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Multifactorial modulation of susceptibility to L-lysine in an animal model of glutaric aciduria type I

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ABSTRACT

Glutaric aciduria type I is an inherited defect in L-lysine, L-hydroxylysine and L-tryptophan degradation caused by deficiency of glutaryl-CoA dehydrogenase (GCDH). The majority of untreated patients presents with accumulation of neurotoxic metabolites – glutaric acid (GA) and 3-hydroxyglutaric acid (3-OHGA) – and striatal injury. Gcdh$^{-/-}$ mice display elevated levels of GA and 3-OH-GA but do not spontaneously develop striatal lesions. L-lysine-enriched diets (appr. 235 mg/d) were suggested to induce a neurological phenotype similar to affected patients. In our hands 93% of mice stressed according to the published protocol remained asymptomatic. To understand the underlying mechanism, we modified their genetic background (F1 C57BL6/Jx129/SvCrl) and increased the daily oral L-lysine supply (235–433 mg). We identified three modulating factors, (1) gender, (2) genetic background, and (3) amount of L-lysine. Male mice displayed higher vulnerability and inbreeding for more than two generations as well as elevating L-lysine supply increased the diet-induced mortality rate (up to 89%). Onset of first symptoms leads to strongly reduced intake of food and, thus, L-lysine suggesting a threshold for toxic metabolite production to induce neurological disease. GA and 3-OH-GA tissue concentrations did not correlate with dietary L-lysine supply but differed between symptomatic and asymptomatic mice. Cerebral activities of glyceraldehyde 3-phosphate dehydrogenase, 2-oxoglutarate dehydrogenase complex, and aconitase were decreased. Symptomatic mice did not develop striatal lesions or intracerebral hemorrhages. We found severe spongiosis in the hippocampus of Gcdh$^{-/-}$ mice which was independent of dietary L-lysine supply. In conclusion, the L-lysine-induced pathology in Gcdh$^{-/-}$ mice depends on genetic and dietary parameters.

1. Introduction

The mitochondrial homotetrameric flavoprotein glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) is required for oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA in the final degradative pathways of the amino acids L-lysine, L-hydroxylysine, and L-tryptophan [1–3]. Quantitatively, L-lysine is the major precursor of glutaryl-CoA. Autosomal recessive GCDH deficiency due to two disease-causing mutations in the *GCDH* gene (gene map locus: 19p13.2) causes glutaric aciduria type I (GA-I), a cerebral organic aciduria biochemically characterized by accumulation of the dicarboxylic metabolites glutaryl-CoA, glutaric acid (GA), 3-hydroxyglutaric acid (3-OH-GA), and glutarylcarnitine (C5DC) [4,5]. Two biochemical groups – so-called high and low excreters – have been arbitrarily delineated based on highly variable GA excretion in patients with or without residual enzyme activity [6,4,7]. The overall estimated incidence of GA-I is about 1 in 100,000 newborns [8], but might be as high as 1 in 200 newborns in high-risk populations [9–13].

Patients are usually asymptomatic at birth or show minor symptoms such as axial muscular hypotonia or asymmetric posturing but if untreated are at high risk to develop irreversible striatal injury between age 3 to 36 months [14,15] and, subsequently, a complex movement disorder with predominant secondary dystonia superimposing on axial hypotonia [16–18,12]. The onset of striatal injury often manifests acutely during an acute encephalopathic crises which is precipitated by catabolism due to infectious diseases, however, may also occur insidiously without such crises [19,20]. There is no known genotype–phenotype correlation [21]. The natural history may be highly variable, even in siblings and in patients with the same *GCDH* gene mutations [8] suggesting an impact of modifier genes and environmental factors on the clinical phenotype. The only known correlation is between the genotype and the biochemical phenotype [7]. Patients with a low excreting phenotype due to high residual GCDH activity have the same risk for

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developing striatal injury as high excreting patients with complete loss of GCDH activity [17]. This is puzzling since at high concentrations glutaryl-CoA, GA, and 3-OH-GA become neurotoxic via inhibition of 2-oxoglutarate dehydrogenase complex [22] and the dicarboxylic acid shuttle [23,24] as well as stimulation of N-methyl-D-aspartate receptors [25], and increased production of reactive oxygen species [26,27]. The striking similarity in the disease course of high and low excreters was suggested to be due to similarly high cerebral concentrations of GA and 3-OH-GA concentrations in both patient groups [28–32]. Cerebral entrapment of these dicarboxylic acid in the brain compartment occurs due to a lacking high capacity transport system for dicarboxylic acids in the blood–brain barrier (BBB) [21,33,34].

Striatal injury can be prevented in the majority of patients by early identification by newborn screening and immediate start of a combined metabolic treatment with low L-lysine diet, carnitine supplementation and intermittent glucose infusion or carbohydrate-rich, L-lysine-free diet during episodes that are likely to induce catabolism [35–43]. Low L-lysine diet and glucose infusion are thought to be most effective in lowering cerebral concentrations of GA and 3-OH-GA [44]. This effect was also demonstrated in *Gcdh*$^{-/-}$ mice [45,46], a transgenic animal model for GA-I with complete loss of Gcdh activity biochemically resembling patients with a high excreting phenotype [47,33]. Supplementation with L-arginine (or homoarginine) which competes with L-lysine for transport across biological membranes such as the BBB (CAT1 transporter) and the inner mitochondrial membrane (ORNT1 transporter) further reduces the cerebral L-lysine influx and thus decreases the cerebral concentrations of GA and 3-OH-GA [45,46]. In analogy, complementary dietary treatment using L-lysine-free, arginine-fortified amino acid mixtures has been associated with a favorable outcome in prospectively followed newborn screening cohorts [38,48].

Gcdh$^{-/-}$ mice on a C57BL/6Crl × 129/SvCrl background do not spontaneously develop a neurological phenotype resembling GA-I, nor can it be precipitated by various means of inducing catabolism [47]. Exposure of *Gcdh*$^{-/-}$ mice on the same genetic background to high protein or high L-lysine diet (L-lysine content in both diets, 4.7%), however, was shown to induce seizures, paralysis, subarachnoidal hemorrhages, and death within 3–6 days in the majority of 4-week-old mice, but not in 8-week-old mice suggesting age-dependent susceptibility similar to the window of vulnerability for striatal injury in GA-I patients [49,46]. Interestingly, the source of L-lysine, protein bound or free amino acids in chow, did not affect the induced phenotype in these studies.

A subgroup of 4-week-old *Gcdh*$^{-/-}$ mice with less strongly elevated GA concentrations, however, remained asymptomatic for unknown reasons. This is similar to the known variable clinical phenotype in siblings and patients with the same *GCDH* gene mutation. Naturally occurring susceptibility to L-lysine or protein (mice) or catabolism (human) suggests an impact of modifier genes and/or environmental factors on the clinical phenotype. The major aim of our study was to validate and to optimize the oral L-lysine loading model for *Gcdh*$^{-/-}$ mice elucidating factors that modify the susceptibility of *Gcdh*$^{-/-}$ mice to the L-lysine-induced phenotype. These factors must be controlled to establish a reliable animal model, but may also help to unravel new therapeutic strategies for this disease.

2. Materials and methods

2.1. Animals

Mice used in this study had a C57BL/6Crl × 129/SvCrl background. Inbreeding of these mice from F1–F8 was used to alter the variability of the genetic background along the generations. Animal breeding and experiments were approved by the governmental review board (Regierungspräsidium Karlsruhe, Germany; No. 35-9185.81/G-72/10).

