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Therapeutic modulation of cerebral L-lysine metabolism in a mouse model for glutaric aciduria type I

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Glutaric aciduria type I, an inherited deficiency of glutaryl-coenzyme A dehydrogenase localized in the final common catabolic pathway of L-lysine, L-hydroxylysine and L-tryptophan, leads to accumulation of neurotoxic glutaric and 3-hydroxyglutaric acid, as well as non-toxic glutarylcarnitine. Most untreated patients develop irreversible brain damage during infancy that can be prevented in the majority of cases if metabolic treatment with a low L-lysine diet and L-carnitine supplementation is started in the newborn period. The biochemical effect of this treatment remains uncertain, since cerebral concentrations of neurotoxic metabolites can only be determined by invasive techniques. Therefore, we studied the biochemical effect and mechanism of metabolic treatment in glutaryl-coenzyme A dehydrogenase-deficient mice, an animal model with complete loss of glutaryl–coenzyme A dehydrogenase activity, focusing on the tissue-specific changes of neurotoxic metabolites and key enzymes of L-lysine metabolism. Here, we demonstrate that low L-lysine diet, but not L-carnitine supplementation, lowered the concentration of glutaric acid in brain, liver, kidney and serum. L-carnitine supplementation restored the free L-carnitine pool and enhanced the formation of glutarylcarnitine. The effect of low L-lysine diet was amplified by add-on therapy with L-arginine, which we propose to result from competition with L-lysine at system y^+ of the blood–brain barrier and the mitochondrial L-ornithine carriers. L-Lysine can be catabolized in the mitochondrial saccharopine or the peroxisomal pipecolate pathway. We detected high activity of mitochondrial 2-aminoadipate semialdehyde synthase, the rate-limiting enzyme of the saccharopine pathway, in the liver, whereas it was absent in the brain. Since we found activity of the subsequent enzymes of L-lysine oxidation, 2-aminoadipate semialdehyde dehydrogenase, 2-aminoadipate aminotransferase and 2-oxoglutarate dehydrogenase complex as well as peroxisomal pipecolic acid oxidase in brain tissue, we postulate that the pipecolate pathway is the major route of L-lysine degradation in the brain and the saccharopine pathway is the major route in the liver. Interestingly, treatment with clofibrate decreased cerebral and hepatic concentrations of glutaric acid in glutaryl-coenzyme A dehydrogenase-deficient mice. This finding opens new therapeutic perspectives such as pharmacological stimulation of alternative L-lysine oxidation in peroxisomes. In conclusion, this study gives insight into the discrepancies between cerebral and hepatic L-lysine metabolism,

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provides for the first time a biochemical proof of principle for metabolic treatment in glutaric aciduria type I and suggests that further optimization of treatment could be achieved by exploitation of competition between L-lysine and L-arginine at physiological barriers and enhancement of peroxisomal L-lysine oxidation and glutaric acid breakdown.

Keywords: lysine metabolism; saccharopine; pipecolic acid; dicarboxylic acids; basic amino acid transporter

Abbreviations: AADAT = aminoadipate aminotransferase; AASDH = aminoadipate semialdehyde dehydrogenase; AASS = 2-aminoadipate semialdehyde synthase; GCDH = glutaryl-CoA dehydrogenase; LOR = lysine 2-oxoglutarate reductase; OGDHc = 2-oxoglutarate dehydrogenase complex

Introduction

Glutaric aciduria type I is a 'cerebral' organic acid disorder first described in 1975 (Goodman *et al.*, 1975). The estimated overall prevalence is 1 in 100 000 newborns (Lindner *et al.*, 2004; Kölker *et al.*, 2007*b*). The disease is caused by inherited deficiency of the homotetrameric mitochondrial flavoprotein glutaryl-CoA dehydrogenase (GCDH; EC 1.3.99.7), which is encoded by the *GCDH* gene mapping to human chromosome locus 19p13.2 (Greenberg *et al.*, 1994). More than 200 disease-causing mutations have been identified (Goodman *et al.*, 1998; Zschocke *et al.*, 2000). GCDH catalyzes the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA (Dwyer *et al.*, 2000) and is a key enzyme in the final degradative pathways of L-lysine, L-hydroxylysine and L-tryptophan. Deficiency of this enzyme results in accumulation of glutaric acid, 3-hydroxyglutaric acid and glutarylcarnitine. L-lysine oxidation is quantitatively the most relevant pathway for production of these metabolites. In mammals, the first steps of L-lysine oxidation are catalyzed by the bifunctional enzyme 2-aminoadipate semialdehyde synthase (AASS) consisting of a lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase subunit being localized in the mitochondrial matrix (Blemings *et al.*, 1994). An alternative route via the pipecolate pathway has been postulated to initiate lysine oxidation in brain (Mihalik and Rhead, 1989; Rao *et al.*, 1993; Ijlst *et al.*, 2000). However, direct evidence for the first steps of cerebral lysine oxidation is still lacking. Subsequently, both pathways converge at the level of 2-aminoadipate semialdehyde that is further broken down by the two cytosolic enzymes 2-aminoadipate semialdehyde dehydrogenase (AASDH) and 2-aminoadipate aminotransferase (AADAT) (Chang *et al.*, 1990; Okuno *et al.*, 1993). The next enzymatic step is 2-oxoglutarate dehydrogenase complex (OGDHc), the rate-limiting enzyme of the tricarboxylic acid cycle catalyzing the conversion of 2-oxoadipate to glutaryl-CoA (Hirashima *et al.*, 1967; Majamaa *et al.*, 1985; Bunik and Pavlova, 1997). In line with this, we demonstrated product inhibition of OGDHc by glutaryl-CoA—in analogy to succinyl-CoA (Sauer *et al.*, 2005). Glutaryl-CoA is then dehydrogenated and decarboxylated to crotonyl-CoA by GCDH.

