da GPx em um período curto de tempo (1 dia) após a infusão de Gli pode ter sido devido ao aumento de expressão gênica dessa enzima atuando como um mecanismo compensatório induzido pelas células a fim de neutralizar o excesso de espécies reativas. Por outro lado, a diminuição das atividades das enzimas CAT, SOD e GPx em períodos

mais longos (5 ou 10 dias) pode ter ocorrido devido à oxidação dos resíduos de aminoácidos que fazem parte da estrutura proteica dessas enzimas pelos radicais livres gerados. É importante salientar aqui que trabalhos prévios que estudaram os efeitos de metabólitos acumulados em outros EIM também evidenciaram efeitos e modulações diferenciais sobre as atividades de enzimas antioxidantes (Costa et al., 2013; da Rosa et al., 2013; Sasso et al., 2014; Viegas et al., 2014).

A seguir, verificamos que a administração de Gli no cérebro de ratos de 5 dias de vida não alterou a homeostase energética e redox 1 ou 5 dias após a injeção do aminoácido. Esses dados evidenciam que o cérebro de animais de 1 dia de vida é mais vulnerável aos efeitos tóxicos causados pela Gli, o que pode ser explicado pelo fato de que animais recém-nascidos possuem um sistema antioxidante que ainda não está totalmente desenvolvido (Ferriero e Miller, 2010; Ikonomidou e Kaindl, 2011). Esses resultados estão aparentemente em desacordo com os dados encontrados no capítulo I que mostraram que a Gli diminuiu as atividades do complexo IV e da CK em ratos de 30 dias de vida. No entanto, deve ser considerado que, apesar de não termos quantificado os níveis de Gli atingidos no cérebro após a injeção dessa substância, podemos estimar através da dose usada e do peso do cérebro dos animais que o cérebro dos ratos jovens foi exposto a maiores concentrações de Gli que o cérebro dos animais de 5 dias vida (concentração de Gli em animais jovens: 8-9 mM; animais neonatos: 6-7 mM), o que explica o fato de a Gli exercer efeitos tóxicos no SNC de ratos jovens.

Na última parte do capítulo III, foram realizados estudos de marcação com imunohistoquímica para as proteínas GFAP, NeuN e S100B em ratos que receberam a injeção de Gli no primeiro dia pós-natal e foram eutanasiados 5 dias depois. A Gli aumentou a marcação para a proteína S100B em córtex cerebral e estriado, enquanto que

um aumento da intensidade da fluorescência para GFAP foi observado em corpo caloso, sugerindo que a Gli induziu reatividade glial em ratos que receberam Gli com 1 dia de vida, uma condição caracterizada pela ativação de astrócitos que tem por objetivo evitar dano neuronal em situações patológicas (Cerutti et al., 2000; Sorci et al., 2010). Enfatize-se aqui que isso está de acordo com análises *postmortem* de pacientes com HNC onde foi mostrada degeneração na substância branca com gliose (Shuman et al., 1978). O tratamento com MEL realizado nos neonatos atenuou o aumento de S100B causado pela Gli em córtex cerebral, sugerindo o envolvimento de espécies reativas na reatividade glial induzida pela Gli. Por outro lado, a injeção de Gli não causou morte neuronal em cérebro de ratos de 1 dia de vida 5 ou 15 dias após a administração, já que essa substância não alterou a imunomarcação com NeuN e a redução de MTT. Dessa forma, podemos concluir que a reatividade glial está acontecendo como uma resposta adaptativa ao insulto causado pela administração da Gli com o intuito de evitar a morte neuronal.

Concluindo o capítulo III, os achados mostraram que altas concentrações de Gli no SNC de ratos neonatos induzem disfunção bioenergética, estresse oxidativo e reatividade glial. Além disso, deve ser enfatizado aqui que essa investigação foi a primeira a estudar os efeitos da Gli em animais recém-nascidos, visando mimetizar o fenótipo clássico da HNC. Portanto, os patomecanismos induzidos pela Gli em cérebro de ratos neonatos reforçam que o acúmulo desse aminoácido possivelmente causa efeitos neurotóxicos no SNC dos pacientes ainda no período fetal ou nos primeiros dias de vida.

