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Acute lysine overload provokes marked striatum injury involving oxidative stress signaling pathways in glutaryl-CoA dehydrogenase deficient mice

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A B S T R A C T

Glutaric acidemia type I (GA I) is a neurometabolic disorder of lysine (Lys) catabolism caused by glutaryl-CoA dehydrogenase (GCDH) deficiency. Patients are susceptible to develop acute striatum degeneration during catabolic stress situations whose underlying mechanisms are not fully established. Thus, in the present work we investigated the effects of a single intrastriatal Lys administration (1.5–4 μmol) to 30-day-old wild type (WT) and GCDH deficient (*Gcdh*−/−) mice on brain morphology, neuronal injury, astrocyte reactivity and myelin structure, as well as signaling pathways of redox homeostasis. We observed a marked vacuolation/edema in striatum and at higher doses also in cerebral cortex of *Gcdh*−/−, but not of WT mice. Lys also provoked a reduction of NeuN and synaptophysin, as well as an increase of astrocytic GFAP, in the striatum of *Gcdh*−/− mice, indicating neuronal loss and astrocyte reactivity. Furthermore, we verified an increase of Nrf2 and NF-κB expression in the nuclear fraction, and a decrease of heme oxygenase-1 (HO-1) content in the striatum of Lys-injected *Gcdh*−/− mice, implying disruption of redox homeostasis. Finally, it was found that Lys provoked alterations of myelin structure reflected by decreased myelin basic protein (MBP) in the cerebral cortex of *Gcdh*−/− mice. Taken together, the present data demonstrate neuronal loss, gliosis, altered redox homeostasis and demyelination caused by acute Lys overload in brain of *Gcdh*−/− mice, supporting the hypothesis that increased brain concentrations of glutaric and 3-hydroxyglutaric acids formed from Lys may be responsible for the acute brain degeneration observed in GA I patients during episodes of metabolic decompensation.

1. Introduction

Glutaric acidemia type I (GA I, OMIM #231670) is an inherited metabolic disorder of lysine (Lys), hydroxylysine and tryptophan catabolism characterized by tissue accumulation of the neurotoxic metabolites glutaric (GA) and 3-hydroxyglutaric (3HGA) acids, particularly in the brain, due to a deficiency of glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) activity (Brismar and Ozand, 1995; Goodman and Frerman, 2001). Most untreated patients develop acute encephalopathic crises precipitated by catabolic events, such as febrile illness, vomiting/diarrhea, fasting and infectious/inflammatory diseases between 3 and 48 months of age, accompanied by acute striatal injury and followed by dystonia, dyskinesia, hypotonia, seizures and spasticity (Boy et al., 2016; Goodman et al., 1977; Kolker et al., 2006; Neumaier-Probst et al., 2004). Progressive cortical leukodystrophy associated with demyelination of the central nervous system with or without

Abbreviations: ANOVA, analysis of variance; GA, glutaric acid; GA I, glutaric acidemia type I; GCDH, glutaryl-CoA dehydrogenase; *Gcdh*−/−, glutaryl-CoA dehydrogenase deficient; GFAP, glial fibrillary acidic protein; HE, hematoxylin and eosin; HO-1, heme oxygenase-1; 3HGA, 3-hydroxyglutaric acid; IkBα, NF-kappa-B inhibitor alpha; Lys, lysine; MBP, myelin basic protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NeuN, neuronal nuclei; PBS, phosphate-buffered saline; WT, wild type

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(insidious) documented metabolic episodes of acute decompensation are also commonly found (Funk et al., 2005; Garbade et al., 2014; Harting et al., 2009, 2015; Neumaier-Probst et al., 2004; Strauss et al., 2007).

In order to better understand the neuropathology of GA I, a genetic mouse model of GA I was developed with complete loss of GCDH activity (*Gcdh*−/−), presenting similar biochemical phenotype including elevated concentrations of GA and 3HGA in brain, blood and urine, but had no apparent striatum damage, a common characteristic of GA I (Koeller et al., 2002). An improvement of this animal model was achieved by giving the animals a high protein or Lys chow, which provoked accumulation of GA and 3HGA and striatum injury apart from marked brain morphological alterations with myelin disruption, gliosis and neuronal loss (Amaral et al., 2015; Zinnanti et al., 2006, 2007). Disruption of energy and redox homeostasis and of the glutamatergic and GABAergic systems, as well as blood–brain barrier breakdown, have also been demonstrated in the cerebral cortex and striatum of *Gcdh*−/− mice exposed to a high protein or Lys chow during variable periods (Amaral et al., 2012a, 2012b, 2015; Busanello et al., 2014; Lagranha et al., 2014; Rodrigues et al., 2015; Seminotti et al., 2012, 2013, 2014; Vendramin Pasquetti et al., 2017; Zinnanti et al., 2014).

Considering that the underlying mechanisms of acute striatum degeneration that follows acute episodes of metabolic decompensation in GA I are still poorly known, in the present work we investigated the effects of brain acute Lys overload achieved by a single Lys injection into the striatum of *Gcdh*−/− mice on important histopathological and biochemical parameters in striatum and cerebral cortex of these animals, as compared to wild type (WT) mice. Cortical and striatal histopathological alterations were determined by hematoxylin and eosin staining, neuronal injury by immunohistochemistry of neuronal nuclei (NeuN) and protein expression of synaptophysin, astrocyte reactivity by immunohistochemistry of glial fibrillary acidic protein (GFAP) and myelination by immunohistochemistry of myelin basic protein (MBP). The protein contents of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), NF-kappa-B inhibitor alpha (IkBα), nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and heme oxygenase-1 (HO-1) were also evaluated.