2.2. Treatment

The high L-lysine diet contained 4.7% (w/w) of L-lysine (Harland Teklad, Indianapolis, IN), the standard diet contained 1.7% (w/w) of L-lysine (Rod18, Las Vendi). To identify age-dependent changes in the susceptibility to high L-lysine diet, *Gcdh*$^{-/-}$ mice received high L-lysine or standard diet for a maximum of two weeks starting at ages of 3, 4, 5, 6, or 8 weeks. Concentration-dependent effects of L-lysine exposure were studied by varying the L-lysine concentrations of the drinking water (from 0 to 6.6% [w/v]) in addition to fixed L-lysine contents (1.7 versus 4.7% [w/w]) in chow. Healthy *Gcdh*$^{-/-}$ and *Gcdh*$^{+/-}$ mice showed an average daily intake of 4 ml drinking water and 3.5 g chow per mouse. Table 1 illustrates the daily L-lysine supply per mouse and day in our experiments.

To test the impact of genetic background on the susceptibility to L-lysine, *Gcdh*$^{-/-}$ mice (C57BL/6 Crl × 129/SvCrl) inbred for up to 8 generations were exposed to increasing amounts of this amino acid in chow and drinking water. As an additional control, we used C57BL/6J mice exposed to the same experimental conditions. *Gcdh*$^{-/-}$ mice have been generated by injecting ES cells derived from 129X1/SvJ mice into blastocytes from C57BL/6J mice and chimeric male animals were crossed to C57Bl/6J females [47]. Thereafter, these mice were crossed to 129X1/SvJ generating the reported (C57BL/6 Crl × 129/SvCrl) background [22,46,47,49].

2.3. Preparation of subcellular fractions and homogenates

For biochemical analyses, symptomatic mice were sacrificed shortly before death and asymptomatic mice after two weeks of treatment. All mice were decapitated and perfused with a solution of phosphate buffered saline and 25 U/ml heparin. Afterwards, tissues (brain, liver) were removed and chilled on ice in a buffer (0.1 ml per 1 mg of tissue) containing 250 mmol/l sucrose, 50 mmol/l KCl, 5 mmol/l $MgCl_2$, and 20 mmol/l Tris–HCl (adjusted to pH 7.4). Homogenates and mitochondrial and cytosolic subcellular fractions of tissues were prepared as described before [45]. Protein concentrations were determined according to Lowry [50] with modifications [51] using bovine serum albumin as a standard.

2.4. Quantitative analysis of GA and 3-OH-GA

The tissue-specific concentrations of GA and 3-OH-GA were determined in tissue homogenates using quantitative gas chromatography/mass spectrometry with stable-isotope dilution assay as previously described [33].

2.5. Quantitative analysis of C5DC

C5DC concentrations were determined in tissue homogenates (600 ×*g* supernatant) and serum by electrospray ionization tandem mass spectrometry according to a previously described method [33].

2.6. Amino acid analysis

Amino acid content of brain homogenates was analyzed by high-performance liquid chromatography as previously described [45].

Table 1
Amount of L-lysine supplied by chow (% w/w) and drinking water in (% w/v) and in mg per mouse and day based on average intake of 3 ml drinking water and 3.5 g chow.

L-lysine (chow %)	1.7	4.7	4.7	4.7	4.7	4.7
L-lysine (water %)	0	0	1.5	3	4.7	6.5
L-lysine [mg/day and mouse]	85	235	280	325	376	433

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2.7. Enzyme analysis

Steady-state activity of enzymes involved in respiratory chain, glycolysis, and tricarboxylic acid cycle (TCA) was determined using a computer-tuneable spectrophotometer (SpectramaxPlus Microplate Reader, Molecular Devices; Sunny Vale, CA) operating in the dual wavelength mode; samples were analyzed in temperature-controlled 96-well plates in a final volume of 300 µl.

2.8. Spectrophotometric analysis of glycolytic enzymes

Enzyme activities in cytosolic fractions were analyzed with and without addition of substrate to subtract unspecific background. Glycolytic enzyme activities were determined as NADP or NAD reduction, or NADH oxidation at λ = 340–400 nm as previously described [52].

2.9. Electron transport chain (ETC) and electron flux

Respiratory chain complexes I–IV and ATP synthetase in mitochondria fractions were investigated as previously described [53,44,52]. The addition of standard respiratory chain inhibitors was used to ascertain the specificity of the enzymatic assays. To study electron flow from complex I or II to complex III mitochondrial fractions were incubated with NADH or succinate and, subsequently, the reduction of cytochrome c was analyzed in the presence of NaCN.

2.10. Spectrophotometric analysis of TCA enzymes

Aconitase activity was determined according to [22], with modifications. Aconitase (0.70 mg) was assayed in a buffer containing 0.7 U IDH, 36 mmol/Tris–HCl, 0.07 mmol/l citric acid, 0.18 mmol/l NADP, 1.3 mmol/l manganese sulfate, 0.8 µmol/l ferrous ammonium sulfate, and 0.08 mmol/l L-cysteine which was adjusted to pH 7.4 (25 C). Aconitase activity was determined as NADP reduction at λ = 340–400 nm.

2-Oxoglutarate dehydrogenase complex (OGDHc) activity was measured according to [22], with modifications. OGDHc (650 mU/ml) was assayed in a buffer containing 35 mmol/l potassium phosphate, 5 mmol/l $MgCl_2$, 0.5 mmol/l EDTA, 0.5 mmol/l NAD, 0.2 mmol/l thiamine pyrophosphate, 0.04 mmol/l CoA-SH, and 2 mmol/l 2-oxoglutarate which was adjusted to pH 7.4 (30 °C). OGDHc activity was determined as NAD reduction at λ = 340–400 nm.

Citrate synthase activity was measure as described previously [22].

2.11. Histology

For histological studies, mice were sacrificed at identical time points as for biochemical analyses, and brains were perfused with 4% paraformaldehyde and post-fixed for 24 h in this solution and embedded in paraffin. Paraffin blocks were cut in slices of 3–4 µm thickness using a microtome (Leica Microsystems, Nussloch GmbH, Nussloch, Germany) and placed onto SuperFrost slides (Thermo Scientific, Dreieich, Germany). Sections were stained with hematoxylin & eosin (HE), Klüver–Barrera (KB), and Periodic acid–Schiff (PAS) according to routine protocols for histologic evaluation. A spongiosis score was applied taking into account the degree of tissue alteration (0 = absent; 1 = mild; 2 = moderate; 3 = severe).

2.12. Statistical analysis

Data are expressed as mean ± SD unless otherwise stated. Experiments were performed at least in triplicates. Tissue concentrations of metabolites and enzyme activities were normalized to the protein content and analyzed by Student's *t*-test or ANOVA. To evaluate the effect of increased L-lysine exposure on survival, binary logistic regression analyzed was applied. These statistical analyses were performed using SPSS for Windows 16.0 Software. Histopathological changes were analyzed by the non-parametric Wilcoxon test using JMP 8.0.1 software (SAS, Cary, NC, USA). $p < 0.05$ was considered significant.

