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Long Lasting High Lysine Diet Aggravates White Matter Injury in Glutaryl-CoA Dehydrogenase Deficient (Gcdh−/−) Mice

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Abstract

Glutaric acidemia type I (GA-I) is a neurometabolic disease caused by deficient activity of glutaryl-CoA dehydrogenase (GCDH) that results in accumulation of metabolites derived from lysine (Lys), hydroxylysine, and tryptophan catabolism. GA-I patients typically develop encephalopatic crises with striatal degeneration and progressive white matter defects. However, late onset patients as well as Gcdh−/− mice only suffer diffuse myelinopathy, suggesting that neuronal death and white matter defects are different pathophysiological events. To test this hypothesis, striatal myelin was studied in Gcdh−/− mice fed from 30 days of age during up to 60 days with a diet containing normal or moderately increased amounts of Lys (2.8%), which ensure sustained elevated levels of GA-I metabolites. Gcdh−/− mice fed with 2.8% Lys diet showed a significant decrease in striatal-myelinated areas and progressive vacuolation of white matter tracts, as compared with animals fed with normal diet. Myelin pathology increased with the time of exposure to high Lys diet and was also detected in 90-day old Gcdh−/− mice fed with normal diet, suggesting that dietary Lys accelerated the undergoing white matter damage. Gcdh−/− mice fed with 2.8% Lys diet also showed increased GRP78/BiP immunoreactivity in oligodendrocytes and neurons, denoting ER stress. However, the striatal and cortical neuronal density was unchanged with respect to normal diet. Thus, myelin damage seen in Gcdh−/− mice fed with 2.8% Lys seems to be mediated by a long-term increased levels of GA-I metabolites having deleterious effects in myelinating oligodendrocytes over neurons.

Keywords Glutaric acidemia type I · Lysine overdose · Myelin alterations · Gcdh−/− mice

Introduction

Glutaric acidemia type I (GA-I, MIM# 231670) is an inherited neurometabolic disease of early infancy caused by mutations in the mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH, MIM# 608801, E.C. 1.3.99.7) that causes severe lack of enzymatic function. Decreased GCDH activity alters the catabolism of L-tryptophan, L-lysine (Lys), and L-hydroxylysine [1–4] causing accumulation of predominantly glutaric and 3-hydroxyglutaric acids in brain and body fluids. Increased concentrations of these organic acids up to millimolar levels are thought to be associated with GA-I clinical features that include acute "encephalopatic crises" followed by chronic motor and neurological sequelae [1, 5]. Loss of striatal neurons, progressive cortical neurodegeneration, and white matter alterations are the characteristic pathological features of GA-I [2, 6]. Interestingly, white matter alterations also occur in the absence of neurological damage as it has been shown in adult onset patients [7–9], suggesting that in GA-I white matter injury and neuronal loss may be independent pathophysiological processes.

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Accordingly, the Gcdh−/− mouse model of the disease, which lacks GCDH activity and accumulates high levels of GA-I metabolites [10, 11], does not suffer spontaneous striatal neurodegeneration or relevant neurological symptoms, but presents a diffuse spongiform myelinopathy similar to that observed in human patients affected by GA-I [10, 11]. As Gcdh−/− mice show elevated levels of GA-I metabolites reproducing the biochemical profiles of the disease [10, 11], we propose that white matter defects might depend on sustained levels of GA-1 metabolites and not strictly on neuron damage.

However, to the best of our knowledge, there are no reports focused on myelin defects over neuronal damage in GA-I. Previous works have shown that white matter defects accompanied different degrees of acute neurological damage when Gcdh−/− mice are metabolically stressed by diets containing high amounts of Lys that raised the levels of glutaric and 3-hydroxyglutaric acids in the brain [6, 12]. These authors reported seizures, paralysis, subarachnoid hemorrhages, and death within 3 to 6 days in the majority of the 4-week old Gcdh−/− mice, as well as white matter lesions and neuronal loss after 6 weeks in 8-week old Gcdh−/− mice, suggesting that myelin defects occur secondarily to neuron death. Nevertheless, these results could not been replicated when using their reported protocol [13, 14] or an alternative model that administers Lys into the drinking water to ensure sustained Lys consumption during 14 days [13]. Thus, there are no confirmatory findings showing that white matter defects depend more on sustained levels of GA-I metabolites rather than on neuron damage.

To test this hypothesis, we have designed an experimental protocol in which moderately high amounts of Lys (2.8%) are administered to weanling Gcdh−/− mice for up to 60 days to ensure sustained high levels of GA-I metabolites, however, at concentrations that did not elicit encephalopatic crises or cause animal death. Therefore, we fed 30-day-old Gcdh−/− and WT mice with a diet containing 2.8% Lys during 3, 30, or 60 days and evaluated striatal myelin damage because the striatum is the most vulnerable brain area to suffer degeneration in patients with GA-I [1, 4, 15, 16] and correspond to the region earlier affected in other murine models of GA-I [17]. Results were compared to Gcdh−/− and WT mice fed with normal Lys content (0.9%).

We found that the moderately high and sustained Lys diet selectively damaged the white matter in Gcdh−/− mice, probably by eliciting endoplasmic reticulum (ER) stress. Interestingly, white matter defects were not associated with significant cellular or neuronal death.