O quarto e último capítulo da tese teve por objetivo estudar os efeitos *in vitro* da Gli em cerebelo de ratos, visto que anormalidades cerebelares são observadas em pacientes com HNC (Applegarth e Toone, 2001; Huisman et al, 2002; Khong et al., 2003). Foi constatado que a Gli aumentou a produção de espécies reativas, induziu lipoperoxidação e

diminuiu as concentrações de GSH em cerebelo, indicando que a Gli induz estresse oxidativo nessa região.

Apesar dos resultados mostrarem que a Gli aumenta a formação de espécies reativas em cerebelo, não foi verificada alteração na liberação de peróxido de hidrogênio, indicando que outras espécies reativas estão envolvidas nos efeitos da Gli. Também não foram encontradas alterações nos parâmetros de dano proteico (formação de carbonilas e conteúdo de sulfidrilas) e nas atividades das enzimas CAT e SOD, sugerindo que as espécies reativas geradas a partir da ação da Gli não são capazes de modificar as proteínas cerebelares.

Com o intuito de elucidar qual espécie reativa poderia estar envolvida na ação tóxica da Gli em cerebelo, testamos a influência *in vitro* da MEL, trolox (TRO; análogo hidrossolúvel da vitamina E), GSH e do MK-801 sobre os efeitos da Gli. A MEL e o TRO preveniram o aumento dos níveis de MDA causado pela Gli, sugerindo que as espécies reativas principalmente envolvidas nesse efeito são os radicais hidroxil e peroxil, uma vez que a MEL e o TRO são sequestradores eficazes desses radicais (Bromme et al., 2000; Garcia et al., 2014). A MEL também foi capaz de atenuar o efeito pró-oxidante da Gli sobre as concentrações de GSH evidenciando mais uma vez o envolvimento do radical hidroxil nos efeitos desse aminoácido. Além disso, o MK-801 preveniu a diminuição das concentrações de GSH induzida pela Gli, mostrando o envolvimento do receptor NMDA nos efeitos da Gli e reforçando os resultados que mostram que altas concentrações desse aminoácido causam excitotoxicidade.

Contudo, a Gli não alterou significativamente a atividade dos complexos da CTE e da enzima CK em cerebelo. Tais dados são controversos, visto que a Gli causou disfunção bioenergética *in vitro* em outras estruturas cerebrais (Busanello et al., 2010). Pode ser especulado que tais efeitos diferenciais podem ter ocorrido devido à expressão de diferentes

subunidades dos complexos da CTE e às grandes concentrações da isoforma cerebral da CK (CKB) no cerebelo, o que torna essa região menos vulnerável que outras estruturas cerebrais à ação da Gli.

Nossos resultados *ex vivo* e *in vitro* em ratos mostram que o acúmulo de Gli no SNC é potencialmente tóxico para diferentes estruturas cerebrais, causando disfunção bioenergética e induzindo excitotoxicidade e estresse oxidativo. No entanto, como já mencioando, observamos que a administração de Gli causa efeitos diferenciais nas regiões cerebrais avaliadas (córtex cerebral, estriado e cerebelo), provavelmente devido à diferente constituição de biomoléculas (proteínas, enzimas, receptores, lipídios de membranas, etc.) característica de cada estrutura. A partir dos resultados *ex vivo* pode ser visto que o estriado é mais vulnerável aos efeitos da Gli sobre a homeostase energética que o córtex cerebral. Já quando comparamos os nossos achados *in vitro* em cerebelo com dados *in vitro* da literatura em córtex cerebral (Busanello et al., 2010), observamos que o córtex cerebral é mais suscetível à disfunção bioenergética induzida pela Gli. Isso está de acordo com o fato de que anormalidades corticais são observadas mais frequentemente nos pacientes portadores de HNC que alterações cerebelares (Bekiesiñiska-Figatowska et al., 2001; Khong et al., 2003; Shin et al., 2012; Tsuyusaki et al., 2012).

Outro achado relevante da presente tese é de que altas concentrações de Gli alteram as vias de sinalização das MAPK em cérebro de ratos. Enfatize-se que a diminuição da fosforilação da p38 pela Gli foi atenuada por MK-801, o que indica a participação do receptor NMDA nas alterações das MAPK. Também verificamos que ocorreu uma diminuição da fosforilação da Tau, o que provavelmente aconteceu como consequência da inativação das MAPK, além de uma diminuição da sinaptofisina. Essas alterações indicam que a Gli causa dano neuronal no cérebro de ratos.