3. Results

3.1. Diet-induced mortality is modulated by age, genetic background, and the initial oral L-lysine dose

It has been described previously that 75% of weanling (4-week-old) *Gcdh*$^{-/-}$ mice on a C57BL/6J × 129/SvEv background (first generation) which received a high L-lysine diet (chow with 4.7% L-lysine [w/w], L-lysine-free drinking water) died within 3–6 days after the start of this treatment, whereas all adult (8-week-old) mice survived [46]. First, we performed experiments using the original conditions to investigate whether L-lysine-induced mortality was similar in our hands. We confirmed that L-lysine exposure of 4-week-old *Gcdh*$^{-/-}$ mice induced a clinical phenotype rapidly progressing to death, but only in a small subgroup (7%). First symptoms were observed as early as 24 h after the start of treatment. The clinical presentation of symptomatic mice started with reduced spontaneous activity and hypothermia and progressed from reduction of food intake, to weight loss (Fig. 1), seizures and, subsequently, death. All 8-week-old mice survived and all *Gcdh*$^{+/-}$ mice (at any age) which were used as controls survived. Compared to the original publication [46], the rate of mortality was much lower in our hands (7% versus 75%).

We observed that *Gcdh*$^{-/-}$ mice changed their eating, but not their drinking behavior during high L-lysine exposure. Therefore, we decided to apply additional L-lysine via drinking water. Calculating the actual daily L-lysine intake based on the amount of chow eaten per mouse, we found that *Gcdh*$^{-/-}$ mice received relevant amounts of L-lysine only during the first 24 h of treatment [lysine intake in % of symptomatic but living *Gcdh*$^{-/-}$ mice on the same diet (Median, Min, Max) 24 h: 28.2, 0, 51.7; 48 h: 0, 0, 32.9; 96 h: 0, 0, 0]. Thereafter, chow and, hence, L-lysine intake was minimal due to onset of first symptoms indicating that the initial L-lysine excess rather than chronic intake was harmful to *Gcdh*$^{-/-}$ mice. Unlike *Gcdh*$^{-/-}$ mice on a hybrid background (i.e., C57BL/6J × 129/SvEv), all 4-week-old *Gcdh*$^{-/-}$ mice on a C57BL/6J inbred background remained asymptomatic and survived the 14-day L-lysine exposure. This suggests that the genetic background modulates the L-lysine-induced mortality. In contrast to *Gcdh*$^{-/-}$ mice, all control mice (*Gcdh*$^{+/-}$ mice) survived, irrespective of their genetic background, the amount of daily L-lysine supply and age at start of treatment. In summary, we showed that L-lysine-induced mortality was specifically induced in *Gcdh*$^{-/-}$ mice, but depended on age and the genetic background. Furthermore, we elucidated that oral lysine loading via chow alone was not reliable, since L-lysine-treated mice changed their eating behavior during exposure.

To better understand the factors modulating L-lysine-induced mortality and to optimize the L-lysine exposure model we (1) modified

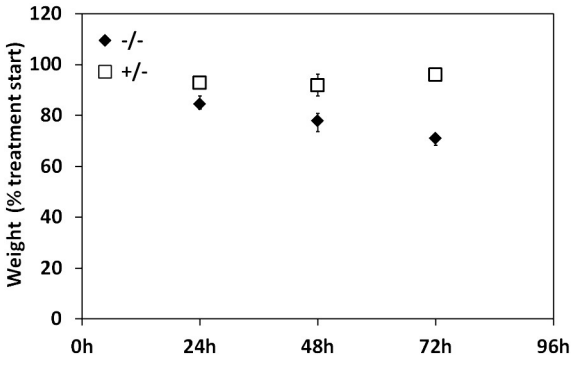

Fig. 1. Body weight under high L-lysine diet. (A) Symptomatic *Gcdh*$^{-/-}$ mice receiving 285 mg L-lysine per day continuously lost weight, whereas the body weight of control mice remained unchanged (n = 9 mice per group).

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the L-lysine content in drinking water (0–6.5% [w/v]; total L-lysine supply, 235–433 mg/d), (2) extended the age spectrum from three to eight weeks, and (3) investigated susceptibilities in female and male mice. Using binary logistic regression analyses we identified three main effects modulating L-lysine-induced mortality in *Gcdh*$^{-/-}$ mice, i.e. (1) gender, (2) the genetic background, and (3) the amount of daily L-lysine supply (Nagelkerke's $r^2 = 0.624$). Of note, we found no interaction between these factors. Male mice showed a higher vulnerability to a high L-lysine exposure than female mice (Fig. 2A; Wald = 8.6, $p < 0.005$). Inbreeding *Gcdh*$^{-/-}$ mice for at least three generations increased their vulnerability to a high lysine diet (Fig. 2B; Wald = 28.9, $p < 0.001$) and abolished their lack of susceptibility to a diet containing 235 mg/d L-lysine. Escalating the daily L-lysine supply up to 433 mg/d also increased mortality in treated mice (Fig. 2C; Wald = 8.7, $p < 0.05$). As described before [49], 8-week-old mice survived a diet containing 235 mg/d L-lysine. However, mortality could be induced in these mice by increasing dietary L-lysine content to 325 mg/d (data not shown). For mice younger than 8 weeks we found no effect of age on the L-lysine-induced phenotype.

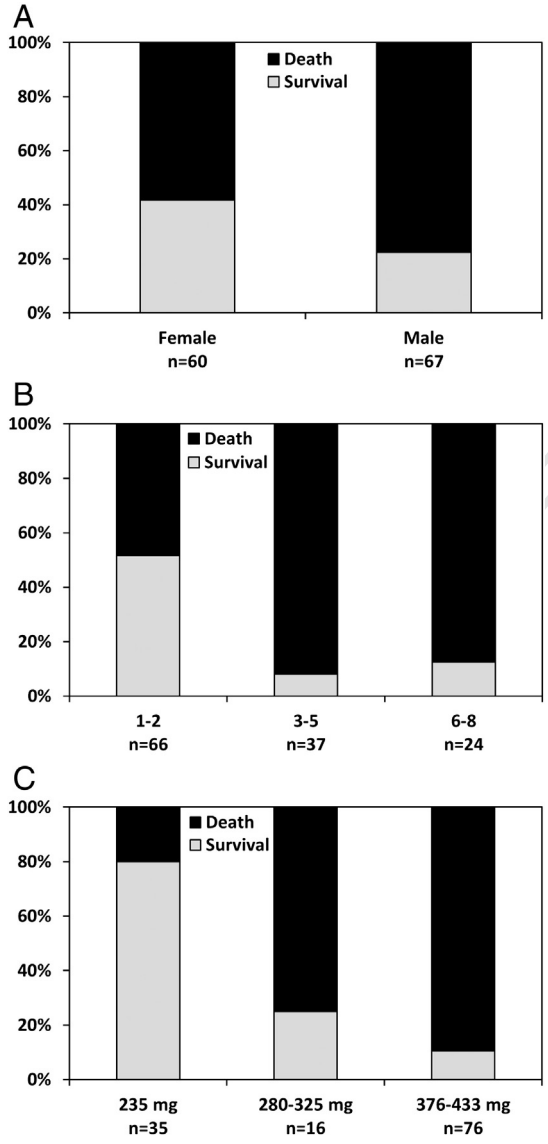

Fig. 2. Stacked column chart of contingency tables for binary logistic regression analysis. (A) Male mice showed a higher vulnerability to a high L-lysine diet than female mice. (B) Inbreeding *Gcdh*$^{-/-}$ mice for at least 3 generations resulted in an increased vulnerability to L-lysine enriched diet. (C) Mortality rate of *Gcdh*$^{-/-}$ mice was also elevated by increasing the daily L-lysine supply up to 433 mg/d.