The initial clinical presentation of affected neonates is usually non-specific. Untreated patients usually develop an acute encephalopathic crisis during a finite period of brain development resulting in permanent brain damage, mainly affecting the striatum and resulting in dystonia (Bjugstad *et al.*, 2000; Strauss *et al.*, 2003; Kyllerman *et al.*, 2004; Kölker *et al.*, 2006*a*). These crises are precipitated by episodes that are likely to induce catabolic state. Some patients, however, develop neurological symptoms without a clinically apparent crisis (Bahr *et al.*, 2002; Külkens *et al.*, 2005; Strauss *et al.*, 2007; Heringer *et al.*, 2010). In addition to acute striatal injury, there is increasing cranial MRI evidence of abnormalities in extrastriatal regions with as yet uncertain clinical relevance and of mental retardation in some patients who have not suffered an encephalopathic crisis (Harting *et al.*, 2009).

Several studies highlight the role of glutaric acid, 3-hydroxyglutaric acid and glutaryl-CoA in the pathogenesis of this disease. Precipitation of excitotoxic mechanisms and oxidative stress (Kölker *et al.*, 2004), imbalance in glutamatergic and GABAergic neurotransmission (Bennett *et al.*, 1973; Stokke *et al.*, 1976; Porciúncula *et al.*, 2000) and impairment of energy metabolism by inhibition of the OGDHc and the dicarboxylic acid shuttle between astrocytes and neurons, have all been indicated as putative synergistic pathomechanism (Sauer *et al.*, 2005, 2006; Yodoya *et al.*, 2006; Stellmer *et al.*, 2007). We have recently identified the blood–brain barrier as playing a central role in the neuropathogenesis of glutaric aciduria type I, by trapping intracerebrally generated glutaric acid and 3-hydroxyglutaric acid due to a low permeability of the blood–brain barrier and the choroid plexus for dicarboxylic acids (Kölker *et al.*, 2006*b*; Sauer *et al.*, 2006, 2010). This hypothesis was based on several *in vitro*, *in vivo* and post-mortem findings. Strikingly, two subgroups of patients called 'high excretors' and 'low excretors', excrete different amounts of glutaric acid and 3-hydroxyglutaric acid but revealed similar brain concentrations in post-mortem brain biopsies (Goodman *et al.*, 1977; Leibel *et al.*, 1980; Bennett *et al.*, 1986; Baric *et al.*, 1999; Funk *et al.*, 2005; Külkens *et al.*, 2005). Furthermore, they share the same risk for brain injury (Christensen *et al.*, 2004; Kölker *et al.*, 2006*a*). *Gcdh*$^{-/-}$ mice display highly increased cerebral levels of glutaric acid and 3-hydroxyglutaric acid (Koeller *et al.*, 2002; Sauer *et al.*, 2006) despite low cerebral GCDH activity in wild-type mice (Woontner *et al.*, 2000; Sauer *et al.*, 2006). Finally, the blood–brain barrier is only weakly permeable and the choroid plexus not permeable for these metabolites *in vitro* (Sauer *et al.*, 2010). These findings have led to the assumption that lowering the L-lysine influx to the brain is a therapeutic means of decreasing the accumulation of neurotoxic dicarboxylic metabolites in the brain.

If infants with glutaric aciduria type I are identified by newborn screening and metabolic treatment is started neonatally, motor dysfunction can be prevented in the majority of patients (Hoffmann *et al.*, 1996; Strauss *et al.*, 2003, 2007; Naughten

et al., 2004; Kölker *et al.*, 2007*b*; Bijarnia *et al.*, 2008; Heringer *et al.*, 2010). Metabolic treatment usually includes: (i) a low L-lysine diet; (ii) L-carnitine supplementation and (iii) intensified emergency treatment to prevent or reverse catabolism during intercurrent illness (Strauss *et al.*, 2003; Kölker *et al.*, 2007*a*). The biochemical proof of principle of these therapeutic strategies, i.e. lowering cerebral concentrations of neurotoxic metabolites, has not yet been shown in patients since glutaric acid and 3-hydroxyglutaric acid cannot be determined by magnetic resonance spectroscopy and CSF, plasma and urine concentrations do not correlate with brain concentrations.

The aim of the present study was to investigate the mitochondrial and peroxisomal branches of lysine metabolism in brain and liver, the biochemical effect of current therapeutic interventions and to develop new treatment strategies by inhibiting lysine transport across biological barriers, i.e. the blood–brain barrier and mitochondrial membrane using arginine supplementation. Furthermore, we tested the modulation of cerebral lysine oxidation as an additional therapeutic target.

Materials and methods

Animals

The *Gcdh*$^{-/-}$ mice used in this study were generated via gene targeting in mouse embryonic stem cells, and have been described previously (Koeller *et al.*, 2002; Sauer *et al.*, 2005, 2006; Zinnanti *et al.*, 2006, 2007). In *Gcdh*$^{-/-}$ mice, the first seven exons of the *Gcdh* gene have been replaced by a β-gal cassette that encodes a modified β-galactosidase enzyme that includes a nuclear localization sequence and is transcriptionally regulated by the *Gcdh* promoter. Mice used in the experiments described below were from the F4 to F6 generation on a C57BL/6J inbred background. Animal care and experiments followed the official governmental guidelines and were approved by the governmental review board (#35-9185.81/G-33/07). As it has been shown previously that *Gcdh*$^{+/+}$ and *Gcdh*$^{+/-}$ mice show the same range of biochemical key parameters in body fluids and tissue homogenates and thus are biochemically indistinguishable with regard to the key metabolites of glutaric aciduria type I (Koeller *et al.*, 2002; Sauer *et al.*, 2006; Zinnanti *et al.*, 2006, 2007), *Gcdh*$^{+/-}$ mice instead of *Gcdh*$^{+/+}$ were used as control animals to reduce the number of animals to be sacrificed for this study. Therefore, the term 'control mice' used in the manuscript indicates *Gcdh*$^{+/-}$ mice. *Gcdh*$^{+/-}$ females were bred with *Gcdh*$^{-/-}$ males. Unless stated otherwise, each experimental group contained five *Gcdh*$^{-/-}$ and five *Gcdh*$^{+/-}$ mice from the same litter.