Materials and Methods

Materials

Sudan III, diamino-2-phenylindole (DAPI), paraformaldehyde, sodium phosphate-based saline solution, and all other chemicals of analytical grade were obtained from Sigma (St. Louis, MO). FluoroMyelin and secondary antibodies were from Molecular Probes (Whaltam, MA). Primary antibodies were purchased to Molecular Probes, abcam (Cambridge, MA), Millipore (Billerica, MA), and Invitrogen (Whaltam, MA).

Ethical Statement

This study was performed in accordance with the Principles of Laboratory Animal Care, National Institute of Health of United States of America, NIH, publication no. 85–23 (2011 revision) and approved by the Ethical Committee for the Care and Use of Laboratory Animals of the Hospital de Clínicas of Porto Alegre, and by the National Committee for Laboratory Animal Care (CNEA) from Uruguay. All efforts were made to minimize suffering, discomfort, and stress to the animals. The number of animals employed in this work was necessary to produce reliable scientific data.

Animals

Wild type (WT, Gcdh+/+) and Gcdh−/− littermates, both of C129SvEv background, were generated from heterozygotes and maintained at the Unidade Experimental Animal of the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) under a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in an air-conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20% (*w*/*w*) protein commercial chow containing 0.9% Lys (SUPRA, Porto Alegre, RS, Brazil).

Animals'diet

Animals were kept with the mother until 30 days of life, where we proceeded to weaning and genotyping to identify WT and Gcdh−/− mice [14]. At this time, half of each background started to be fed with normal diet (ND) containing 20% protein containing 0.9% Lys, whereas the other half received a chow with 20% protein with 2.8% Lys as previously reported [14, 18].

Animal Processing and Tissue Preparation

After 3, 30, and 60 days of feeding animals with ND (0.9% Lys) or 2.8% Lys, WT and Gcdh−/− animals were anesthetized with ketamine/xilazine (90:10 mg/kg) and submitted to intracardiac perfusion with a volume of phosphate buffered saline solution (PBS) containing commercial anticoagulants and then with 4% paraformaldehyde (PFA) in 10 mM, pH 7.4 PBS. After fixation, brains were quickly removed, post-fixed with 4% PFA overnight at 4 °C, and then maintained at 4 °C in PBS until sectioning. A 1000S Leica (Buffalo Grove, IL) vibratome was used to obtain 30 μm thick

consecutive coronal series containing the striatal region. Sections containing the striatum were stored either free-floating at 4 °C to perform myelin histochemistry or mounted to do immunostaining against myelin basic protein (MBP), APC product gene (CC-1 protein), NeuN or GRP78/BiP protein.

Myelin Histochemistry

Coronal brain sections of WT and Gcdh−/− mice fed with either ND or 2.8% Lys were permeabilized with 0.3% X-100 Triton during 20 min, immersed in 70% ethanol for 2 min, and then incubated with 2% Sudan III for 30 min at room temperature [17]. The reaction was stopped with 70% ethanol and further with distilled water. Stained sections were then adhered to glass slides, left drying overnight at room temperature, and mounted in glycerol.

Brain sections containing the striatum at equivalent levels from all experimental conditions were placed on clean coverslips, delimited with Pap Pen and directly stained with 1:300 dilution of the stock solution of FluoroMyelin Green during 20 min at room temperature. Sections were then rinsed, mounted in glycerol, and imaged in a confocal FV300 Olympus microscope using a 488-nm laser. Size of striatal areas positive to Sudan III or FluoroMyelin was measured by using the Image J or the FIJI (NIH, Bethesda, MD) software in WT and Gcdh−/− mice fed with ND or 2.8% Lys along the whole dieting period. A ratio between myelinated areas in animals of the same background was obtained by dividing the values obtained in animals fed with 2.8% Lys per those obtained in age-matched animals fed with ND. Around five to seven animals of each background were analyzed per each diet condition and age.

Immunohistochemistry

For each animal and staining procedure, 5–7 equivalent sections covering the striatum were employed. Anatomical landmarks (aspect, size and position of the anterior commissures, corpus callosum, lateral ventricles, striatum, and nucleus accumbens; [19]) were used to ensure that parameters were analyzed at similar levels within and between groups. Commercial antibodies chosen were specific and allowed recognizing MBP, APC (CC-1) that labels mature oligodendrocytes (OLs), and the pan-neuronal marker NeuN, all previously employed successfully [17, 20]. A polyclonal antibody was employed to evaluate GRP78/BiP signal as an indicator of ER stress [21]. DAPI that labels all cells was employed to determine cellular density in the striatum. All the assays were performed on free-floating sections that were washed, permeabilized with 0.1–0.3% Triton X-100 in 10 mM PBS during 20 min, then blocked during 30 min with PBS containing 0.3% Triton X-100 plus 5% bovine serum albumin. Afterward, at 4 °C overnight incubation with a single antibody or a pair of antibodies was performed in a wet chamber: anti-MBP (1:500, abcam), anti-APC product gene (1:300, abcam), anti-NeuN (1:500, abcam), or anti-GRP78/BiP (1:500, abcam). Then sections were washed and incubated for 90 min with 1:800 dilutions of 1 mg/mL secondary antibodies conjugated to Alexa fluorescent probes (Molecular Probes). After three washes, sections were mounted in glycerol containing 1 μg/mL DAPI. As negative controls, the primary or secondary antibodies were omitted.