In summary, we showed that L-lysine induced mortality is also dependent on the genetic background of *Gcdh*$^{-/-}$ mice. The variation caused by the genetic background, however, can be overcome by increasing the amount of daily L-lysine supply of *Gcdh*$^{-/-}$ mice. Moreover, the gender also modulates the induced phenotype.

3.2. Correlation between dicarboxylic compounds and the clinical presentation

Since L-lysine is thought to be the major amino acid precursor for toxic dicarboxylic metabolites in GA-I and *Gcdh*$^{-/-}$ mice, we hypothesized that the dose-dependent increase in mortality after high L-lysine exposure was associated with a dose-dependent increase in cerebral and hepatic concentrations of toxic GA and 3-OH-GA. In fact, cerebral and hepatic concentrations of both GA and 3-OH-GA increased during L-lysine exposure above that of mice receiving the standard diet (i.e., 85 mg/d). However, cerebral and hepatic concentrations of GA reached a plateau between 235 and 376 mg/d L-lysine and those of 3-OH-GA between 325 and 376 mg/d L-lysine (Fig. 3A,B). Similarly, L-lysine concentrations of brain and liver tissue also reached a plateau at a daily L-lysine supply of 325 mg/d (L-lysine concentration (nmol/mg): 85 mg/d L-lysine supply, brain 11 ± 8, liver 15 ± 9; 325 mg/d L-lysine supply, brain 25 ± 4, liver 54 ± 16).

Next, a statistical comparison of all tested control mice (i.e. *Gcdh*$^{-/-}$ mice receiving standard diet) with all tested asymptomatic and symptomatic *Gcdh*$^{-/-}$ mice (i.e. *Gcdh*$^{-/-}$ mice receiving a high lysine diet showing no symptoms and symptoms respectively) was performed. As expected asymptomatic and symptomatic *Gcdh*$^{-/-}$ mice had higher cerebral and hepatic GA concentrations than control mice. More importantly, symptomatic *Gcdh*$^{-/-}$ mice could be distinguished from asymptomatic mice by a more pronounced increase in GA concentrations in brain and liver (Fig. 3C). For 3-OH-GA, cerebral but not hepatic concentrations differed from asymptomatic mice, whereas asymptomatic mice displayed the same concentrations in both organs as controls (Fig. 3D). These results support the neurotoxicological hypothesis of GA-I and confirm previous results [25,48,49,46]. To gain a reliable L-lysine-inducible clinical phenotype, we performed all following experiments in 4-week-old *Gcdh*$^{-/-}$ mice on a C57BL/6 Crl × 129/SvCrl inbred background using chow (4.7%, w/w) and drinking water (4.7%, w/v; total L-lysine supply, 376 mg/d) enriched with L-lysine unless otherwise stated.

3.3. Ammonia detoxification remains unaffected during L-lysine exposure

Glutaryl-CoA inhibits the TCA cycle [22], whereas GA [24] and – to a lesser extent – 3-OH-GA [23] both inhibit the dicarboxylic acid shuttle between astrocytes and neurons which relies on highly active pyruvate carboxylase in astrocytes and effective sodium-dependent dicarboxylic acid carriers 1 and 2. As a consequence, the flux and availability of TCA cycle intermediates is thought to be impaired in GA-I [33,48]. One important TCA cycle intermediate is 2-oxoglutarate. This metabolite is a substrate of many enzymes such as the 2-oxoglutarate dehydrogenase complex in the TCA cycle. In addition, it provides the carbon backbone for the synthesis of L-glutamine by L-glutamate dehydrogenase and L-glutamine synthetase and thus for the fixation of ammonia. 2-Oxoglutarate is also required as a substrate for the formation of saccharopine within the so-called saccharopine pathway of L-lysine oxidation [45]. The formation of saccharopine is the first irreversible step of L-lysine oxidation via this pathway. Since it was suggested that L-lysine exposure might limit the availability of 2-oxoglutarate in *Gcdh*$^{-/-}$ mice [46] and that 3-OH-GA and – less pronounced – GA might increase the concentration of ammonia in 3D-organotypic rat brain cell cultures [54], we wondered whether L-lysine exposure would affect ammonia detoxification and the formation of L-glutamine and urea in *Gcdh*$^{-/-}$ mice. Hyperammonemic crises are known to Q7

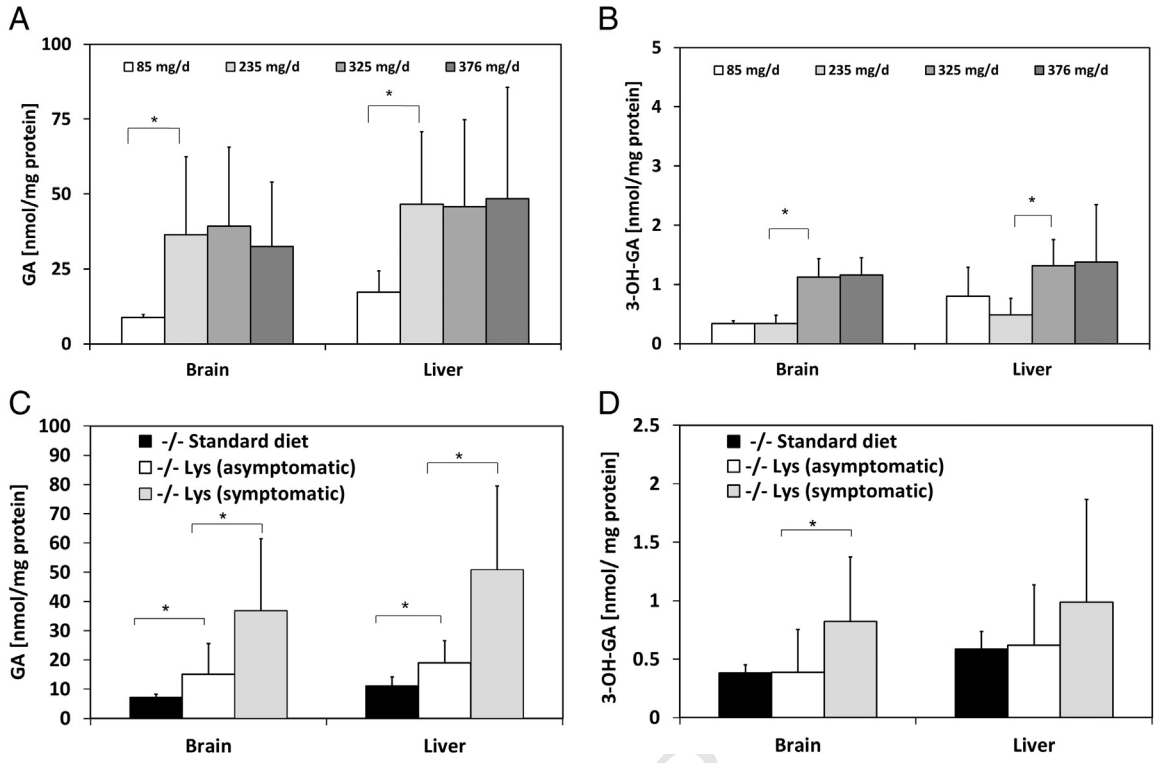

Fig. 3. Changes in metabolic parameters following high L-lysine exposure. GA levels (A) were elevated in *Gcdh*$^{-/-}$ mice receiving 235 mg L-lysine per day. Increasing the dietary L-lysine supply above 235 mg/d did not further increase the GA concentrations. The same pattern was found for 3-OH-GA (B) except that 325 mg L-lysine per day enhanced formation of this metabolite (ANOVA with contrasts, *$p \leq 0.05$). Next, we compared the cerebral and hepatic concentrations of GA (C) and 3-OH-GA (D) in symptomatic [−/− Lys (symptomatic); n = 14] and asymptomatic [−/− Lys (asymptomatic); n = 18] *Gcdh*$^{-/-}$ mice receiving high L-lysine diet as well as in *Gcdh*$^{-/-}$ mice on a standard diet [−/− standard diet; n = 9]. GA and 3-OH-GA concentrations were significantly increased in brain and liver of *Gcdh*$^{-/-}$ mice after high L-lysine exposure. Of note, symptomatic mice displayed higher cerebral and hepatic levels of GA and 3-OH-GA than asymptomatic mice (ANOVA, *$p \leq 0.05$).