Treatments used in *Gcdh*$^{-/-}$ mice

All treatment studies were started at P28 and were continued until P42. Mice were given *ad libitum* access to food and water. Before the start of treatment studies mean food and water intake was monitored for 7 days, revealing a mean intake of 4 g food per day and of 3.5 ml water per day.

Low L-lysine diet

Mice were fed with either a standard diet containing 1.7% (w/w) L-lysine (Altromin, C1069) or a low L-lysine diet. All diets are based on amino acid formulas to calculate amino acid intake. Low L-lysine diets contained 0.4, 0.2 and 0.1% L-lysine, respectively, with 0.2% being the minimal maintenance requirement of mice. In analogy to humans, we hypothesized that a diet containing the minimal L-lysine requirement for mice (i.e. a diet with 0.2% L-lysine) would reduce the concentrations of glutaric acid, 3-hydroxyglutaric acid and glutarylcarnitine effectively, but not affect weight gain. Note that the absolute amount of L-lysine intake used in the low L-lysine diet in mice is different from that recommended for children with glutaric aciduria type I (Kölker *et al.*, 2007*b*). This discrepancy is explained by species-dependent minimal L-lysine requirements of mice and humans. However, as a basic therapeutic strategy low L-lysine diets in both mice and humans aim to reduce L-lysine intake to the minimal requirements of each species.

L-Arginine supplementation

L-Lysine and L-arginine compete for system y^+ localized in the blood–brain barrier to enter the brain. We hypothesized that L-arginine supplementation should decrease the cerebral accumulation of glutaric acid and 3-hydroxyglutaric acid due to reduced cerebral influx of L-lysine. To test this hypothesis *Gcdh*$^{-/-}$ mice were fed on a standard diet or on a low L-lysine diet (0.2%). These diets contained 1.3% L-arginine as the standard maintenance requirement of mice. In parallel experiments, mice received standard L-arginine intake only or were additionally supplemented with 2 or 3% (w/w, referring to dietary L-arginine intake) L-arginine, which was added to drinking water, corresponding to 1.5- and 2-fold increases of daily L-arginine intake compared to maintenance requirements.

L-Carnitine supplementation

L-Carnitine supplementation has two aims: (i) to amplify the physiological detoxification of glutaryl-CoA via glutarylcarnitine and (ii) to prevent secondary L-carnitine depletion (Kölker *et al.*, 2007*a*). To evaluate L-carnitine supplementation, mice were fed a standard diet supplemented with L-carnitine via drinking water. Treatment was started with 100 mg/kg body weight per day, which did not affect glutaric acid or 3-hydroxyglutaric acid levels (brain: glutaric acid 116 ± 15% of control, 3-hydroxyglutaric acid 99 ± 16% of control; liver: glutaric acid 89 ± 40% of control, 3-hydroxyglutaric acid 74 ± 39% of control) in tissue and body fluids of *Gcdh*$^{-/-}$ mice. Therefore, L-carnitine supplementation was further increased to an excess dosage (500 or 1000 mg/kg body weight per day) to identify whether supraphysiological concentrations were able to lower glutaric acid and 3-hydroxyglutaric acid levels. The standard diet did not contain L-carnitine.

Clofibrate

In higher eukaryotes, L-lysine is not only degraded via the mitochondrial saccharopine but also via the L-pipecolate pathway (Mihalik and Rhead, 1989; Rao *et al.*, 1993) that is thought to be localized in peroxisomes. Therefore, we wondered whether induction of peroxisomal proliferation by clofibrate could modulate the concentrations of glutaric acid and 3-hydroxyglutaric acid. Mice were fed with a standard diet supplemented with 0.5% clofibrate (800 mg/kg body weight per day) via food. Catalase activity was determined as a marker for peroxisomal proliferation. Since clofibrate also induces the expression of enzymes of the mitochondrial fatty acid oxidation (Djouadi and Bastin, 2008), we tested the activity of medium-chain acyl-CoA dehydrogenase that is known to be upregulated by clofibrate. Furthermore, we studied whether clofibrate may induce GCDH activity. Medium-chain acyl-CoA dehydrogenase and GCDH activity were detected as previously described (Sauer *et al.*, 2005). Mice did not show any adverse effects of clofibrate treatment.

Preparation of tissue homogenates

Mice were sacrificed at P42, i.e. at the end of the treatment interval and perfused with a solution of phosphate buffered saline and 25 U/ml heparin. Afterwards, tissues (brain, liver and kidneys) were removed and chilled on ice in buffer A (0.1 ml per 0.1 mg of tissue) containing 250 mmol/l sucrose, 50 mmol/l KCl, 5 mmol/l $MgCl_2$, 20 mmol/l Tris/HCl (adjusted to pH 7.4). Tissues were homogenized with a Potter Elvehjem system using a pestle with a size that allows disruption of cell membranes but not organelles, and subcellular fractions were prepared. For preparation of mitochondrial enriched fractions, homogenates were centrifuged at 600*g* for 10 min at 4°C and the resulting supernatants were centrifuged at 3000*g* for 10 min at 4°C. The pellet of this centrifugation step was used as mitochondria-enriched fractions, since mitochondrial fractions prepared at a higher *g* are contaminated with peroxisomes as well as particles of the endoplasmic reticulum and Golgi apparatus. Mitochondria-enriched fractions were washed twice with buffer A. For preparation of mitochondrial membrane and matrix fractions the 3000*g* pellet was sonicated and centrifuged at 10 000*g* for 10 min at 4°C. The 3000*g* supernatant or the so-called light mitochondrial fraction was centrifuged at 8000*g* for 10 min at 4°C to minimize contamination with mitochondria. The resulting supernatant was used as peroxisomal enriched fractions. Due to the lack of methods to prepare brain peroxisomes at a sufficient quality and quantity, we decided to use this fraction for measuring cerebral peroxisomal enzymes. For the preparation of liver peroxisomes, the peroxisome-enriched fraction was centrifuged at 30 000*g* for 20 min. Cytosolic fractions of liver and brain homogenates were prepared by centrifuging the peroxisome-enriched fraction at 30 000*g* for 20 min.

