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

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ABSTRACT

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



## 1. Introduction

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

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

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

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

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

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

## 2. Materials and methods

### 2.1. Chemicals

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

### 2.2. Animals

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

### 2.3. Ethical statement

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

### 2.4. Tissue preparation

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

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

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

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

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

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

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

### 2.6. Determination of mitochondrial respiratory parameters by oxygen consumption

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

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

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

### 2.8. Spectrophotometric analysis of creatine kinase (CK) activity

CK activity was measured in total homogenates according to Hughes [46] with slight modifications. Briefly, the reaction mixture consisted of 50 mM Tris buffer, pH 7.5, containing 7.0 mM phosphocreatine, 7.5 mM $MgSO_4$, and 0.5–1.0 μg protein in a final volume of 0.1 mL. The reaction was then started by addition of 4.0 mM ADP and stopped after 10 min by addition of 0.02 mL of 50 mM p-hydroxy-mercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes [46]. The color was developed by the addition of 0.1 mL 20% α-naphtol and 0.1 mL 20% diacetyl in a final volume of 1.0 mL and read after 20 min at $\lambda =$ 540 nm. Results were calculated as μmol of creatine. $min^{-1}$ mg $protein^{-1}$.

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

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

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

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

### 2.11. Protein determination

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

### 2.12. Statistical analysis

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

## 3. Results

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

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