induce severe cerebral injury in intoxication type metabolic disorders such as urea cycle disorders, but have never been reported in patients with GA-I.

Serum and brain ammonia concentrations did not differ in *Gcdh*$^{-/-}$ and *Gcdh*$^{+/-}$ mice after L-lysine exposure (mean ± SD: serum 105 ± 38 μmol/l [*Gcdh*$^{-/-}$] vs. 105 ± 28 μmol/l [*Gcdh*$^{+/-}$]; brain 115 ± 15 μmol/l [*Gcdh*$^{-/-}$] vs. 147 ± 18 μmol/l [*Gcdh*$^{+/-}$]), whereas serum urea concentrations were higher in *Gcdh*$^{-/-}$ (183 ± 89 mg/dl; $p = 0.036$) than in *Gcdh*$^{+/-}$ mice (33 ± 8 mg/dl). In analogy, urea concentrations in brain and liver of *Gcdh*$^{-/-}$ mice were also elevated (Fig. 4A). Uremia due to renal failure was excluded by analysis of serum creatinine and cystatin C which both remained in the normal range (creatinine, *Gcdh*$^{+/-}$ 0.5 ± 0.4 mg/dl, *Gcdh*$^{-/-}$ 0.9 +/− 0.5 mg/dl; cystatin C, *Gcdh*$^{+/-}$ 0.3 ± 0.5 mg/l, *Gcdh*$^{-/-}$ 0.1 +/− 0.1 mg/l). In contrast to urea, L-glutamate which is used for transient fixation of ammonia by L-glutamine synthetase in liver and brain remained unchanged in both tissues (Fig. 4B). Similarly, tissue-specific L-glutamine concentrations also remained unchanged following L-lysine exposure (Fig. 4C). These results show that L-lysine exposure induces enhanced but effective detoxification of ammonia via the urea cycle, but do not support the notion of hyperammonemia playing an important role in the neuropathogenesis of GA-I. The most likely cause of increased ammonia is induction of catabolism following L-lysine exposure.

3.4. Changes in energy metabolism: focus on glycolysis and the TCA cycle

Previous studies have highlighted that impairment of brain energy metabolism and formation of reactive oxygen species can be induced by accumulating toxic dicarboxylic metabolites and thus is thought to play a key role in the pathogenesis of GA-I [55,48,27]. So far, OGDHc and the dicarboxylate shuttle have been identified as major targets [23,22,24]. We therefore wondered whether L-lysine exposure resulted in changes of the major routes of energy metabolism.

L-lysine exposure induced changes in the activity of some glycolysis and TCA cycle enzyme. Specifically, the activities of GAPDH and – less pronounced – of PFK2, PGM2, PK LA, and LDH were specifically decreased in the brain but not in the liver of *Gcdh*$^{-/-}$ mice compared to control mice, whereas hexokinase activity was slightly increased in the liver (Table 2A). In the TCA cycle, activities for aconitase (liver and brain) and OGDHc (liver) were lower in *Gcdh*$^{-/-}$ mice compared to *Gcdh*$^{+/-}$ mice (Table 2B). In contrast, citrate synthase activity did not differ between *Gcdh*$^{-/-}$ and *Gcdh*$^{+/-}$ mice (Table 2B) suggesting that the decreased energy supply via glycolysis and TCA cycle is not counterbalanced by increased mitochondrial proliferation. The activities of respiratory chain enzyme complexes I–IV and ATP synthase remained unchanged (Table 2C). In comparison to a previous study focusing on direct metabolite–enzyme interactions [22], these findings do not reflect toxic metabolite-induced enzyme inhibition, but adaptive changes following L-lysine exposure.

3.5. Hippocampal pathology is most prominent but is not influenced by L-lysine exposure

The main neuropathological finding in symptomatic GA-I patients is striatal necrosis which may manifest acutely during encephalopathic crisis or insidiously in infancy [29,20]. In addition, extrastriatal changes have been found both in symptomatic and asymptomatic patients. This includes putatively reversible temporal hypoplasia and dilated Sylvian fissures, T_2 hyperintensity of white matter changes reflecting spongiform myelinopathy as well as T_2 hyperintensities in dentate

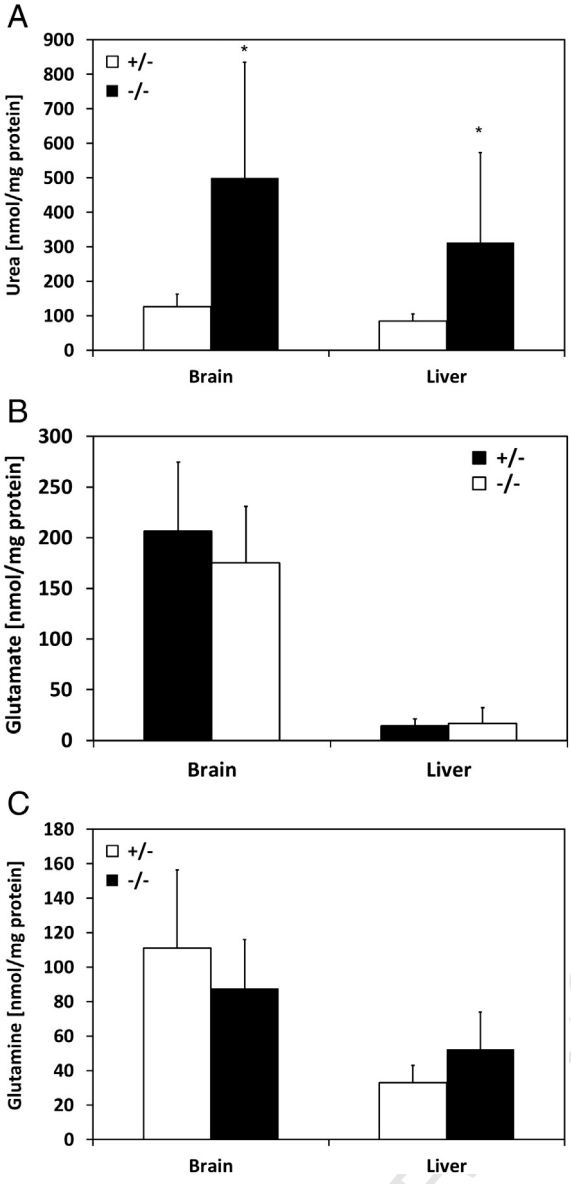

Fig. 4. Effect of L-lysine exposure on urea, glutamate, and glutamine. (A) *Gcdh*$^{-/-}$ mice (−/− Lys, n = 8) showed an increase in urea concentrations of brain and liver compared to controls (+/− Lys, n = 10) after treatment with high L-lysine diet (Student's *t*-test, *$p \leq 0.005$). Glutamine (B) and glutamate (C) levels were similar in both groups.