Purity of the fractions was tested by assaying organelle-specific enzymes, i.e. the mitochondrial matrix enzyme citrate synthetase and the mitochondrial membrane component cytochrome *c* oxidase according to previously described protocols (Sauer *et al.*, 2005), and peroxisomal catalase activity. Protein was determined according to Lowry (Lowry *et al.*, 1951) with modifications (Helenius and Simons, 1972) using bovine serum albumin as a standard.

Quantitative analysis of glutaric acid and 3-hydroxyglutaric acid

Glutaric acid and 3-hydroxyglutaric acid were detected in tissue homogenates (600*g* supernatant) and serum from mice as previously described (Sauer *et al.*, 2006) using quantitative gas chromatography/mass spectrometry with stable-isotope dilution assay.

Quantitative analysis of glutarylcarnitine

Glutarylcarnitine was determined in tissue homogenates (600 *g* supernatant) and serum by electrospray ionization tandem mass spectrometry according to a modified method as previously described (Sauer *et al.*, 2006).

Amino acid analysis

Amino acid content of brain homogenates was analysed by high-performance liquid chromatography (LC3000 Eppendorf-Biotronik). To evaluate the impact of low L-lysine diet and L-arginine supplementation on brain amino acid levels, we grouped amino acids into: (i) (non-basic) non-essential amino acids (L-alanine, L-glycine, L-serine, L-asparagine, L-aspartic acid, L-glutamic acid and L-glutamine); (ii) (non-basic) essential amino acids (L-phenylalanine, L-valine, L-threonine, L-methionine, L-isoleucine, L-leucine, L-histidine and L-tryptophan) and (iii) (essential or non-essential) basic amino acids (L-lysine, L-arginine, L-ornithine and L-citrulline). Basic amino acids were grouped separately as described since they might have been influenced directly (L-lysine, L-arginine) or indirectly (L-ornithine, L-citrulline) by dietary treatment (for concentrations of single amino acids see Supplementary Table 1).

Enzyme analysis

2-Aminoadipate semialdehyde synthase

AASS is a bifunctional enzyme with LOR (EC 1.5.1.8) and saccharopine dehydrogenase (EC 1.5.1.9) activity. LOR and saccharopine dehydrogenase activity were determined as described previously (Blemings *et al.*, 1994).

2-Aminoadipate semialdehyde dehydrogenase

AASDH was prepared as previously described (Sadilkova *et al.*, 2009). AASDH (EC 1.2.1.31) activity was assayed in buffer A additionally containing 1 mmol/l NAD and 0.5 mmol/l aminoadipate semialdehyde at pH 8.5.

2-Aminoadipate aminotransaminase

AADAT (EC 2.6.1.39) activity was recorded in buffer A additionally containing 10 mmol/l oxoadipate, 1 mmol/l glutamate, 1 mmol/l alanine, 0.5 mmol/l NADH and 1 U/ml of alanine transaminase and lactate dehydrogenase (both Sigma Aldrich).

2-Oxoglutarate dehydrogenase complex assay

OGDHc (EC 1.2.4.2, 2.3.1.61, 1.8.1.4) activity was determined as previously described (Sauer *et al.*, 2005).

Catalase assay

Catalase activity was assayed in a buffer containing 50 mmol/l, 0.05% sodium deoxycholate and 15% H_2O_2 adjusted to pH 7 (25°C). Catalase activity was determined after the addition of 3 µg protein of liver homogenate at $\lambda = 240$ nm.

Pipecolate oxidase assay

The organellar localization (mitochondria, peroxisomes) and activity of L-pipecolic acid oxidation differs in mammalian species (Mihalik and Rhead, 1991) and the localization of this enzyme in mouse brain is still uncertain. Therefore, we assayed pipecolate oxidase activity in mitochondrial and peroxisomal enriched fractions of brain homogenates of control mice using L- and D-pipecolate. Pipecolate oxidase activity was assayed in a buffer consisting of 50 mmol/l Bis-Tris-propane, 1 mmol/l aminopyridine, 6 mmol/l tribromohydroxybenzoate, 0.1% Triton X-100, 10 mmol/l NaN_3, 25 µmol/l flavin adenine dinucleotide, 3.5 U/ml horseradish peroxidase and 50 mmol/l L- or D-pipecolate at 37°C (Ijlst *et al.*, 2000). For assays in the mitochondrial fractions pH was adjusted to 7.4 and for peroxisomal fractions to 8.4. For the characterization of a putative mitochondrial pipecolate oxidase, additional enzyme studies were performed in a buffer containing 250 mmol/l sucrose, 50 mmol/l KCl, 5 mmol/l $MgCl_2$ and 20 mmol/l Tris/HCl at pH 7.4 using functional mitochondria (mitochondrial enriched fraction) or mitochondria disrupted by detergent (0.01% [v/v] Triton X-100 or 0.1% [v/v] laurylmaltoside) or sonicated. Various electron acceptor systems were tested to assay pipecolate oxidase activity using 0.006 µmol/l phenazin methosulfate and 0.6 mmol/l

iodo-nitro-tetrazolium, 0.06 mmol/l dichlorophenol indophenol, 0.04 mmol/l ubiquinone and 0.06 mmol/l dichlorophenol indophenol, or by coupling of pipecolate oxidase activity to cytochrome *c* reduction by complex III of the respiratory chain by adding 0.04 mmol/l ubiquinone, 0.1 mmol/l cytochrome *c* and 1 mmol/l NaCN to mitochondria in analogy to a previously described proline dehydrogenase assay (Hu *et al.*, 2007). Under all experimental conditions specific activity was detected with and without the addition of substrate to calculate the baseline activity. The following wavelengths were used: (i) tribromo-hydroxybenzoate, $\lambda = 510$ nm; (ii) iodo-nitro-tetrazolium, $\lambda = 500$–750 nm; (iii) dichlorophenol indophenol, $\lambda = 610$–750 nm and (iv) cytochrome *c*, $\lambda = 540$–550 nm.