Image Acquisition

Light microscopy images were acquired by using an inverted IX61 Olympus microscope attached to a DSP71 monochromatic camera and its attached software. Immunofluorescence 2048 × 2048 images were obtained in a FV300 Olympus confocal microscope provided with 405, 488, 546, and 633 nm lasers. Microphotographs of representative areas were taken with all acquisition parameters identical for animals fed with ND or 2.8% Lys (PMT below to 650 V, 0% gain, 0 V offset, negative controls, and maximum pixel size). Once pictures were obtained, cells positive to DAPI and each specific marker were counted in 5–7 fields of each striatal section. Fifteen to 21 slices from five to seven animals per condition were analyzed at equivalent striatal levels.

Data Analysis

Statistical analysis was performed with Origin 8.5 or free downloaded GraphPad Prism software programs. Cell numbers or densities as well as myelinated areas comparing two or many groups were analyzed by using unpaired Student's *t* test or one-way ANOVA followed by Tukey or Tukey–Kramer post hoc analysis if necessary. All results are presented as mean ± SD; $p \leq 0.05$ was considered significant.

Results

Myelin Alterations in Gcdh−/− Mice Fed with 2.8% Lys Diet Immediately after weaning, 30-day-old WT and Gcdh−/− mice started to be fed with ND containing 0.9 or 2.8% Lys during 3, 30 or 60 days, respectively. Under ND, striatal myelin of WT and Gcdh−/− animals did not show significant differences both in the microscopic morphology or in total areas up to 60 days old (Fig. 1a). At 90 days old, Gcdh−/− mice showed ~ 15 and 20% decrease in myelinated areas related to 30-day-old Gcdh−/− and to 90-day-old WT mice, respectively (Fig. 1b), suggesting a delayed demyelinating process.

Diet containing 2.8% Lys did not cause significant signs of myelin damage along the whole treatment in WT animals (left

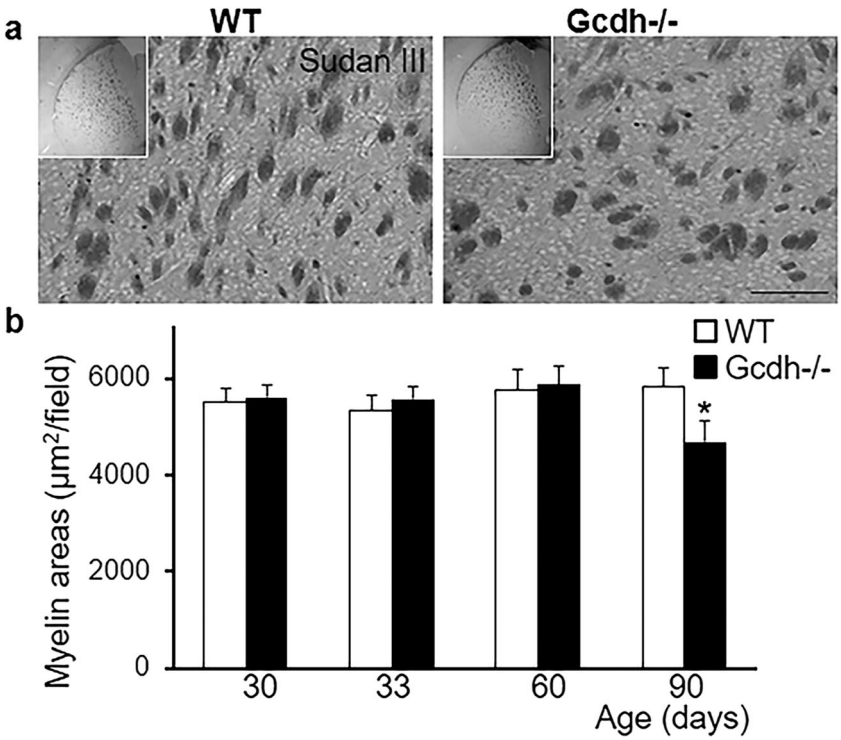

Fig. 1 Striatal myelinated areas in WT and Gcdh−/− mice along age. a Sudan III histochemistry showing no significant differences in the general appearance of striatal myelin in 30-day-old animals immediately after weaning. Insets show the general appearance of the whole striatum in WT and Gcdh−/− mice. Calibration bar = 100 µm. b Quantitation of total striatal myelinated areas after feeding WT and Gcdh−/− mice with ND, indicating a significant decrease in 90-day-old Gcdh−/− animals. As expected, with age, WT mice showed a tendency to increase myelinated areas. Data are the media ± SD of results obtained in three independent experiments with 3–5 animals per condition. (*) means statistical significant difference at $p < 0.05$

images Fig. 2a, Fig. 2 b, c). In contrast, Gcdh−/− mice fed with 2.8% Lys displayed a progressive decrease in myelinated areas by 30 and 55% at 30 and 60 days of treatment, respectively (Fig. 2a, right images, Fig. 2b, c), suggesting that Lys intake aggravated the loss of myelin in Gcdh−/− mice.

Defective striatal myelinated bundles in Gcdh−/− mice fed with 2.8% Lys typically displayed pathological vacuolation assessed by Sudan III, FluoroMyelin, and MBP immunoreactivity (Figs. 2a and Fig. 3). Vacuoles appeared as big "holes" inside the striatal axonal packages (Fig. 4a, white arrows) whose number increased with duration of 2.8% Lys feeding and widespread progressively throughout many axonal packages (Fig. 4b, c).