nuclei, substantia nigra, and the pontine medial lemniscus [56,14,57,58]. In *Gcdh*$^{-/-}$ mice receiving high protein or high L-lysine diet, the striatal pathology was thought to be similar to GA-I patients [49,46]. In these studies, however, striatal cell loss was only significantly increased in asymptomatic 8-week-old animals after a high L-lysine diet for 6 weeks but not in symptomatic 4-week-old animals after 3 days of L-lysine exposure. Both treatment groups, however, showed pronounced cortical cell loss and, additionally, subarachnoidal, subdural and intraventricular hemorrhages as well as BBB breakdown. Subarachnoidal and intraventricular hemorrhages have not yet been reported in GA-I patients, whereas subdural hygromas resembling non-accidental head trauma are sometimes found, usually as an incidental finding in MRI studies [14,15].

In our hands, symptomatic *Gcdh*$^{-/-}$ mice receiving optimized high L-lysine diet as described in detail above did not produce a significant striatal pathology (Fig. 5). Furthermore, we did not find evidence for BBB breakdown or intracranial hemorrhages (not shown). In contrast to GA-I patients, the major pathology in *Gcdh*$^{-/-}$ mice was identified in the hippocampus and – less pronounced – in the cortex showing massive focal spongiosis and focal neuronal damage within these areas. Neuronal damage consisted among other features of neuronal pyknosis, nuclear chromatin condensation, and dark neurons indicating very early (i.e. 1–48 h) signs of neuronal damage. These changes, however, appeared in both *Gcdh*$^{-/-}$ mice on a standard and on a high L-lysine diet.

Statistical analysis confirmed that hippocampal spongiosis was increased in *Gcdh*$^{-/-}$ mice compared to *Gcdh*$^{+/-}$ mice, but that it did not depend on the amount of L-lysine in the diet (Fig. 5). In analogy to previous studies, we also found an increased rate of spongiosis in the cortex (Fig. 5). No striking pathology was observed in the white matter.

4. Discussion

The major aim of our study was to validate and to optimize the previously published L-lysine-induced mouse model for GA-I [46] and to identify modifiers that influence the induced clinical phenotype. High L-lysine exposure in weanling (4-week-old) but not in adult (8-week-old) *Gcdh*$^{-/-}$ mice induced an acute clinical phenotype, but only in small group of animals. Initial symptoms are reduced spontaneous activity and hypothermia that progress over weight loss and seizures to death within 24–96 h. Despite these similarities to previous findings [46], we observed significant obstacles in the reliability and reproducibility of this diet-induced model and raised questions on whether the induced neuropathology is similar to that of GA-I patients. In the following, we will discuss our major findings that support the need of a critical revision of this model.

4.1. Genetic background

In our study the mortality rate of *Gcdh*$^{-/-}$ mice on a high L-lysine diet was strongly dependent on the genetic background. Mice of the F1 generation (C57Bl6/Crlx129/SvCrl; as used in the study of [49,46] as well as mice of F2 generation on a 4.7% (i.e., 235 mg/d) L-lysine diet had 93% survival rate. In contrast, inbreeding of *Gcdh*$^{-/-}$ mice strongly increased their susceptibility to L-lysine exposure and improved the reliability of the L-lysine-induced phenoytpe. *Gcdh*$^{-/-}$ mice of F6–8 generations showed neurological symptoms and a high mortality (~71%) receiving 235 mg L-lysine/d. These findings suggest that susceptibility to high L-lysine diet is based on modifier genes of mice on a mixed C57Bl6/Crl × 129/SvCrl background whose phenotypic impact changes during inbreeding. Whether increased susceptibility to L-lysine exposure from the F1 to the F8 generation reflects an increasing impact of genes which amplify L-lysine-induced toxicity or the decreasing impact of neuroprotective factors remain to be elucidated. Nevertheless, it is well known that inbreeding generally decreases the fitness of mice and their susceptibility to different stress conditions (the so-called inbreeding depression).

Moreover, we demonstrate that male mice have a higher risk to develop neurological symptoms than female mice on a high L-lysine diet. This is similar to a variety of murine models for neurological disorders such as stroke [59].

4.2. L-lysine exposure

Susceptibility to a high L-lysine diet (235 mg/d) in F1 and F2 generations could be increased by escalating the daily L-lysine supply (280–433 mg/d). All *Gcdh*$^{-/-}$ mice of F6–8 generation receiving at least 376 mg L-lysine per day developed neurological symptoms and died within 96 h, whereas 22% of F1–2 generation mice survived on this diet. Since L-lysine exposure induced a decreased food intake, we hypothesize that the peak L-lysine intake during the first 24 h of the start of L-lysine exposure is most important for inducing a clinical phenotype

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Table 2
Tissue-specific effects of L-lysine exposure on glycolysis, TCA cycle, and respiratory chain.
Activities of enzymes of energy metabolism in control and *Gcdh*$^{-/-}$ mice receiving a modified high L-lysine diet (n = 10 per group).