Mitochondrial uptake assay of Carbon-14-L-lysine

To study mitochondrial lysine uptake, cerebral and hepatic mitochondrial fractions were incubated with ^{14}C-L-lysine (1 mmol/l; specific activity, 0.1 μCi/nmol) in buffer A for 10 min at 37°C. Afterwards, mitochondria were washed twice with ice cold buffer A. The amount of intramitochondrial ^{14}C-L-lysine was measured using a scintillation counter (Beckmann LS6500).

Statistical analysis

All experiments were performed in ≥5 *Gcdh*$^{-/-}$ and control mice. Data are expressed as mean ± SD unless otherwise indicated. Tissue concentrations and enzyme activities were normalized to the protein concentration of the same sample. For statistical analysis we divided our experiments into experiments with and without an *a priori* hypothesis. In case of special diets we postulated that the treatment reduces the levels of toxic metabolites and therefore results were evaluated with ANOVA and repeated contrasts. The following contrasts were calculated: (i) Contrast A = comparison between control group and experimental group 1; (ii) Contrast B = comparison between experimental groups 1 and 2 and (iii) Contrast C = comparison between experimental groups 2 and 3. When lysine reduction was combined with arginine supplementation, two-factorial ANOVA was calculated to identify the contribution of each diet to the overall effect. Statistical parameters (*t* and *f* values) and exact *P*-values are described in Supplementary Table 2. Enzymatic studies were evaluated with ANOVA with Bonferroni adjustment for multiple comparisons or Student's *t*-test. All statistical analyses were performed by SPSS for Windows 16.0 Software. $P < 0.05$ was considered significant.

Results

Low L-lysine diet reduces glutaric acid and 3-hydroxyglutaric acid concentrations in *Gcdh*$^{-/-}$ mice

First, we investigated whether reduction of oral L-lysine intake for two weeks (P28 to P42) lowered glutaric acid and 3-hydroxyglutaric acid concentrations. *Gcdh*$^{-/-}$ and control mice were fed a standard diet containing 1.7% L-lysine or on a low L-lysine diet containing 0.4% (Group 1), 0.2% (minimal L-lysine requirement; Group 2) or 0.1% L-lysine (below minimal L-lysine requirement; Group 3). *Gcdh*$^{-/-}$ mice fed on standard diet showed strongly elevated glutaric acid (brain: 8.0 ± 1.4 nmol/mg protein or 1200 ± 210 μmol/l; liver: 14.8 ± 2.9 nmol/mg protein or 5286 ± 1036 μmol/l) and 3-hydroxyglutaric acid concentrations (brain: 0.3 ± 0.05 nmol/mg protein or 45 ± 8 μmol/l; liver: 0.4 ± 0.04 nmol/mg protein or 143 ± 14 μmol/l) compared to control mice [glutaric acid (brain; liver): 0.08 ± 0.02 nmol/mg protein or 12 ± 3 μmol/l; 0.1 ± 0.03 nmol/mg protein or 36 ± 11 μmol; 3-hydroxyglutaric acid (brain; liver): 0.06 ± 0.01 nmol/mg protein or 9 ± 2 μmol/l; 0.05 ± 0.01 nmol/mg protein or 18 ± 4 μmol/l). Reducing the L-lysine content of the diet resulted in a dose-dependent decrease in glutaric acid concentrations in brain and liver (Fig. 1A), whereas 3-hydroxyglutaric acid levels remained unaffected (Fig. 1B). Glutaric acid and 3-hydroxyglutaric acid levels in control mice on these diets remained unchanged (data not shown).

Animals started on the amino acid-based standard diet showed a transient drop of weight during the first week (87 ± 4% of P28), but subsequently gained weight again until P42 (90 ± 7% of P28).

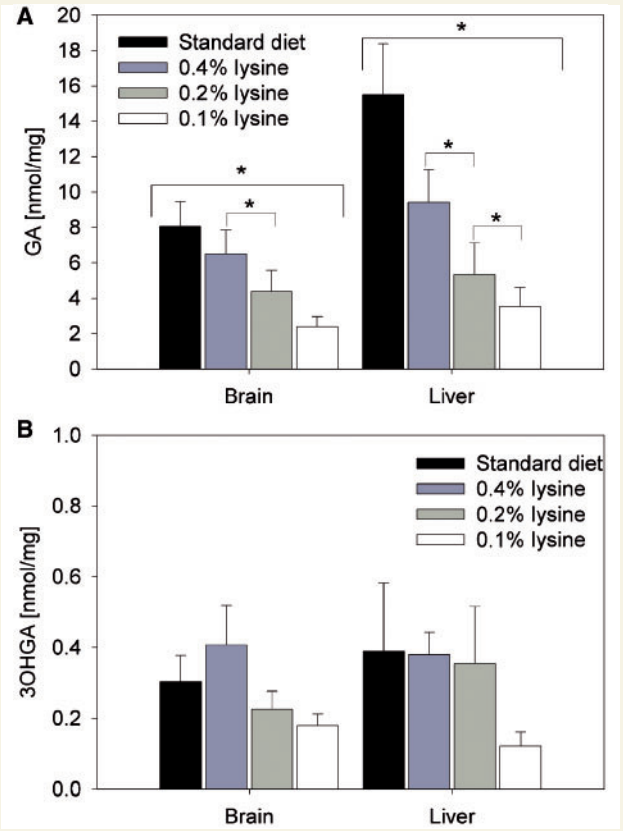

Figure 1 Low L-lysine diet reduces glutaric acid concentrations in brain and liver. *Gcdh*$^{-/-}$ mice were fed on a standard diet containing 1.7% L-lysine or on a low lysine diet containing 0.4%, 0.2% (minimal L-lysine requirement) or 0.1% L-lysine. Dietary reduction of L-lysine levels decreased the concentrations of glutaric acid (GA) in liver and brain in a dose-dependent manner (**A**), whereas 3-hydroxyglutaric acid (3OHGA) concentrations remained virtually unchanged (**B**). Data are expressed as mean ± SD in nmol/mg protein ($n = 5$ mice per group; *$P < 0.05$, ANOVA and repeated contrasts).