2. Material and methods

2.1. Reagents

Unless otherwise stated, reagents were purchased from Sigma (St Louis, MO, USA). Solutions were prepared on the day of the experiments in the appropriate buffers, with pH adjusted to 7.2–7.4.

2.2. Animals

Thirty-day-old WT and *Gcdh*−/− mice, both of C129SvEv background, were maintained at Unidade de Experimentação Animal from Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) in a colony room with constant temperature (22 ± 1 °C) and 12:12 h light/dark cycle (lights on 07:00-19:00 h). Animals had free access to water and to a 20% (w/w) protein commercial chow containing 0.9% Lys (Nuvilab, Porto Alegre, RS, Brazil).

Procedures performed with animals in this investigation were conducted in accordance with the Principles of Laboratory Animal Care, National Institute of Health of United States of America, NIH, publication nº 85-23, revised in 2011, the International Guiding Principles for Biomedical Research Involving Animals and approved by the Ethical Committee for the Care and Use of Animals of the Hospital de Clínicas de Porto Alegre (nº 160264).

2.3. Intrastriatal administration of lysine (Lys)

WT and *Gcdh*−/− mice were anesthetized with isoflurane (45 mg/kg) and thereafter placed on a stereotaxic frame (Stoelting Co., Wood Dale IL, USA). Then, they received an intrastriatal injection of 1 μL of phosphate-buffered saline (PBS), pH 7.4, or 1 μL of 1.5, 2.5 or 4 M Lys solution (1.5, 2.5 and 4 μmol, respectively) dissolved in PBS into both hemispheres, using the coordinates: 0.0 mm anteroposterior to bregma, +2.6 mm lateral to bregma and −2.4 mm dorsoventral to the dura (Amaral et al., 2018; Paxinos and Franklin, 2012). Animals were euthanized 24 or 48 h after injection.

2.4. Sample preparation for histological analyses

Hematoxylin and eosin (HE) staining and immunohistochemical analyses were performed in brains post-fixed in 10% formaldehyde buffered solution (pH 7.00–7.05) and sectioned transversely (3 μm thick) at the level of dorsal striatum on a Microtome (MICROM HM 360), as previously reported (Amaral et al., 2018).

2.5. Histological analyses

HE staining and immunohistochemistry analyses of the striatal sections were performed according to da Rosa et al. (2015), Amaral et al. (2018) and van Tilborg et al. (2017). The following primary antibodies were used: anti-GFAP (glial fibrillary acidic protein, 1:400 dilution, Cell Marque, clone: SP78), anti-NeuN (neuronal nuclei, 1:400 dilution, Zeta Corporation, clone: A60) and anti-MBP (myelin basic protein polyclonal antibody, 1:100 dilution, Cell Marque). Three sections were prepared per mice and five fields per section analyzed utilizing the Q Capture Pro Software (Olympus) (magnification of 100× and 400×). Medians obtained from five randomly selected fields per animal were used for the statistical analyses. Quantification of the numbers of vacuoles, GFAP and NeuN immunoreactive cells, as well as the percentage of MBP stained area (magnification of 400×) were performed by Image J software. Three to five mice were used per group.

2.6. Sample preparation for Western blot analyses

For the measurement of synaptophysin and heme oxygenase-1 (HO-1) protein content, striatum from WT and *Gcdh*−/− mice was homogenized, centrifuged and the supernatants obtained for the analyses, as previously reported (Seminotti et al., 2019). On the other hand, for the determination of NF-κB, Nrf2 and IκBα, striatum was homogenized and centrifuged to obtain cytosolic and nuclear fractions as described by Seminotti and collaborators (2016). Protein concentrations were measured by the method of Lowry et al. (1951).

2.7. Western blot analyses

Western blot experiments were performed according to Seminotti et al. (2016) and Seminotti et al. (2019), using equal amounts of protein (30 μg/well). The primary antibodies anti-HO-1 (1:500, Abcam® ab13248), anti-synaptophysin (1:500, Abcam® ab8049), anti-NF-κB p-65 (1:500, Cell Signaling® D14E12), anti-Nrf2 (1:500, Abcam® ab31163) and anti-IκBα (1:500, Cell Signaling® L35A5) were used. All results were expressed as a ratio relative to the β-actin (1:1000, Sigma-Aldrich® A1978) or lamin B1 (1:1000, Abcam® ab133741) internal control. Three mice were used per group.

2.8. Statistical analysis

Unless otherwise stated, results are presented as mean ± standard deviation. Data from HE staining and immunohistochemistry were analyzed by two-way analysis of variance (ANOVA), considering the factors: 1) genotype: WT or *Gcdh*−/−; 2) treatment: PBS or Lys; 3) interaction between genotype x treatment. F values are presented when differences between groups were rated significant at $P < 0.05$. The post-hoc Tukey's test was performed when means from three or more