Decreased Density of APC (CC-1) Positive OLs in Gcdh−/− Mice Fed with 2.8% Lys Diet Although the number of striatal APC (CC-1) positive OLs was low, Gcdh−/− mice fed with 2.8% Lys evidenced a 35 and 60% decreased density of mature positive OLs after 30 and 60 days of treatment as compared to WT animals fed with the same chow (Fig. 5 a, c). APC (CC-1) positive OLs in Gcdh−/− mice fed with 2.8% Lys also was 25 and 40% minor than age matched Gcdh−/− mice fed with ND (Fig. 5a, c). APC (CC-1) OLs appeared swollen (~ 15% increases in body diameters; Fig. 5a, white arrow) which may suggest increased ER stress [17, 22]. Accordingly, increased signal of the ER stress marker GRP78/BiP (Fig. 5b, d) was found in Gcdh−/− mice fed with 2.8% Lys during 60 days. Quantitative analysis revealed a general increase in GRP78/BiP signal in several striatal cell types of Gcdh−/− mice fed with 2.8% Lys during 60 days (Fig. 5d). However, no significant decrease in total striatal nuclear density (99 ± 10% in 2.8% Lys diet compared to ND was found in 90-day-old mice fed with 2.8% Lys during 60 days), suggesting that Lys intake may elicit ER stress but did not cause significant cell death.

Absence of Significant Neuronal Death in Gddh−/− Mice Submitted to 2.8% Lys

Since ER stress may provoke neuronal death [23], we analyzed NeuN staining in the striatum and cortex to assess the survival of local neurons and of cortical neurons projecting to the striatum. No significant differences in the density of NeuN signal was found in any experimental conditions discarding that even the Gcdh−/− mice fed with 2.8% Lys during 60 days suffer significant neuronal death (Figs. 5e, Supplementary 1). Moreover, no evident changes were observed in NeuN labeling neither in the striatum nor in frontal cortex (Figure Supplementary 1), thus discarding significant neuronal death.

Discussion

In the present study, we found that a long lasting and sustained increased Lys content in a daily diet triggers a significant white matter injury in the striatum of Gcdh−/− mice without affecting neuronal survival or inducing systemic or