		Brain [mU/mg protein]	Liver [mU/mg protein]
Table 2A. Glycolysis			
Hexokinase	*Gcdh*$^{+/-}$	4.9 ± 1.2	6.3 ± 1.1
	Gcdh$^{-/-}$	4.9 ± 0.8	7.4 ± 0.9
	p-Value (*t*-test)	0.881	0.000
Phosphofructokinase	*Gcdh*$^{+/-}$	2.5 ± 1.3	43.0 ± 8.4
	Gcdh$^{-/-}$	1.8 ± 0.9	39.0 ± 12.0
	p-Value (*t*-test)	0.032	0.1790
Triosephosphate isomerase	*Gcdh*$^{+/-}$	892.2 ± 227.6	770.6 ± 126.5
	Gcdh$^{-/-}$	834.3 ± 134.5	751.0 ± 100.7
	p-value (*t*-test)	0.254	0.534
Glyceraldehyde 3-phosphate dehydrogenase	*Gcdh*$^{+/-}$	294.9 ± 153.3	38.2 ± 2.6
	Gcdh$^{-/-}$	146.4 ± 72.2	40.5 ± 4.3
	p-Value (*t*-test)	0.001	0.0880
Phosphoglycerate mutase	*Gcdh*$^{+/-}$	242.2 ± 35.3	211.4 ± 40.3
	Gcdh$^{-/-}$	217.5 ± 26.3	207.6 ± 32.0
	p-Value (*t*-test)	0.005	0.701
Enolase	*Gcdh*$^{+/-}$	518.3 ± 79.3	536.3 ± 126.3
	Gcdh$^{-/-}$	533.1 ± 115.2	561.2 ± 113.2
	p-Value (*t*-test)	0.599	0.4560
Pyruvate kinase	*Gcdh*$^{+/-}$	379.3 ± 101.3	834.3 ± 186.1
Low affinity form	*Gcdh*$^{-/-}$	298.3 ± 54.6	795.3 ± 167.3
	p-Value (*t*-test)	0.004	0.507
Pyruvate kinase	*Gcdh*$^{+/-}$	126.2 ± 33.6	469.8 ± 83.1
High affinity form	*Gcdh*$^{-/-}$	118.9 ± 22.4	441.9 ± 77.9
	p-Value (*t*-test)	0.344	0.2150
Lactate dehydrogenase	*Gcdh*$^{+/-}$	918.3 ± 301.2	623.7 ± 86.1
	Gcdh$^{-/-}$	766.1 ± 133.6	618.6 ± 90.7
	p-Value (*t*-test)	0.018	0.839
Table 2B. TCA cycle			
Citrate synthase	*Gcdh*$^{+/-}$	85.4 ± 15.5	17.3 ± 5.3
	Gcdh$^{-/-}$	92.9 ± 19.3	16.5 ± 4.3
	p-Value (*t*-test)	0.137	0.535
Aconitase	*Gcdh*$^{+/-}$	2.49 ± 1.34	0.97 ± 0.65
	Gcdh$^{-/-}$	1.02 ± 0.68	0.38 ± 0.21
	p-Value (*t*-test)	0.000	0.0000
2-Oxoglutarate dehydrogenase complex	*Gcdh*$^{+/-}$	7.7 ± 1.6	7.5 ± 3.8
	Gcdh$^{-/-}$	8.1 ± 2.5	3.4 ± 2.8
	p-Value (*t*-test)	0.464	0.000
Table 2C. Respiratory chain			
Complex I	*Gcdh*$^{+/-}$	11.7 ± 3.3	22.8 ± 5.4
	Gcdh$^{-/-}$	11.8 ± 2.6	23.0 ± 6.4
	p-Value (*t*-test)	0.939	0.912
Complex II	*Gcdh*$^{+/-}$	9.4 ± 2.5	13.0 ± 4.6
	Gcdh$^{-/-}$	9.0 ± 2.6	13.7 ± 4.7
	p-Value (*t*-test)	0.568	0.617
Complex III	*Gcdh*$^{+/-}$	39.4 ± 6.0	22.5 ± 11.7
	Gcdh$^{-/-}$	39.9 ± 7.4	21.6 ± 7.5
	p-Value (*t*-test)	0.797	0.736
Complex IV	*Gcdh*$^{+/-}$	1172.6 ± 530.1	583.5 ± 315.0
	Gcdh$^{-/-}$	1123.9 ± 227.1	579.6 ± 176.0
	p-Value (*t*-test)	0.654	0.955
ATP synthase	*Gcdh*$^{+/-}$	54.9 ± 13.2	19.7 ± 6.8
	Gcdh$^{-/-}$	53.7 ± 8.9	20.7 ± 7.6
	p-Value (*t*-test)	0.701	0.635
Flux complex I–III	*Gcdh*$^{+/-}$	31.9 ± 7.5	132.4 ± 50.9
	Gcdh$^{-/-}$	31.4 ± 6.3	121.4 ± 53.2
	p-Value (*t*-test)	0.815	0.453
Flux complex II–III	*Gcdh*$^{+/-}$	2.6 ± 0.8	11.7 ± 6.4
	Gcdh$^{-/-}$	2.6 ± 1.3	11.5 ± 3.0
	p-Value (*t*-test)	0.985	0.838

in these mice. This may be associated with an increase in neurotoxic dicarboxylic metabolites that induce the acute clinical phenotype.

4.3. Threshold for neurotoxicity

For GA-I patients, there is no known correlation between the genotype and clinical phenotype or the biochemical and the clinical phenotypes, whereas the genotype correlates with the biochemical phenotype [7,29,17]. Furthermore, there is a high variability in the severity of the disease course, even in siblings and in other patients with the same *GCDH* gene mutations [17]. Considering the toxic metabolite and trapping hypotheses it is tempting to speculate that the variability of the natural history of GA-I patients might be based on different intracerebral concentrations of neurotoxic metabolites and that such differences might be influenced by the genetic background and environmental factors.

We therefore wondered whether symptomatic and asymptomatic *Gcdh*$^{-/-}$ mice differed in their GA and 3-OH-GA concentrations during

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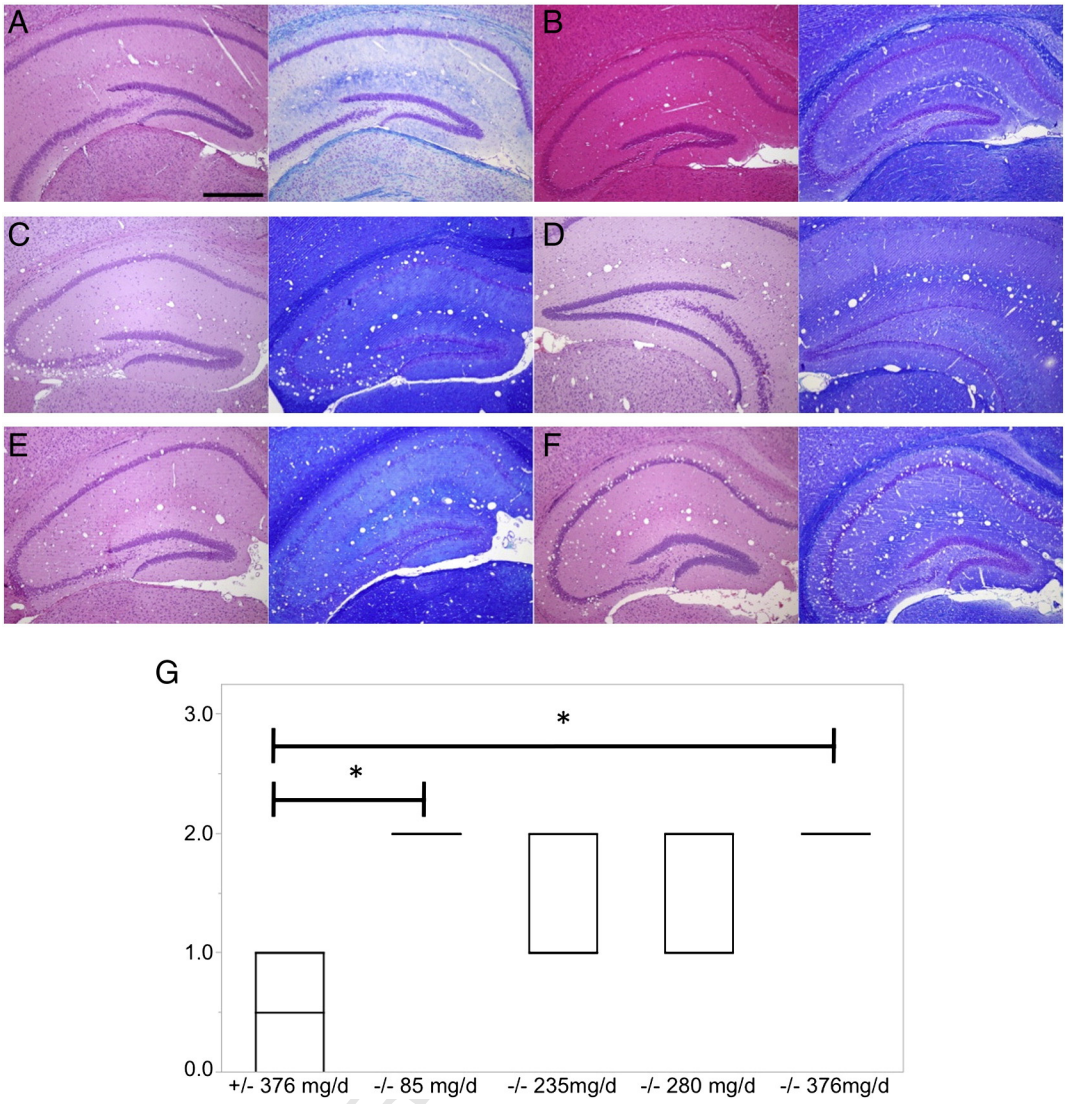