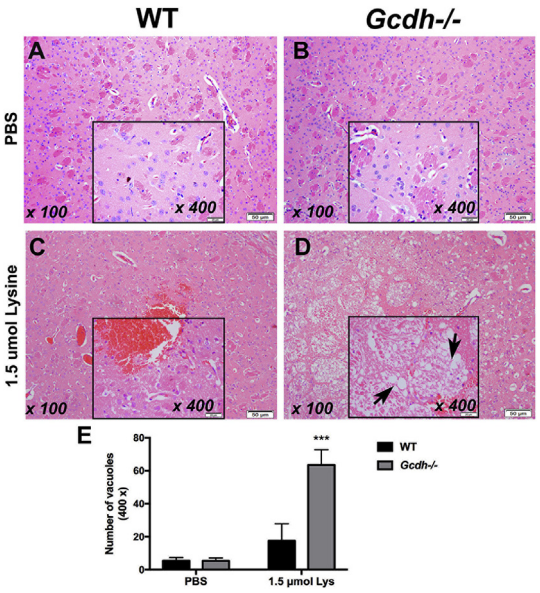

Fig. 1. Light microscopic images showing hematoxylin and eosin (HE) staining of striatum from developing (30-day-old) wild type (WT) (A and C) and glutaryl-CoA dehydrogenase deficient (*Gcdh−/−*) (B and D) mice submitted to an intrastriatal injection of PBS (A and B) or 1.5 µmol lysine (Lys) (C and D). Mice were euthanized 48 h after intrastriatal administration. Representative images were obtained from four to five independent experiments (animals) per group. HE staining was visualized with magnification of 100× and 400×. Arrows in panel D indicate vacuolation. (E) Quantification of the number of vacuoles was performed with 400× magnification by using the median of five randomly selected fields from three striatal sections per brain. Values are mean ± standard deviation for four to five mice per group. Two-way ANOVA was performed as described in the text. ***$P < 0.001$ compared to Lys-injected WT mice (Tukey's test).

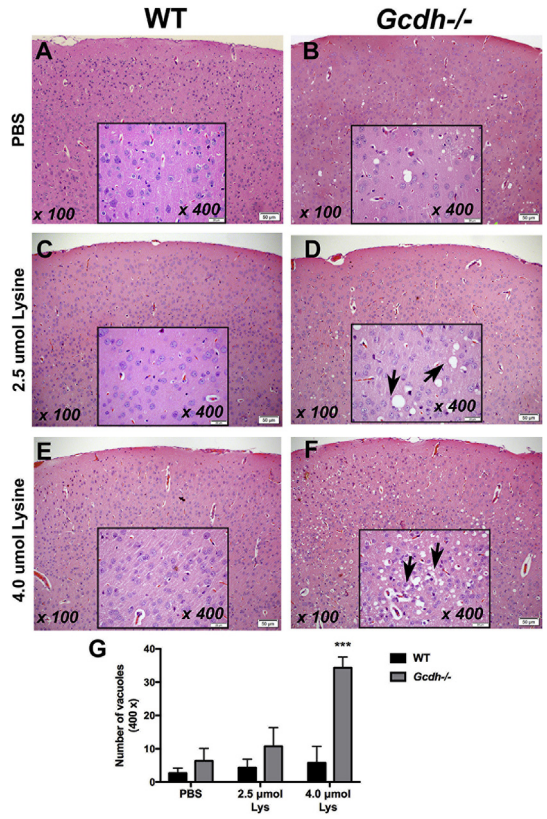

Fig. 2. Light microscopic images showing hematoxylin and eosin (HE) staining of cerebral cortex from developing (30-day-old) wild type (WT) (A, C and E) and glutaryl-CoA dehydrogenase deficient (*Gcdh−/−*) (B, D and F) mice submitted to an intrastriatal injection of PBS (A and B), 2.5 µmol lysine (Lys) (C and D) or 4.0 µmol Lys (E and F). Mice were euthanized 48 h after intrastriatal administration. Representative images were obtained from three to four independent experiments (animals) per group. HE staining was visualized with magnification of 100× and 400×. Arrows in panels D and F indicate vacuolation. (G) Quantification of the number of vacuoles was performed with 400× magnification by using the median of five randomly selected fields from three cortical sections per brain. Values are mean ± standard deviation for three to four mice per group. Two-way ANOVA was performed as described in the text. ***$P < 0.001$ compared to Lys-injected WT mice (Tukey's test).

groups were compared. Protein content by western blotting was analyzed by Student's *t*-test for unpaired samples to compare means between two different groups. All statistical analyses were carried out using the GraphPad Prism 7 software.

3. Results

3.1. Acute lysine (Lys) brain overload provokes extensive vacuolation in striatum and cerebral cortex of glutaryl-CoA dehydrogenase deficient (Gcdh−/−) mice

We first evaluated the histopathological findings in striatum and cerebral cortex of WT and *Gcdh−/−* mice by HE staining 48 h after Lys injection. Statistical analysis (two-way ANOVA) showed marked vacuolation associated with edema in striatum of *Gcdh−/−* mice injected with 1.5 µmol of Lys (Fig. 1) (interaction between treatment x genotype: $F_{(1,13)} = 48.45$, $P < 0.001$). Furthermore, separate influences of the *Gcdh−/−* genotype ($F_{(1,13)} = 48.03$, $P < 0.001$) and Lys treatment ($F_{(1,13)} = 112$, $P < 0.001$) on striatum vacuolation were observed (Fig. 1).

In contrast, intrastriatal administration of 1.5 µmol of Lys caused no vacuolation or other histopathological findings in cerebral cortex of both WT and *Gcdh−/−* mice after 48 h (results not shown). However, a few vacuoles were identified with 2.5 µmol Lys and intense vacuolation 4.0 µmol of Lys injection in cerebral cortex of *Gcdh−/−* mice after 48 h, as compared to WT injected with Lys at the same tested doses (interaction between treatment x genotype: $F_{(2,14)} = 18.4$, $P < 0.001$). Again, separate influences of *Gcdh−/−* genotype ($F_{(1,14)} = 48.34$, $P < 0.01$) and Lys treatment ($F_{(2,14)} = 26.46$, $P < 0.001$) on vacuolation were observed (Fig. 2).