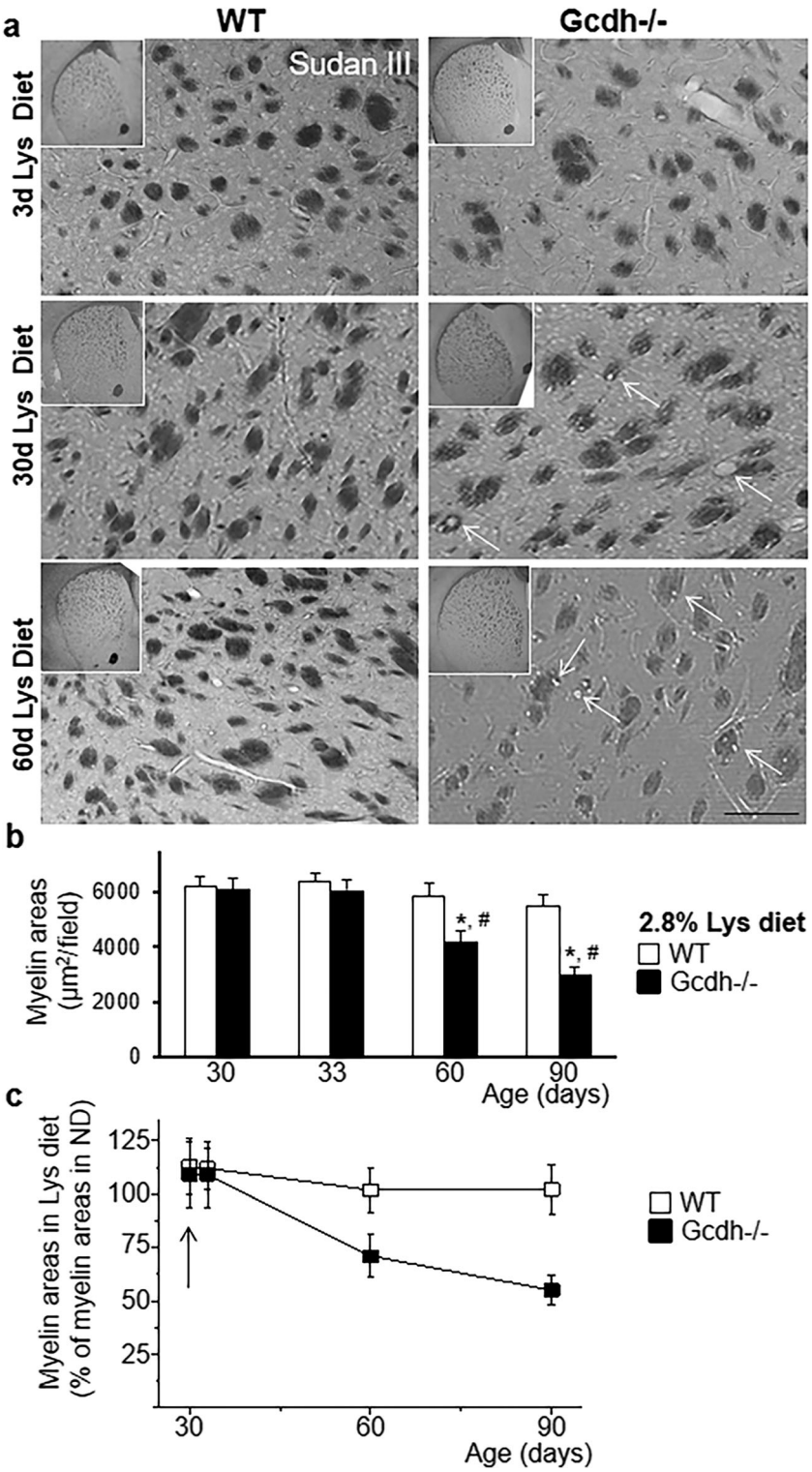

Fig. 2 Sudan III signal in the striatum of WT and Gcdh−/− mice upon 2.8% Lys treatment. a Light microscopy images of Sudan III histochemistry of transverse brain sections evidencing that at 30 and 60 days of Lys diet, myelin in Gcdh−/− mice show significant vacuolation (white arrows). Insets show representative pictures of the whole striatum of each experimental condition evidencing that the typical striatal architecture was preserved in spite of myelin vacuolation in Gcdh−/− mice upon Lys diet. Magnification is × 40 for the full pictures and × 4 for insets, respectively. Calibration bars = 100 μm. b Total area of striatal myelin in WT (white columns) and Gcdh−/− mice (black columns) fed with 2.8% Lys chow. Note that Lys diet accelerated loss of myelinated areas in Gcdh−/− mice. c Ratio of myelin loss in WT and Gcdh−/− mice due to dietary Lys. Data are the media ± SD of results obtained in three independent experiments with 3–5 animals per condition. (*) means statistical difference between Gcdh−/− and WT animals of the same age fed with 2.8% Lys and (#) between Gcdh−/− mice fed with 2.8% Lys and those fed with normal chow

neurological alterations. The abnormalities observed in Gcdh −/− animals fed with high Lys exceeded in much those shown in age-matched Gcdh−/− mice fed with a normal diet. As high Lys diet contains approximately threefold levels of Lys with the same amount of protein, the injuring myelin effects found in Gcdh−/− mice can be directly attributed to Lys or more likely, to the accumulation of the Lys- by-products, glutaric acid, or/and 3-hydroxyglutaric acid.

In our experimental model, 2.8% Lys intake was sufficient to accelerate myelin damage in Gcdh−/− mice without causing significant cell death. Lys levels in dietary chow were lower than those used by Zinnanti et al. [6], which caused a three-