Além disso, demonstramos que a Gli causa neurotoxicidade no cérebro de ratos neonatos, o que mimetiza, ao menos parcialmente, o fenótipo encontrado nos pacientes com HNC clássica. A partir disso pode ser presumido que o aumento agudo dos níveis de Gli no cérebro em desenvolvimento compromete a homeostase desse órgão e possivelmente causa danos mais graves, visto que nesse período o SNC é mais vulnerável a insultos.

Tomados em conjunto os nossos resultados, podemos concluir que excitotoxicidade, alterações na homeostase energética e redox, bem como nas vias das MAPK causadas por elevadas concentrações de Gli podem contribuir para a fisiopatologia da disfunção neurológica observada na HNC. Além disso, visto que antioxidantes e substratos energéticos preveniram os efeitos neurotóxicos da Gli, temos a perspectiva de que a inclusão de tais compostos neuroprotetores ou fármacos similares como adjuvantes no tratamento da HNC possa melhorar a qualidade de vida dos pacientes afetados por essa doença.

III.2. CONCLUSÕES

- A injeção ICV de Gli diminuiu a produção de CO_2, e as atividades da CS, IDH e complexo I-III da cadeia respiratória em estriado, e a atividade do complexo IV em córtex cerebral. A Gli também diminuiu a atividade da tCK e da mCK em córtex cerebral e estriado de ratos jovens. Os antioxidantes NAC e CR, e o antagonista do receptor NMDA MK-801 preveniram o efeito inibitório da administração de Gli sobre a atividade da CK e dos complexos I-III e IV. A injeção ICV de Gli também diminuiu a fosforilação da p38, ERK, JNK e Tau, e o conteúdo de sinaptofisina em córtex cerebral e estriado. Além disso, o MK-801 atenuou a diminuição da fosforilação da p38 em estriado;

- A administração ICV de Gli diminuiu a atividade do complexo IV e da enzima CK no córtex cerebral de ratos neonatos (1 dia de idade). A administração de Gli também aumentou a oxidação de DCFH e os níveis de MDA, e diminuiu as concentrações de GSH em córtex cerebral. Foi verificado ainda que a Gli modulou as atividades das enzimas SOD, CAT e GPx na mesma estrutura cerebral. A Gli também aumentou a imunomarcação positiva para GFAP em corpo caloso e para S100B em córtex cerebral e estriado, sendo que o tratamento IP com MEL preveniu tanto o efeito na imunomarcação de S100B quanto a inibição das atividades do complexo IV e da CK, a diminuição das concentrações de GSH, e o aumento nos níveis de MDA;

- A Gli aumentou a oxidação de DCFH e os níveis de MDA, e diminuiu as concentrações de GSH *in vitro* em cerebelo de ratos jovens. Os antioxidantes MEL e TRO, e o antagonista do receptor NMDA MK-801 preveniram o aumento dos níveis de MDA e a diminuição das concentrações de GSH causados pela Gli em cerebelo.

III.3. PERPECTIVAS

- Avaliar o efeito da administração intracerebelar de Gli sobre a homeostase energética e redox em cerebelo de ratos jovens;
- Investigar o padrão de mielinização através de marcadores imunohistoquímicos em cérebro de ratos com diferentes idades após a injeção ICV de Gli. Para tanto, pretendemos utilizar a sonda fluoromielina e marcadores de oligodendrócitos e pré-oligodendrócitos, tais como a proteína básica da mielina (MBP), a proteína associada à glicoproteína (MAG), a proteína CC-1 (produto do gene APC), neuroglicano 2 (NG2) e o receptor alfa para o fator de crescimento derivado de plaquetas (PDGFRa);
- Avaliar os efeitos *in vitro* da exposição de Gli em culturas primárias de astrócitos e neurônios em córtex cerebral e estriado de ratos Wistar. Pretendemos analisar parâmetros de estresse oxidativo, viabilidade e morte celular, potencial de membrana mitocondrial e o imunoconteúdo das subunidades (NR1, NR2A e NR2B) do receptor glutamatérgico NMDA;
- Estudar os efeitos da injeção ICV de Gli sobre a captação de glutamato em cérebro de ratos jovens;
- Avaliar os efeitos do antagonista do receptor NMDA MK-801 sobre a diminuição da fosforilação da ERK1/2 e da JNK causada pela injeção ICV de Gli em cérebro de ratos jovens.

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