Taken together, these data demonstrate that Lys administration provokes extensive histopathological alterations predominantly in striatum of *Gcdh−/−* mice, as well as in cerebral cortex at higher doses, indicating that brain of knockout mice is more susceptible to the toxic effects of acute Lys overload that results in GA and 3HGA accumulation (Seminotti et al., 2012; Zinnanti et al., 2006, 2007). Thereafter, we searched for the type of neural cells (neurons or astrocytes) this toxicity was directed in striatum of Lys-treated *Gcdh−/−* mice.

3.2. Acute lysine (Lys) brain overload provokes neuronal loss in striatum of glutaryl-CoA dehydrogenase deficient (Gcdh−/−) mice

We determined whether Lys intrastriatal injection could damage neuronal cells by measuring NeuN immunohistochemistry and synaptophysin content in brain of *Gcdh−/−* and WT mice. Intrastriatal Lys infusion caused a significant neuronal loss (decreased NeuN) in striatum of *Gcdh−/−* mice 48 h after injection at concentrations as low as 1.5 µmol (Fig. 3) (Lys treatment: $F_{(1,12)} = 30.25$, $P < 0.001$; *Gcdh−/−* genotype: $F_{(1,12)} = 23.72$, $P < 0.001$), with no significant interaction between treatment and genotype (two-way ANOVA). In contrast, cerebral cortex of *Gcdh−/−* mice injected with PBS or Lys presented no alterations of neuronal viability (results not shown). Furthermore, synaptophysin content was not altered in striatum from both genotypes after 1.5 µmol Lys injection (results not shown), but was

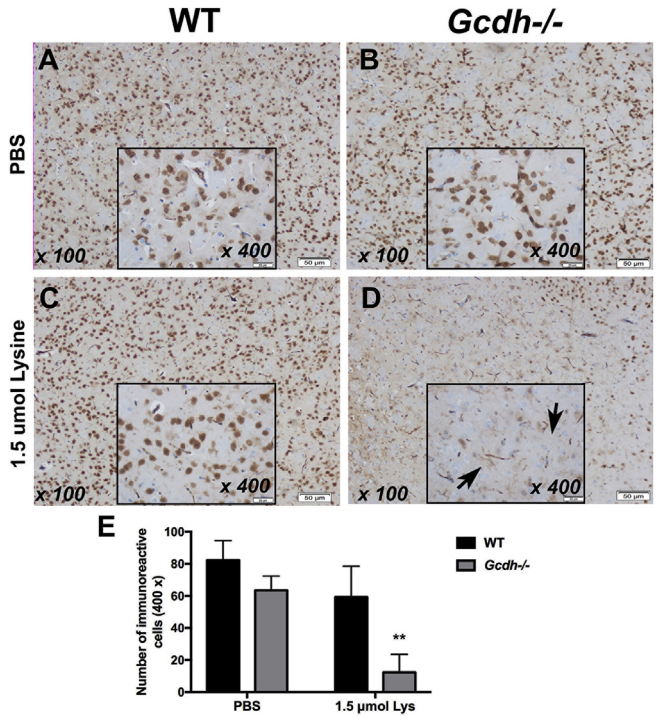

Fig. 3. Light microscopic images showing NeuN immunohistochemistry staining of striatum from developing (30-day-old) wild type (WT) (A and C) and glutaryl-CoA dehydrogenase deficient (*Gcdh*−/−) (B and D) mice submitted to an intrastriatal injection of PBS (A and B) or 1.5 µmol lysine (Lys) (C and D). Mice were euthanized 48 h after intrastriatal administration. Representative images were obtained from three to four independent experiments (animals) per group. NeuN immunohistochemical staining was visualized with magnification of 100× and 400×. Arrows in panel D indicate neuronal loss. (E) Quantification of the number of immunoreactive cells (viable neurons) was performed with 400× magnification by using the median of five randomly selected fields from three striatal sections per brain. Values are mean ± standard deviation for three to four mice per group. Two-way ANOVA was performed as described in the text. $^{**}P < 0.01$ compared to Lys-injected WT mice (Tukey's test).