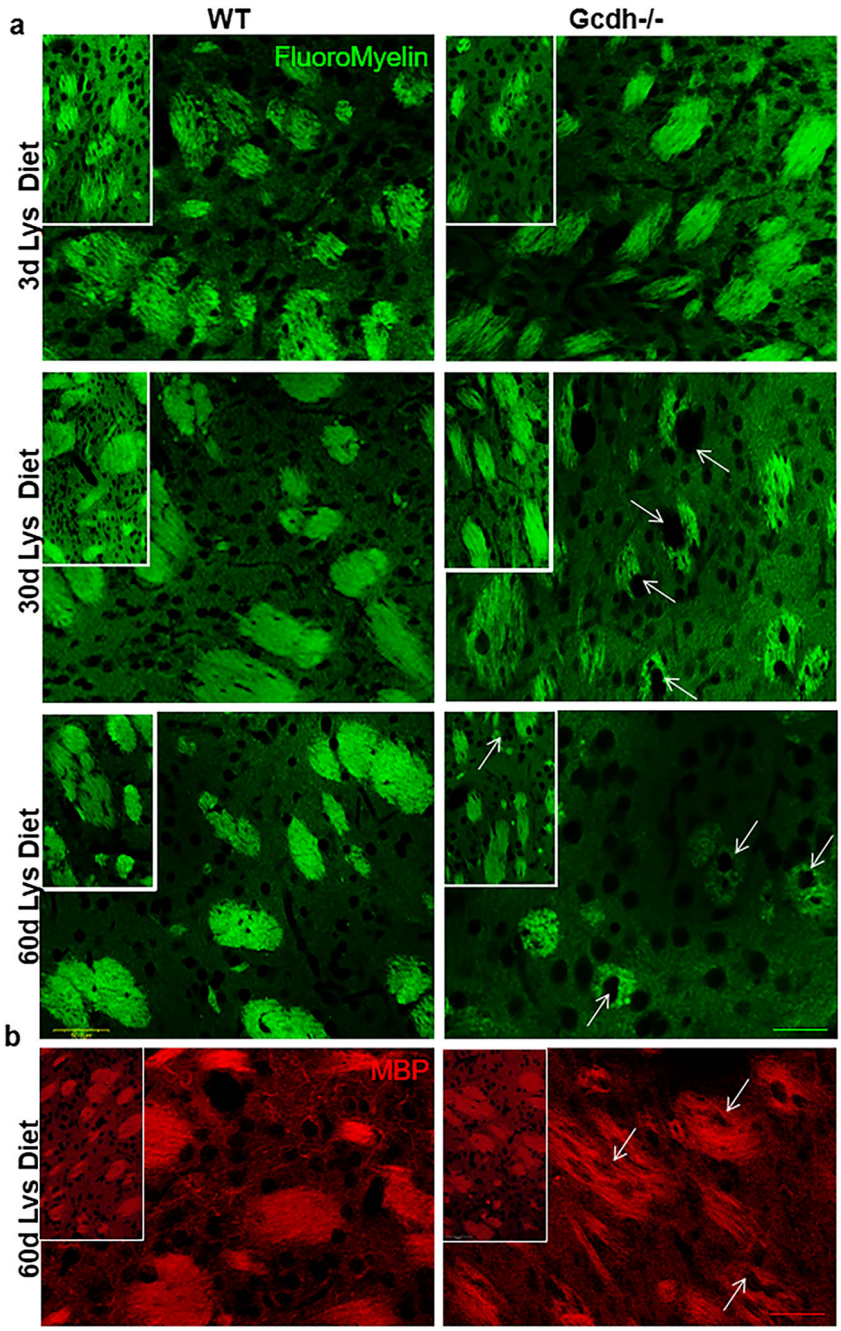

Fig. 3 Alterations in striatal myelin of Gcdh−/− but not in WT mice upon sustained 2.8% Lys diet. a FluoroMyelin Green histochemistry evidencing the presence of abundant vacuoles that appear as black holes into the myelinated striatal bundles (white arrows) upon feeding Gcdh−/− animals with 2.8% Lys. Inset shows FluoroMyelin signal in animals of the same age submitted to ND. Under this treatment, only 90-day-old Gcdh−/− mice showed spare vacuolation (white arrow into the inset). b MBP immunoreactivity in 90-day-old WT and Gcdh−/− mice fed with 2.8% Lys. Note that MBP signal appears disrupted in areas of vacuolated myelin in Gcdh−/− mice and looked normal in WT animals. Calibration bar = 50 μm

and twofold increase in brain Lys- and glutaric acid concentrations, respectively, 48 h after diet start and intermediate between the 1.7 and 4.7% used by Sauer et al. [13]. The age of animals at the start of 2.8% Lys diet (30 days old) was chosen due to previous reports showing severe toxic effects of Lys overload during early post-natal development to Gcdh −/− mice [14, 24, 25] together with the time-course of striatal myelination in rodents [26]. Duration of the treatment covered the experimental periods reported on acute high Lys intake [6, 12], medium-term treatment (2 weeks, [13]), and chronic Lys feeding (45 days, [27]), thus likely reproducing pathological conditions observed in adult onset GA-I.

The most important signs of striatal myelin injury in Gcdh−/− mice fed with 2.8% Lys include decreased myelinated areas associated with abnormal vacuolation of axonal bundles, resembling the white changes with myelin splitting observed in the neuropathological findings of GA-1 patients [28, 29]. In this sense, vacuolation is highly suggestive of direct damage to myelin sheath as previously shown in some genetic diseases such as Canavan's disease, which is also related to metabolic insult [30]. While myelin histopathological alterations have been previously reported in older Gcdh−/− mice fed with normal diet [10, 11], the long-lasting Lys intake seems to accelerate myelin damage.