The same was found for the 0.2 and 0.4% L-lysine diets. In contrast, mice fed on a 0.1% L-lysine diet continued to lose weight during the whole feeding period (P35: 83 ± 7% of weight at P28; P42: 80 ± 10%). Therefore, the 0.1% L-lysine diet was discontinued and for all following experiments the 0.2% L-lysine diet was used.

L-Arginine supplementation amplifies the biochemical effect of low L-lysine diet

Since L-arginine competes with L-lysine at dibasic amino acid transporters, such as cationic amino acid transporters belonging to system y^+ at the blood–brain barrier (O'Kane *et al.*, 2006), we tested whether L-arginine supplementation alone or in combination with low L-lysine diet lowered the cerebral glutaric acid and 3-hydroxyglutaric acid concentrations. Oral administration of L-arginine (3%) to *Gcdh*$^{-/-}$ mice receiving standard diet mimicked the biochemical effects of low L-lysine diet and decreased glutaric acid concentrations (brain: 74 ± 13% of *Gcdh*$^{-/-}$ mice fed on standard diet; liver: 68 ± 15%; $P < 0.05$). A combination of low L-lysine diet (0.2% L-lysine and 1.3% L-arginine; Group 1) and additional L-arginine supplementation [total L-arginine content: 2% (Group 2) or 3% (Group 3)] additively decreased cerebral and hepatic glutaric acid (Fig. 2A) and 3-hydroxyglutaric acid concentrations (Fig. 2B) in *Gcdh*$^{-/-}$ mice. However, increasing the L-arginine content of the diet from 2 to 3% did not further decrease glutaric acid and 3-hydroxyglutaric acid concentrations in brain or liver. Two-factorial ANOVA revealed main effects for L-lysine reduction and L-arginine supplementation on brain and liver glutaric acid and 3-hydroxyglutaric acid levels. Serum and kidney concentrations were not reduced by L-arginine supplementation (Supplementary Fig. 1). Glutaric acid and 3-hydroxyglutaric acid levels of control mice remained unaffected by these diets (Supplementary Fig. 2).

L-Arginine inhibits mitochondrial L-lysine uptake

Since L-arginine supplementation and low L-lysine diet additively reduced the cerebral and hepatic concentrations of glutaric acid and 3-hydroxyglutaric acid in *Gcdh*$^{-/-}$ mice, we wondered whether competition between L-arginine and L-lysine at the mitochondrial ornithine carriers 1 and 2 might contribute to the additive effect of L-arginine supplementation. The human mitochondrial ornithine carriers 1 and 2 (SLC25A15, SLC25A2) mediate the mitochondrial uptake of basic amino acids (Fiermonte *et al.*, 2003), such as L-lysine, L-arginine and L-ornithine. We therefore studied the uptake of ^{14}C-L-lysine (1 mmol/l; specific activity, 0.1 μCi/nmol) in cerebral and hepatic mitochondria of control and *Gcdh*$^{-/-}$ mice and subsequently investigated if mitochondrial uptake was blocked by L-arginine. In fact, L-arginine (2 mmol/l) strongly reduced mitochondrial L-lysine uptake in brain and liver mitochondria of control and *Gcdh*$^{-/-}$ mice (reduction of mitochondrial ^{14}C-L-lysine uptake by L-arginine: brain, 85 ± 6%; liver, 52 ± 1%; $P = 0.000$).

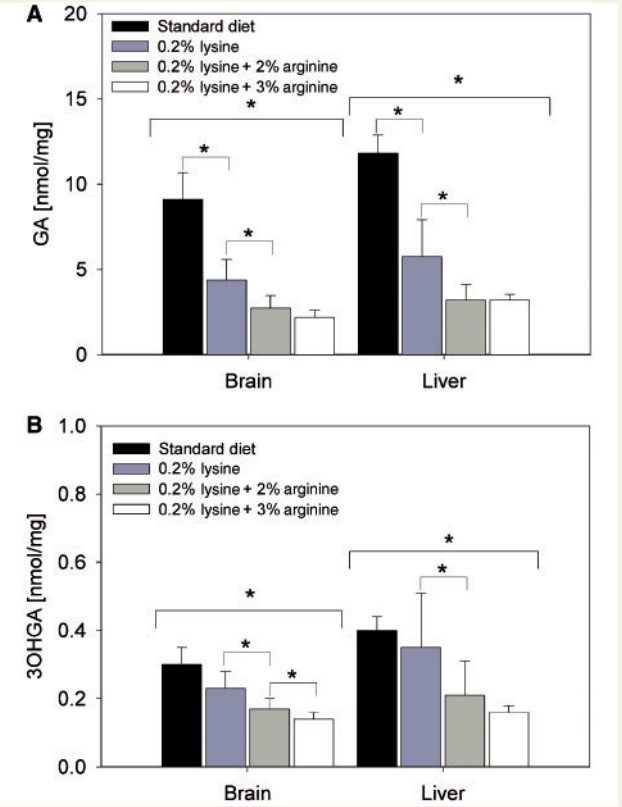

Figure 2 L-Arginine supplementation amplifies the effect of low L-lysine diet. A combination of low L-lysine diet (0.2%; containing 1.3% L-arginine) and L-arginine supplementation (total content: 2 or 3%) amplified the biochemical effect of a low L-lysine diet and thus further decreased the cerebral and hepatic glutaric acid (GA) concentrations (**A**) and 3-hydroxyglutaric acid (3OHGA) concentrations (**B**). Data are expressed as mean ± SD in nmol/mg protein ($n = 5$ mice per group; $^*P < 0.05$, ANOVA and repeated contrasts).