**Table 1**
PCR primers design.

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

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

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

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

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

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

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

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

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

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

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

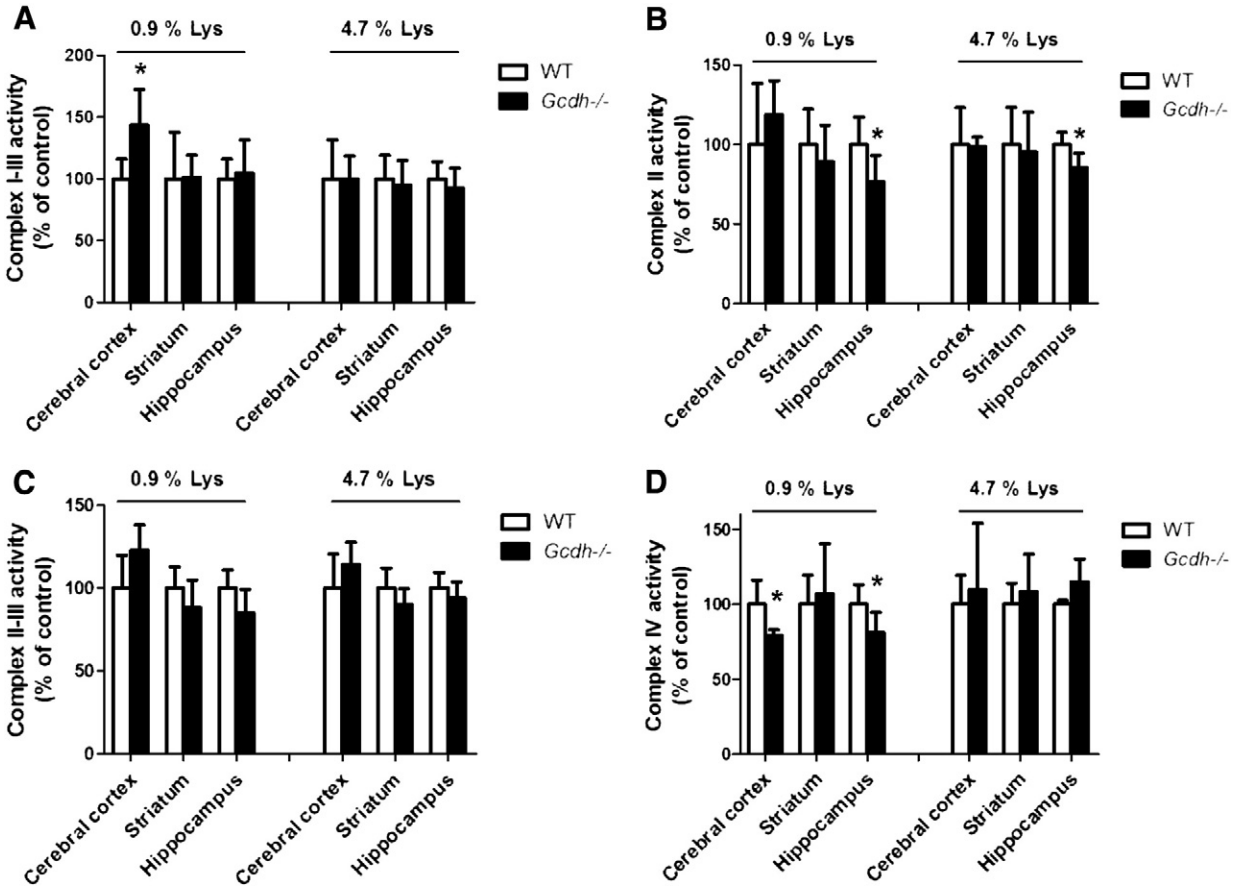


**Fig. 1.** Respiratory chain complexes I–IV activities in rat cerebral cortex, striatum and hippocampus from glutaryl-CoA dehydrogenase deficient knockout mice (*Gcdh* −/−) under a baseline (0.9% Lys) or a high Lys (4.7%) diet. The activities of complexes I-III (A) were calculated as nmol cytochrome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

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

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

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

## 4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

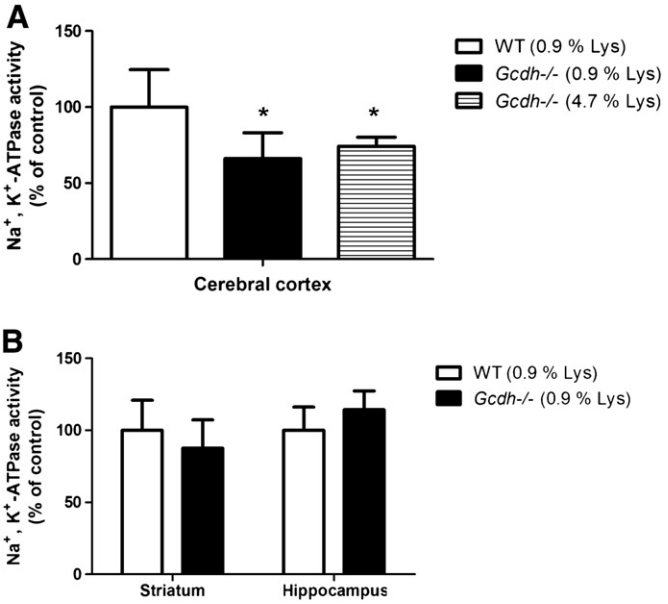


**Fig. 2.** $Na^+$, $K^+$-ATPase activity in rat cerebral cortex (A), striatum and hippocampus (B) from glutaryl-CoA dehydrogenase deficient knockout mice (*Gcdh* −/−). Animals received a baseline (0.9% Lys) or a high Lys (4.7%) diet. Values are mean ± standard deviation of four to five independent experiments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

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

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

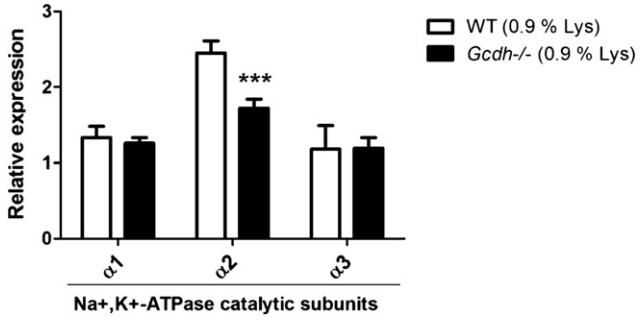


**Fig. 3.** Relative gene expression profile of the Na+,K+ −ATPase catalytic subunits in cerebral cortex from glutaryl-CoA dehydrogenase deficient knockout mice *(Gcdh −/−)* under baseline diet (0.9% Lys). RT-qPCR analysis was used for these experiments. Values are mean ± standard deviation for four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

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

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

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

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

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

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

## 5. Conclusions

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

## Conflicts of interest statement

There are no conflicts of interest between the authors.

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