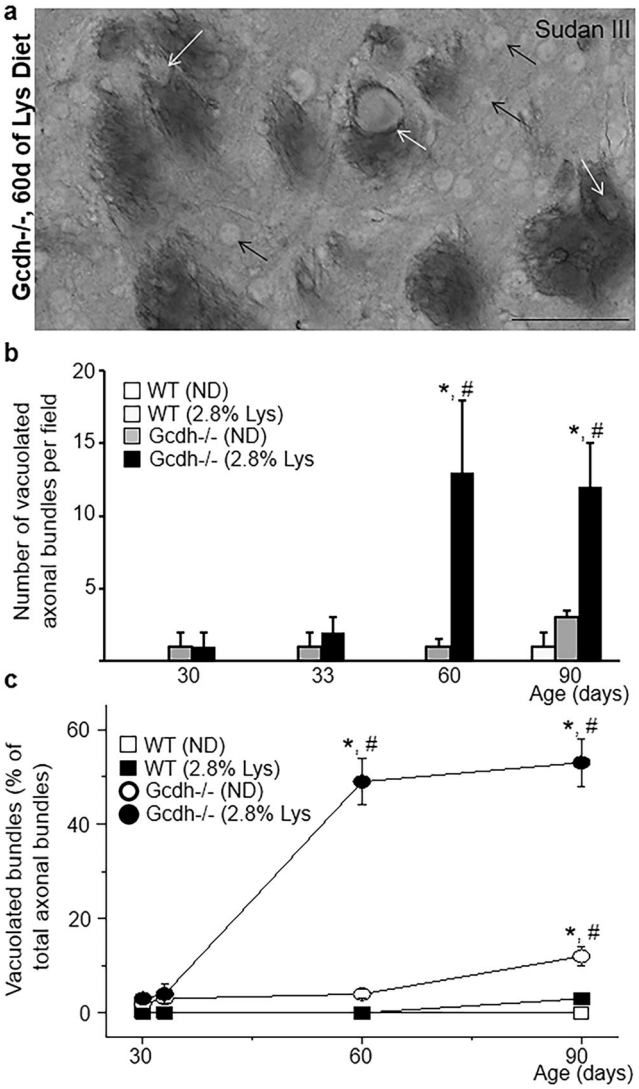

Fig. 4 Assessment of vacuolation in striatal myelin of animals fed with ND or 2.8% Lys diet. a A representative image of many vacuoles (white arrows) found in the striatal myelinated axonal bundles of 60-day-old Gcdh−/− mice fed with 2.8% Lys during 30 days. Note that at light microscopy, vacuoles appear as pale holes that are bigger than cells that are negatively stained with Sudan III (black arrows). Calibration bar = 50 μm. b Number of vacuoles in WT and Gcdh−/− mice submitted to ND or 2.8% Lys diet showing a dramatic increase in Gcdh−/− mice when compared to age-matched WT animals fed with the same chow (*) or to Gcdh−/− mice fed with ND (or to Gcdh−/− mice fed with ND (#). c Percentage of vacuolated axonal bundles related to total bundles in WT and Gcdh−/− mice along the whole period of dieting with ND or 2.8% Lys. Black arrow indicates the beginning of the diet. Note the significant increase upon 30 and 60 days of feeding Gcdh−/− mice with 2.8% Lys. Data are the media ± SD of results from three independent experiments with 3–5 animals per condition. (*) means statistical difference between Gcdh−/− and WT animals of the same age fed with 2.8% Lys whereas (#) indicates the statistical difference between Gcdh−/− mice fed with 2.8% Lys and those fed with ND

Interestingly, Harting et al. [31] correlated long-lasting elevation of GA and 3-OHGA to intramyelinic edema leading to myelin splitting in late onset GA-1 patients. These authors proposed that sustained levels of GA-1 metabolites promote a reduced flux through the citric acid cycle [32], decreasing the uptake of anaplerotic substrates [33], and finally leading to low ATP generation that impedes normal Na+/K + -ATPase functioning. Decrease of this activity deregulate the K+ siphoning system with consequent accumulation of paranodal K+ leading to myelin edema.

High Lys diet also caused a significant decrease in MBP immunoreactivity from striatal axonal bundles in 90-day old Gchd−/− mice, further confirming myelin vulnerability to sustained accumulation of GA-I metabolites produced by continuous feeding with 2.8% Lys diet. MBPs are integral myelin components that contribute to myelin stability and integrity [34, 35], playing a role in OL differentiation [36], cytoskeleton assembly, mediation of signaling pathways, and signal transduction in OLs and myelin [34]. They also contribute to the maintenance of calcium and cell homeostasis [35], thus being a sensitive marker of OL function and myelination status.

A minor density of APC (CC-1)-positive mature OLs was also relevant in Gcdh−/− mice fed with 2.8% Lys, suggesting that OLs are vulnerable to GA-I metabolites formed from defective Lys metabolism in Gcdh−/− mice. Such a loss of APC (CC-1) positive OLs could account, at least partially, for the decreased myelinated areas, since APC (CC-1) is a protein that regulates the adhesive properties of OLs [37] and myelin formation. APC also seems to participate in the demyelination process [38] and altered communication between axons. Moreover, the few OLs that preserve the positive signal to APC (CC-1) displayed significant body swelling and expressed increased levels of GRP78/BiP, suggesting that OLs were undergoing cytopathology and ER stress as described previously [17, 30]. Of note, GRP78/BiP protein is a marker of ER stress, mediating the unfolded protein response (UPR) induced by unfolded/misfolded proteins while dissociating from ER stress sensors [22, 39]. Because myelination involves a high demand on the protein synthesis machinery [26], it may elicit a deleterious UPR response further increasing OL vulnerability and myelin defects. In this regard, ER stress is also involved in other myelopathies related to perturbation of the OL protein secretory pathway such as Vanishing White Matter disease, Pelizaeus-Merzbacher disease, and multiple sclerosis [22, 30, 40]. Therefore, we propose ER stress as an underlying mechanism of myelin defects found in Gcdh−/− animals fed with 2.8% Lys.

In spite of myelin and OL damage, high-Lys diet caused no apparent change in striatal and cortical neuronal density in Gcdh−/− mice as denoted by the pan-neuronal marker NeuN. However, some degree of ER stress in striatal neurons surrounding the axonal bundles that did not compromise neuronal survival at the experimental times employed was evidenced by increased GRP78/BiP signals. Absence of significant neuronal death is in accordance with previous reports