Effect of dietary treatment on brain amino acid levels

Next we studied the effect of dietary treatment on brain amino acid levels. Low L-lysine diet (containing 0.2% L-lysine) did not significantly change brain levels of amino acids in general. Similarly, a combination of low L-lysine diet and L-arginine supplementation (2%) had no significant effect on brain amino acids, whereas at a higher dosage (3%) L-arginine supplementation caused a mild decrease of brain non-essential and essential amino acids. Brain levels of L-lysine were not affected by low L-lysine diet but were decreased by L-arginine supplementation (Supplementary Fig. 3).

Divergent therapeutic modulation of tissue-specific glutarylcarnitine and free L-carnitine concentrations

Glutarylcarnitine concentrations were significantly elevated in brain, liver and serum of *Gcdh*$^{-/-}$ mice (Fig. 3; Supplementary Fig. 4)

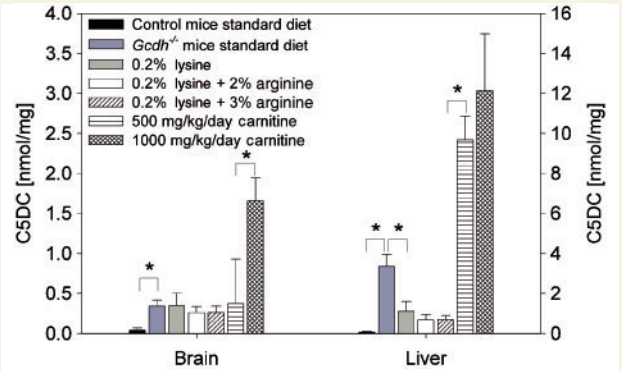

Figure 3 Divergent therapeutic modulation of glutarylcarnitine concentrations. Glutarylcarnitine (C5DC) concentrations were increased in brain and liver of *Gcdh*$^{-/-}$ mice compared to control mice. Low L-lysine diet (0.2%) alone or in combination with L-arginine supplementation (2 or 3%) decreased glutarylcarnitine concentrations in liver but not in brain. Carnitine supplementation (500 or 1000 mg/kg body weight) caused a massive and dose-dependent increase of glutarylcarnitine in liver and—less pronounced—in brain of *Gcdh*$^{-/-}$ mice. Data are expressed as mean ± SD in nmol/mg protein (brain, primary *y*-axis; liver secondary *y*-axis; $n = 5$ mice per group; $^{*}P < 0.05$, ANOVA and repeated contrasts).

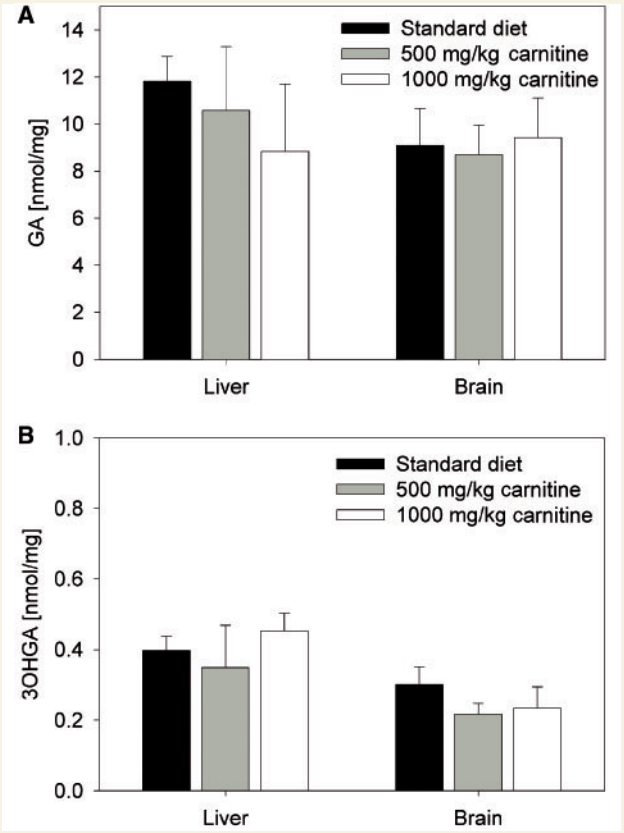

Figure 4 L-Carnitine supplementation does not affect glutaric acid and 3-hydroxyglutaric acid (3OHGA) concentrations. Even at high doses, carnitine supplementation (500 or 1000 mg/kg body) did not reduce hepatic and cerebral glutaric acid (GA) (**A**) and 3-hydroxyglutaric acid (**B**) concentrations in *Gcdh*$^{-/-}$ mice. Data are expressed as mean ± SD in nmol/mg protein.

compared to control mice. Low L-lysine diet [0.2% L-lysine (Group 1)] alone or in combination with L-arginine [2% (Group 2) or 3% L-arginine (Group 3)] decreased glutarylcarnitine concentrations in liver and serum but not in brain (Fig. 3; Supplementary Fig. 4). Two-factorial ANOVA revealed a main effect of low L-lysine diet on liver and serum glutarylcarnitine levels.

L-Carnitine supplementation [500 mg/kg body weight (Group 1) or 1000 mg/kg body weight (Group 2)] caused a massive increase of glutarylcarnitine in tissue and serum of *Gcdh*$^{-/-}$ mice (Fig. 3; Supplementary Fig. 4) but not in control mice (Supplementary Fig. 5), confirming that L-carnitine supplementation stimulates the formation of glutarylcarnitine in *Gcdh*$^{-/-}$ mice. Despite the increased formation of glutarylcarnitine and thus removal of glutaryl-CoA, cerebral and hepatic concentrations of glutaric acid (Fig. 4A) and 3-hydroxyglutaric acid (Fig. 4B) remained virtually unchanged after L-carnitine supplementation in *Gcdh*$^{-/-}$ mice fed on a standard diet, highlighting that L-carnitine supplementation alone even at high doses is insufficient to lower glutaric acid and 3-hydroxyglutaric acid levels. In control mice, dietary treatment and L-carnitine supplementation did not significantly influence tissue-specific glutarylcarnitine concentrations (Supplementary Fig. 5).