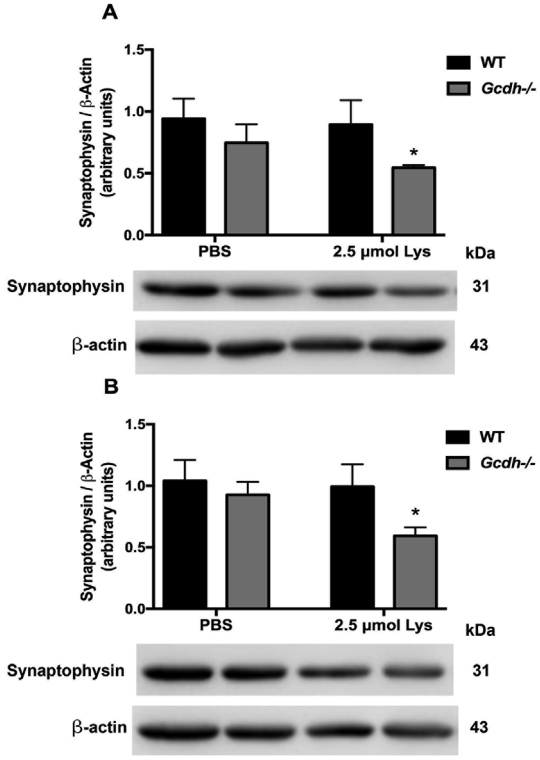

Fig. 4. Immunoblot and densitometric analysis of synaptophysin levels in striatum from developing (30-day-old) wild type (WT) and glutaryl-CoA dehydrogenase deficient (*Gcdh*−/−) mice submitted to an intrastriatal injection of PBS or 2.5 µmol lysine (Lys). Mice were euthanized 24 (A) and 48 (B) hours after intrastriatal administration. Representative immunoblots are shown as mean ± standard deviation for three independent experiments (animals) normalized by β-actin levels. $^{*}P < 0.05$, compared to Lys-injected WT mice (Student's *t*-test for unpaired samples).

diminished in striatum of *Gcdh*−/− mice 24 ($t_{(3)} = 2.371$, $P < 0.05$) and 48 ($t_{(4)} = 3.56$, $P < 0.05$) hours after 2.5 µmol of Lys infusion (Fig. 4), implying neuronal death in this cerebral structure. Interestingly, it can be also seen in this figure that there was no difference in synaptophysin content between the PBS-injected WT and *Gcdh*−/− mice, ruling out a genotype effect in animals not receiving Lys. Taken together, the present data indicate that Lys overload was critical to cause acute neuronal damage in the knockout mice.

3.3. Acute lysine (Lys) brain overload induces reactive astrogliosis in striatum of glutaryl-CoA dehydrogenase deficient (Gcdh−/−) mice

Next, we determined GFAP staining in brain of *Gcdh*−/− and WT mice injected with Lys in order to evaluate reactive astrogliosis. We verified a significant increase of GFAP 48 h after administration of 1.5 µmol Lys in striatum of *Gcdh*−/− mice, as compared to WT (interaction between treatment x genotype: $F_{(1,13)} = 9.272$, $P < 0.01$) (Fig. 5). The statistical analysis (two-way ANOVA) also showed an influence of Lys treatment ($F_{(1,13)} = 9.272$, $P < 0.01$) on GFAP staining. Since GFAP is predominantly expressed by activated astrocytes, our data indicate that Lys causes reactive astrogliosis in striatum of *Gcdh*−/− mice. In contrast, no alterations in GFAP staining were observed in the cerebral cortex of *Gcdh*−/− and WT mice following Lys administration (results not shown).

3.4. Acute lysine (Lys) brain overload increases NF-κB and Nrf2 and decreases HO-1 in striatum of glutaryl-CoA dehydrogenase deficient (Gcdh−/−) mice

In order to investigate the signaling pathways that are possibly involved in the striatum neuronal loss and astrocyte activation in Lys-injected *Gcdh*−/− mice, we measured the protein expression of nuclear NF-κB, cytosolic IkBα and nuclear Nrf2 that regulate pro-inflammatory (Muriach et al., 2014) and antioxidant responses (Gan and Johnson, 2014). We also determined HO-1 enzyme activity, which promotes cytoprotective effects against oxidative stress (Ryter et al., 2006). It was verified an increased expression NF-κB-p65 ($t_{(2)} = 3.156$, $P < 0.05$) and Nrf2 ($t_{(4)} = 2.168$, $P < 0.05$), with no changes of the NF-κB inhibitor IkBα in the striatum of *Gcdh*−/− mice 24 h after Lys (1.5 µmol) injection (Fig. 6). Moreover, Lys (2.5 µmol) provoked a decrease of HO-1 in the striatum of *Gcdh*−/− mice 24 ($t_{(4)} = 3.419$, $P < 0.05$) and 48 ($t_{(4)} = 3.651$, $P < 0.05$) hours after injection, as compared to WT (Fig. 7), although a lower dose (1.5 µmol Lys) caused no alteration of this parameter (results not shown). The figures also show that in general no influence of the genotype was found in the levels of NF-κB and HO-1, although striatum of PBS-injected *Gcdh*−/− mice presented higher Nrf2 content ($t_{(4)} = 2.689$, $P < 0.05$). These findings demonstrate the involvement of signaling pathways involving these proteins in Lys-induced disruption of redox homeostasis that may possibly underlie the striatum vulnerability of *Gcdh*−/− mice to Lys toxicity.