Fig. 5. Brain pathology of *Gcdh*$^{-/-}$ mice. Histological analysis of hippocampal sections of (A) WT control, (B) +/− 376 mg/d Lys, (C) −/− 85 mg/d Lys, (D) −/− 235 mg/d Lys, (E) −/− 280 mg/d Lys and (F) −/− 376 mg/d Lys mice are depicted (for each condition HE (left) and Klüver–Barrera (right) stainings are depicted). In comparison to WT control and +/− mice, *Gcdh*$^{-/-}$ mice display CNS spongiosis which is most prominent in the hippocampus (scale bar = 500 μm for all images). (G) Box-plots of different genetic and dietary conditions are depicted. Statistical analysis was performed using non-parametric Wilcoxon test. A significance level of alpha = 0.05 was selected for all tests (* = $p < 0.05$). (+/− 376 mg/g Lys: n = 4; −/− 85 mg/d Lys: n = 4; −/− 235 mg/d Lys: n = 5; −/− 280 mg/d: n = 3; −/− 376 mg/d: n = 3).

L-lysine exposure. In fact, symptomatic mice showed a much higher increase in cerebral and hepatic GA and – less pronounced – in cerebral 3-OH-GA concentrations than asymptomatic mice.

4.4. Oral L-lysine loading induced pathology results in enhanced catabolism

Due to reduced food intake oral L-lysine loading leads to a catabolic state of symptomatic animals and, thereby, increases the breakdown of L-lysine and other amino acids. Symptomatic *Gcdh*$^{-/-}$ mice showed weight loss upon start of L-lysine exposure as well as increased production of urea secondary to increased ammonia production. Normal serum cystatin C and creatinine concentrations virtually exclude renal insufficiency as cause of elevated urea concentrations upon treatment.

Zinnanti and colleagues put forward the idea that a high L-lysine diet diminishes 2-oxoglutarate levels due to increased saccharopine pathway activity [46]. However, in our study we did not find evidence for decreased L-glutamate or L-glutamine levels as a consequence of 2-oxoglutarate depletion. Our data also do not support the suggestion that hyperammonemia plays a pathomechanistic role [54] since we did not find increased cerebral L-glutamine or ammonia concentrations. In analogy, hyperammonemic episodes have never been reported for patients with GA-I.

4.5. L-lysine diet induced changes of energy metabolism

Evaluation of energy metabolism in symptomatic and asymptomatic mice identified functional impairment and secondary changes of enzyme activities in glycolysis and TCA cycle, whereas activities of respiratory chain complexes remained unchanged. Most notably, activities of GAPDH and aconitase activities were markedly decreased in the brain of *Gcdh*$^{-/-}$ mice after L-lysine exposure. No increase of citrate synthase activity was found in *Gcdh*$^{-/-}$ mice upon L-lysine exposure suggesting that mitochondrial proliferation is not induced to compensate for the reduced metabolite flux in glycolysis and TCA cycle. Of note, the used enzymatic assays record steady state activity and do not reflect inhibitory effects of accumulating metabolites. In a previous study we have shown that GA, 3-OH-GA, or glutaryl-CoA do not have a direct inhibitory effect on proteins of energy metabolism except for an inhibition of OGDHc by glutaryl-CoA [22]. Therefore, the observed changes in our

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study are most likely due to metabolic stress induced by increased L-lysine intake.

GAPDH, OGDHc, and aconitase have shown to be highly vulnerable for oxidative stress decreasing their catalytic activity [60–63]. In concert with the direct inhibition of OGDHc by glutaryl-CoA these findings indicate that impairment of energy homeostasis plays an important role in neuropathogenesis of GA-I.

4.6. Hippocampal spongiosis is a major neuropathological finding in $Gcdh^{-/-}$ mice but does not depend on dietary L-lysine supply

Histological evaluation of $Gcdh^{-/-}$ mice following L-lysine exposure revealed no considerable pathology of the striatum. In contrast, $Gcdh^{-/-}$ mouse brains showed increased spongiosis, most prominently within the hippocampus. Although hippocampal spongiosis was significantly increased in $Gcdh^{-/-}$ mouse brain as compared to $Gcdh^{+/-}$ mice, no considerable differences were detected between symptomatic and asymptomatic mice. Further, neuropathological changes were independent of the amount of L-lysine supplied. This is in contrast to a previous study which suggested that the high L-lysine diet induces a striatal pathology similar to that of GA-I patients [49].

Hippocampal neurons are highly vulnerable to various toxins and ROS and thus are involved in many neurodegenerative diseases [64]. An in vitro study has demonstrated that hippocampal rat neurons are susceptible to 3-OH-GA and ROS formation [55]. Chronic damage of these neurons may occur in $Gcdh^{-/-}$ mice due to long-term exposure to high cerebral GA and 3-OH-GA concentration during postnatal and maybe also prenatal brain development. Selective spongiosis in hippocampus and cortex has been found in neurodegenerative diseases such as Alzheimer's disease [65–67]. PAS staining indicates that spongiosis occurs due to loss of neuronal cells as it has been suggested before by Zinnanti et al. [46] and been demonstrated in asymptomatic $Gcdh^{-/-}$ mice on a standard diet [47]. In GA-I patients, however, no evidence of a significant hippocampal pathology has been found yet [29]. This may reflect species-dependent differences

4.7. $Gcdh^{-/-}$ mice as an animal model for GA-I

The acute clinical phenotype in $Gcdh^{-/-}$ mice induced by a high L-lysine depends on various parameters such as the age at start of treatment, gender, genetic modifiers, and the amount of oral L-lysine supply. These parameters, as far as they are known, need to be carefully controlled to achieve a reliable and reproducible murine model for GA-I. Although this model resembles the biochemical phenotype of GA-I patients with a high excreting phenotype, neuropathological changes were significantly discrepant to the human phenotype. Specifically, major changes were observed in the murine hippocampus, whereas the striatum remained virtually unaffected. In addition, the same hippocampal changes were observed in $Gcdh^{-/-}$ mice both on a standard diet or on a high L-lysine diet and thus are likely induced by chronic rather than acute neurotoxicity. Since both, the spontaneously developing and the high L-lysine diet-induced clinical phenotype, differ from that of GA-I patients, clinical endpoints for therapeutic studies on $Gcdh^{-/-}$ mice should be carefully chosen. Of note, the lack of apparent neuropathology in the sensitive mice is suggestive of toxicity that affects only a relatively minor population of neurons. In contrast to the global injury reported by Zinnanti and others, this is more consistent with the observed phenotype in patients with GA-I. Future studies have to identify the subpopulation of affected neurons which will further help to evaluate the suitability of this animal model.

For the first time, we have demonstrated that the genetic background and gender plays an important role in the clinical phenotype. This needs to be addressed more specifically in future studies in order to elucidate factors that influence cerebral L-lysine oxidation and the susceptibility to neurotoxic metabolites and thus might be used for the development of new therapeutic strategies.

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