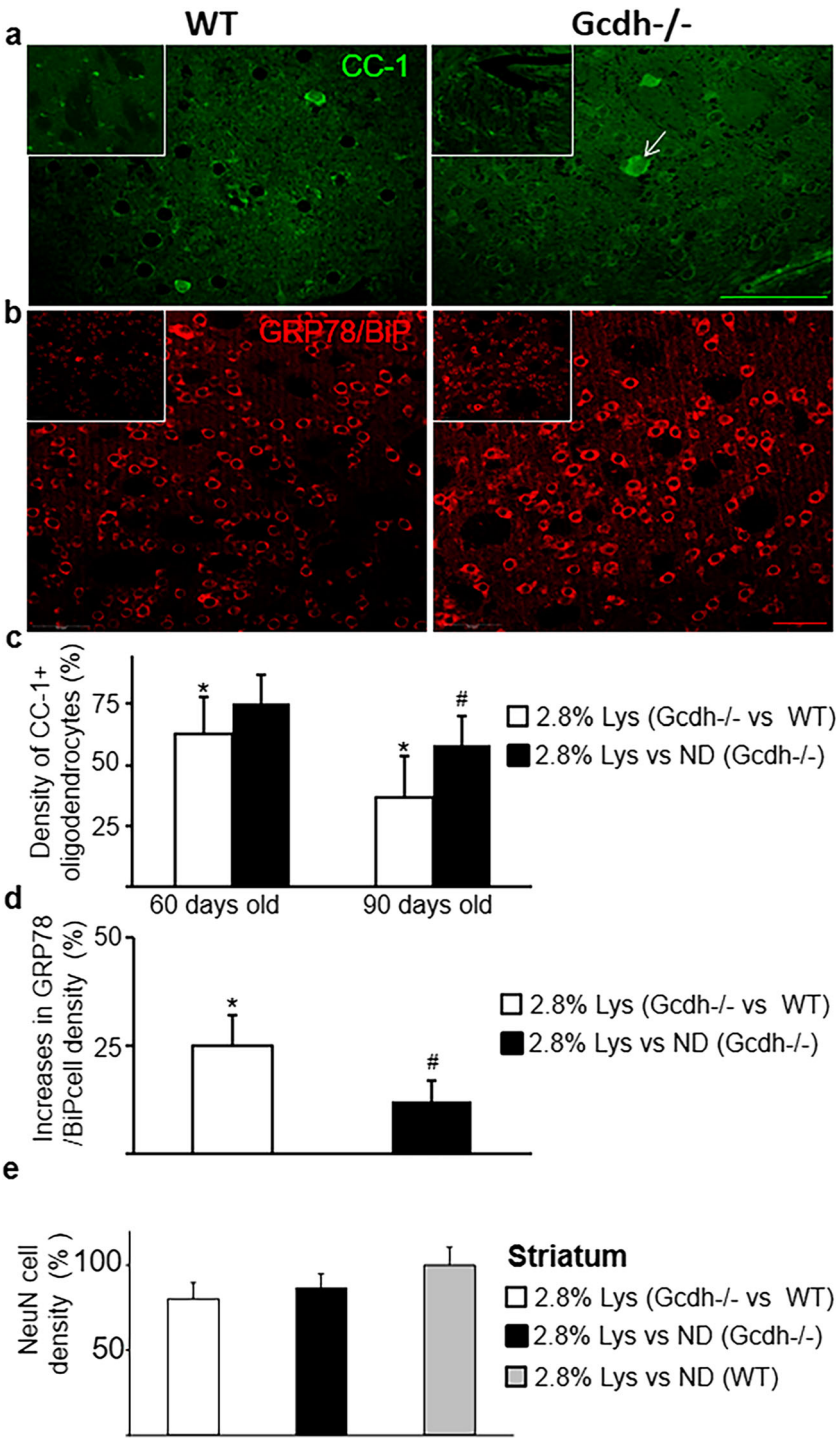

Fig. 5 Effects of Lys diet in striatal OLs and neurons from WT and Gcdh−/− mice. a Decreased population of APC (CC-1) positive cells in Gcdh−/− vs WT mice both fed with 2.8% Lys (white column) or to Gcdh−/− mice fed with ND. b Striatal GRP78/BiP signal showing more positive cells in Gcdh−/− mice fed with 2.8% Lys. Calibration bars = 30 (a) and 50 (b) μm, respectively. c Decreased density of CC-1 positive OLs upon 30 and 60 days of feeding Gch−/− mice with 2.8% Lys. d Increases in GRP78/BiP density of positive cells determined when comparing 90-day old Gcdh−/− mice fed with 2.8% Lys (white columns) or with different diets (black columns). e Density of striatal neurons positive to NeuN discarding significant death at the end of feeding Gcdh−/− mice with 2.8% Lys. Data are the media ± SD of results from three independent experiments with 3–5 animals per condition. * and # mean significant difference at $p < 0.05$ between Gcdh−/− and WT animals of the same age fed with 2.8% Lys and between Gcdh −/− mice fed with 2.8% Lys vs those fed with ND, respectively

showing low neuronal vulnerability in Gcdh−/− mice [10, 11]. Although doses are not similar, it also agrees with the results showed by Sauer et al. [13] in which variable Lys doses did not affect neuronal population in Gcdh−/− animals. Therefore, the present data show evidence that long-term high Lys intake in Gcdh−/− animals results in a damage restricted to OLs and white matter with a preserved neuronal survival. These pathologic features are comparable to those described in GA-1 late-onset patients in which white matter is selectively affected but neurological symptoms are negligible [41, 42].

Finally, the underlying mechanisms of selective OL injury over neurons have not yet been elucidated in humans and in Gcdh−/− mice. It can be produced due to the higher accumulation of GA-1 metabolites in white mater areas of late onset GA-1 patients [31] that may cause a selective damage through one or more of the pathological mechanisms already proposed

in GA-1 [1, 2, 14, 27]. In fact, the combination of a complex differentiation program, very high metabolism, high intracellular iron, low glutathione concentrations together with functional NMDA and P2X7 ATP receptors, makes OLs particularly vulnerable to multiple pathomechanisms like ER stress, oxidative damage, excitotoxic injury, or mitochondrial dysfunction [22, 26, 30, 43]. Moreover, oxidative damage can be amplified not only by the iron Fenton reaction but also by the sphingomyelinase/ceramide pathway that is very important because ceramide is a major lipid component of myelin [43].

In summary, although we cannot at this point clearly determine the major mechanism (s) leading to the alterations observed in this work, we propose that our present model of moderately high Lys intake may be important to delineate the mechanisms underlying the selective damage of glial cells over neurons in Gcdh−/− mice and the progressive white matter defects in GA-I.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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