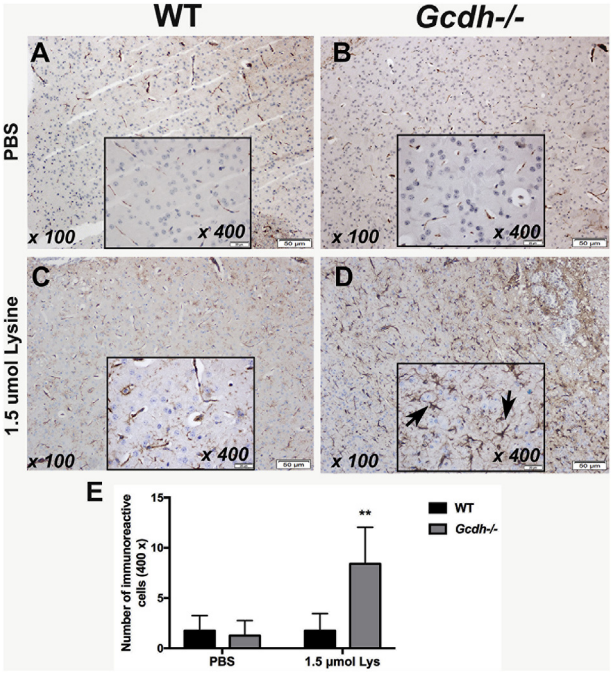

Fig. 5. Light microscopic images showing GFAP immunohistochemistry staining of striatum from developing (30-day-old) wild type (WT) (A and C) and glutaryl-CoA dehydrogenase deficient (*Gcdh*−/−) (B and D) mice submitted to an intrastriatal injection of PBS (A and B) or 1.5 µmol lysine (Lys) (C and D). Mice were euthanized 48 h after intrastriatal administration. Representative images were obtained from three to five independent experiments (animals) per group. GFAP immunohistochemical staining was visualized with magnification of 100× and 400×. Arrows in panel D indicate presence of reactive astrocytes. (E) Quantification of the number of immunoreactive cells (reactive astrocytes) was performed with 400× magnification by using the median of five randomly selected fields from three striatal sections per brain. Values are mean ± standard deviation for three to five mice per group. Two-way ANOVA was performed as described in the text. $^{**}P < 0.01$ compared to Lys-injected WT mice (Tukey's test).

3.5. Acute lysine (Lys) brain overload causes demyelination in cerebral cortex of glutaryl-CoA dehydrogenase deficient (Gcdh−/−) mice

Since progressive cortical leukodystrophy associated with demyelination are common findings in GA I patients, we assessed whether acute Lys administration could alter myelin content in cerebral cortex of *Gcdh*−/− mice by measuring the percentage of MBP stained area. Statistical analysis calculated by two-way ANOVA (Lys treatment: $F_{(2,12)} = 4.04$, $P < 0.05$) revealed that Lys injection at the highest dose (4 µmol) significantly decreased myelination and provoked alterations in myelin structure in the cerebral cortex of the *Gcdh*−/− but not of the WT mice, indicating demyelination of this cerebral structure only in the knockout mice (Fig. 8). In contrast, 2.5 µmol Lys injection did not significantly alter this parameter (Fig. 8).

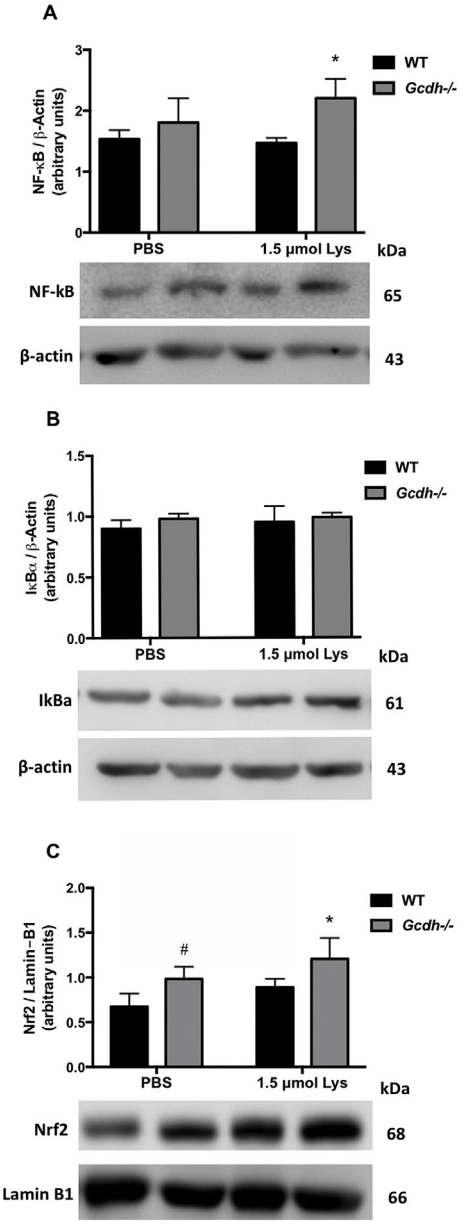

Fig. 6. Immunoblot and densitometric analysis of nuclear NFκB (A), cytosolic IκBα (B) and nuclear Nrf2 (C) protein levels in striatum from developing (30-day-old) wild type (WT) and glutaryl-CoA dehydrogenase deficient (*Gcdh*−/−) mice submitted to an intrastriatal injection of PBS or 1.5 µmol lysine (Lys). Mice were euthanized 24 h after intrastriatal administration. Representative immunoblots are shown as mean ± standard deviation for three independent experiments (animals) normalized by β-actin (NFκB and IκB) or Lamin-B1 (Nrf2) levels. $^{\#}P < 0.05$, compared to PBS-injected WT mice; $^{*}P < 0.05$, compared to Lys-injected WT mice (Student's *t*-test for unpaired samples).

4. Discussion

Patients affected by GA I are very susceptible to develop acute striatum degeneration during episodes of metabolic decompensation, accompanied by acute encephalopathy that significantly aggravates their prognosis. Interestingly, during these crises there is increased brain generation of GA and 3HGA from Lys catabolism (Goodman and Frerman, 2001; Jafari et al., 2011; Olivera-Bravo et al., 2015), implying neurotoxicity of these accumulating metabolites. This is in line with the observations of striatum injury following a high Lys chow for 3 days or longer periods in *Gcdh*−/− mice that was associated with GA and 3HGA accumulation (Olivera-Bravo et al., 2019; Zinnanti et al., 2006, 2007). However, to the best of our knowledge there is no report on whether an acute Lys overload could cause striatum damage in *Gcdh*−/− mice. Thus, in the present study we evaluated whether a single intrastriatal injection of Lys could provoke brain morphological changes and/or neuronal injury, astrocyte activation and alterations of myelination in striatum and cerebral cortex of developing *Gcdh*−/− mice.

We first observed intense vacuolation and edema in the striatum but not in the cerebral cortex of *Gcdh*−/− mice 48 h after 1.5 µmol Lys administration. Similar histopathological alterations were observed in *post mortem* examination of basal ganglia of GA I patients (Goodman et al., 1977) and *Gcdh*−/− mice fed a high Lys chow (Zinnanti et al., 2006). Furthermore, 2.5 µmol Lys administration provoked few

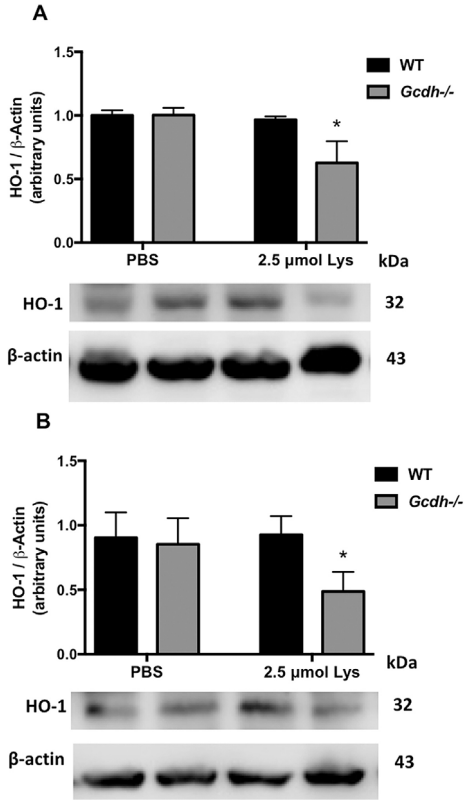

Fig. 7. Immunoblot and densitometric analysis of heme oxygenase-1 (HO-1) levels in striatum from developing (30-day-old) wild type (WT) and glutaryl-CoA dehydrogenase deficient (*Gcdh*−/−) mice submitted to an intrastriatal injection of PBS or 2.5 µmol lysine (Lys). Mice were euthanized 24 (A) or 48 (B) hours after intrastriatal administration. Representative immunoblots are shown as mean ± standard deviation for three independent experiments (animals) normalized by β-actin levels. *$P < 0.05$, compared to Lys-injected WT mice (Student's *t*-test for unpaired samples).

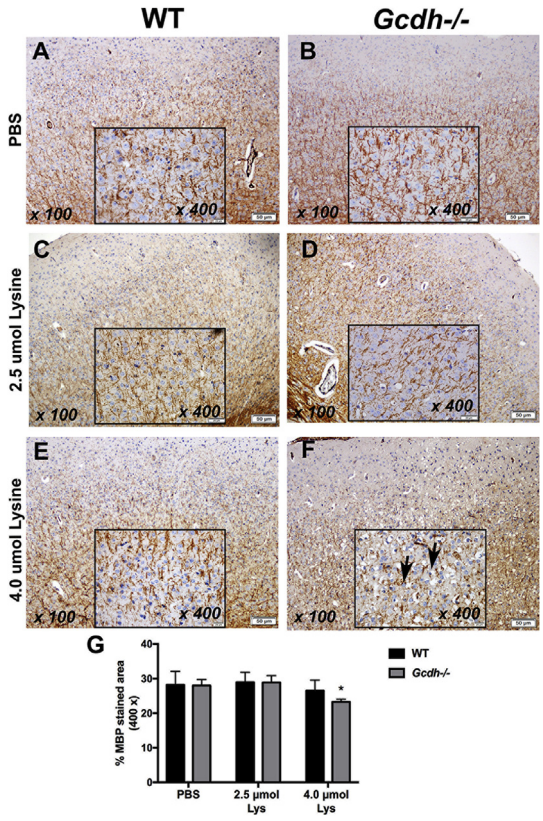

Fig. 8. Light microscopic images showing MBP immunohistochemistry staining of cerebral cortex from developing (30-day-old) wild type (WT) (A, C and E) and glutaryl-CoA dehydrogenase deficient (*Gcdh*−/−) (B, D and F) mice submitted to an intrastriatal injection of PBS (A and B), 2.5 µmol lysine (Lys) (C and D) or 4.0 µmol Lys (E and F). Mice were euthanized 48 h after intrastriatal administration. Representative images were obtained from three independent experiments (animals) per group. MBP immunohistochemical staining was visualized with magnification of 100× and 400×. Arrows in panel F indicate alterations of myelin structure. (G) Quantification of the percentage (%) of MBP stained area was performed with 400× magnification by using the median of five randomly selected fields from three striatal sections per brain. Values are mean ± standard deviation for three mice per group. Two-way ANOVA was performed as described in the text. *$P < 0.05$ compared to PBS-injected *Gcdh*−/− mice (Student's *t*-test for unpaired samples).

vacuoles, whereas 4.0 µmol Lys injection caused marked vacuolation and edema in cerebral cortex of *Gcdh*−/− mice, suggesting diffusion of this amino acid through the cerebral cortex. These cortical histopathological alterations might alternatively be due to corticostriatal connections (Hintiryan et al., 2016), which is in accordance with previous observations showing that the characteristic neuronal abnormalities in striatum and substantia nigra of patients with Huntington's disease and Parkinson's disease, respectively, also appear in multiple cortex areas as the diseases progress (Hintiryan et al., 2016; Nelson and Kreitzer, 2014; Rebec, 2018; Shepherd, 2013).

In contrast, striatum and cerebral cortex of WT mice administered with various doses of Lys showed no histopathological alterations, indicating that by-products (GA and 3HGA), rather than Lys itself, provoked these lesions probably by intoxication. This is supported by previous studies showing that GA induces similar morphological alterations in rat pups submitted to a single intracerebroventricular injection of this organic acid (Olivera-Bravo et al., 2011, 2014; Olivera et al., 2008).

We also found that Lys overload induces marked decreases of NeuN and synaptophysin content, the most abundant presynaptic vesicle protein, only in striatum of *Gcdh*−/− mice 48 h after its injection, reflecting therefore neuronal loss (Janz et al., 1999; Tarsa and Goda, 2002). In this scenario, it is stressed that reduction of synaptophysin has been related to neuronal dysfunction in various neurodegenerative disorders (Rao et al., 2012).

Other novel finding of the present investigation was the increased GFAP content detected in the striatum of Lys-injected *Gcdh*−/− mice, indicating reactive astrogliosis. (Pekny and Pekna, 2014). Noteworthy, astrogliosis is usually triggered to protect the surrounding neurons after an insult, but, when exacerbated, it may cause neuronal damage (De Keyser et al., 2008; McGeer and McGeer, 2008; Nagele et al., 2004). These results corroborate previous works suggesting a role for astrocytes in the neuropathology of GA I (Jafari et al., 2013; Olivera-Bravo and Barbeito, 2015; Olivera-Bravo et al., 2011, 2015; Rodrigues et al., 2017).

The observed vacuolation associated with neuronal death and astrogliosis found in the striatum of the knockout mice were previously seen in *Gcdh*−/− mice fed a high Lys chow (Zinnanti et al., 2006, 2007) and probably secondary to oxidative stress and a pro-inflammatory state, as recently shown in GA I patients (Guerreiro et al., 2018). This assumption is supported by earlier findings from our lab showing that Lys overload provokes a marked increase of 2′,7′-dichlorofluorescin (DCFH) oxidation and malondialdehyde (MDA) levels (reflecting increased ROS generation and lipid peroxidation), besides impairing the antioxidant defenses, as revealed by reduction of GSH levels and altered antioxidant enzyme activities, including increased SOD activity, in striatum and cerebral cortex of Lys-treated *Gcdh*−/− mice (Seminotti et al., 2013). Furthermore, the decreased content of HO-1 accompanied by elevated levels of the transcription factors Nrf2 and NF-kB further corroborate the hypothesis that disruption of redox

homeostasis may underlie some of the histopathological alterations detected in the brain of *Gcdh−/−* mice, since this enzyme has a potent antioxidant role and is associated with the expression of genes involved in the antioxidant response and anti-inflammatory processes (Gan and Johnson, 2014; Johnson et al., 2008; Kensler et al., 2007; Shih et al., 2003; Ryter et al., 2006). This is also in line with other findings demonstrating that GA and 3HGA induce oxidative stress in vitro and in vivo in rat brain (de Oliveira Marques et al., 2003; Latini et al., 2007; Latini et al., 2002; Latini et al., 2005).

Finally, we observed that myelination was severely compromised in the cerebral cortex of the Lys injected *Gcdh−/−* mice, although neuronal loss or astrocyte activation was not found in this cerebral structure, indicating different underlying pathomechanisms for these processes. Of note, a recent work described demyelination in *Gcdh−/−* mice submitted to a long-term Lys overload, and this was linked to increased GRP78/BiP immunoreactivity of oligodendrocytes and neurons, denoting endoplasmic reticulum stress (Olivera-Bravo et al., 2019). Altogether, the present and previous studies may contribute to clarify the cortical atrophy associated with hypomyelination/demyelination observed in brain of GA I patients (Harting et al., 2009, 2015).

In conclusion, we demonstrate for the first time that a single intrastriatal injection of Lys provokes marked damage in the striatum and to a lesser degree in the cerebral cortex of the genetic animal model of GA I (*Gcdh−/−* mice), with no changes in the WT mice. This acute treatment induced vacuolation associated with edema, as well as neuronal loss and reactive astrogliosis, similarly to the acute basal ganglia degeneration observed in the affected patients and *Gcdh−/−* mice during catabolic events with encephalopathy characterized by dramatic increase of the accumulating metabolites (Goodman et al., 1977; Zinnanti et al., 2006). Alteration of signaling pathways associated with redox homeostasis in the striatum, as well as reduced myelination was also observed in the cerebral cortex of *Gcdh−/−* mice submitted to Lys injection and may possibly be involved with the brain abnormalities here shown. It is feasible that these deleterious effects were caused by GA and 3HGA that originate from Lys in brain of the *Gcdh−/−* mice (Seminotti et al., 2012; Zinnanti et al., 2006, 2007).

5. Conclusion

In conclusion, our results support the hypothesis that brain accumulation of GA and 3HGA derived from increased Lys catabolism selectively induces acute striatum degeneration commonly observed in GA I patients following catabolic events. It is conceivable that a single Lys intrastriatal injection in the *Gcdh−/−* mice may serve as a valuable model to study the neuropathology and signaling pathways involved in the brain damage in GA I.

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuint.2019.104467.

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