When fed on a standard diet, free carnitine concentrations were significantly lower in brain and serum of *Gcdh*$^{-/-}$ mice than in control mice, whereas the hepatic free L-carnitine concentration was similar in both groups (Fig. 5; Supplementary Fig. 4). Low L-lysine diet (0.2%) and L-arginine supplementation (2 and 3%) did not change free L-carnitine concentrations in brain, liver and serum of *Gcdh*$^{-/-}$ mice (Fig. 5; Supplementary Fig. 4). In contrast, L-carnitine supplementation restored tissue-specific free L-carnitine concentrations in *Gcdh*$^{-/-}$ (Fig. 5; Supplementary Fig. 4**)** and

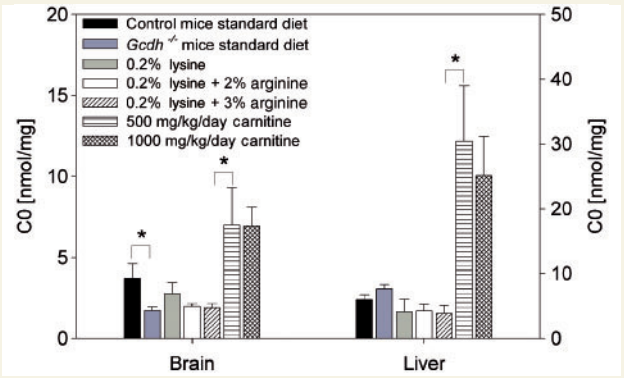

Figure 5 Therapeutic modulation of the free carnitine pool. Free carnitine (C0) concentrations were significantly lower in the brain of *Gcdh*$^{-/-}$ mice than in control mice, whereas the hepatic free L-carnitine concentrations were similar in both groups. Carnitine supplementation increased free L-carnitine concentrations in *Gcdh*$^{-/-}$ mice. In contrast, low L-lysine diet (0.2%) alone or in combination with L-arginine supplementation (2 or 3%) did not restore the decreased free L-carnitine concentrations. Data are expressed as mean ± SD in nmol/mg protein (brain, primary *y*-axis; liver secondary *y*-axis; $n = 5$ mice per group; $^{*}P < 0.05$, ANOVA and repeated contrasts).

increased free L-carnitine concentrations above the normal range in control mice (Supplementary Fig. 6).

Discrepant role of mitochondrial and peroxisomal pathways of L-lysine oxidation in brain and liver

To identify new treatment strategies for glutaric aciduria type I, we investigated cerebral L-lysine metabolism in *Gcdh*$^{-/-}$ and control mice. It has been reported that dietary L-lysine supply modulates the activity of AASS (Blemings *et al.*, 1998). Upregulation of this bifunctional enzyme (LOR/saccharopine dehydrogenase) would counteract the aim of a low L-lysine diet, whereas pharmacological inhibition of this enzyme could be a new therapeutic option for glutaric aciduria type I. Therefore, we determined LOR and saccharopine dehydrogenase activity in the mitochondrial matrix fractions of liver and brain of *Gcdh*$^{-/-}$ and control mice fed on a low L-lysine diet (0.2%) with or without L-arginine supplementation. Hepatic activity of LOR and saccharopine dehydrogenase was similar in control and *Gcdh*$^{-/-}$ mice and was not influenced by dietary treatment (Fig. 6A and B). In brain, however, LOR and saccharopine dehydrogenase activities were below the detection limit of the method. Neither mitochondrial membrane fractions nor cytosolic or peroxisomal enriched fractions of liver and brain displayed LOR or saccharopine dehydrogenase activity underlining the purity of our fractions. In addition to enzymatic studies, we compared AASS messenger RNA expression in different brain regions (striatum, cortex and cerebellum) with liver tissue. Messenger RNA expression of AASS was very low, if at all (Supplementary Fig. 7). We wondered whether the lack of cerebral LOR and saccharopine dehydrogenase activity reflects a low L-lysine oxidation capacity of the brain in general. Therefore, we measured the subsequent enzymatic steps of L-lysine oxidation, i.e. AASDH, AADAT and OGDHc. In contrast to AASS we found significant activities of AASDH and AADAT in cytosolic fractions of liver and brain of mutant and control mice (Fig. 6C). We further identified OGDHc activity in the mitochondrial matrix fraction of liver and brain tissue. Cerebral OGDHc activity was slightly higher in control than in mutant mice ($P = 0.049$), whereas hepatic OGDHc activity did not differ (Fig. 6C). In line with decreased cerebral OGDHc activity in *Gcdh*$^{-/-}$ mice, concentrations of 2-aminoadipate were elevated in brain tissue compared to control mice (*Gcdh*$^{-/-}$ mice: 0.27 ± 0.02 nmol/mg protein; control: 0.18 ± 0.05 nmol/mg protein; $P = 0.012$) suggesting accumulation of metabolites upstream of OGDHc.

Since it is an ongoing debate whether OGDHc catalyzes the conversion of 2-oxodipate to glutaryl-CoA (Sherman *et al.*, 2008), we tested if 2-oxoadipate is a substrate of OGDHc. In our hands, 2-oxoadipate was degraded by OGDHc using purified enzyme from porcine heart (Sigma Aldrich) and in liver mitochondria from control mice. Using equimolar concentrations of 2-oxoadipate and 2-oxoglutarate (1 mmol/l), 2-oxoadipate dehydrogenase activity of OGDHc was estimated to be three times lower than its 2-oxoglutarate dehydrogenase activity as previously described (Supplementary Fig. 8; Hirashima *et al.*, 1967; Majamaa *et al.*, 1985; Bunik and Pavlova, 1997).

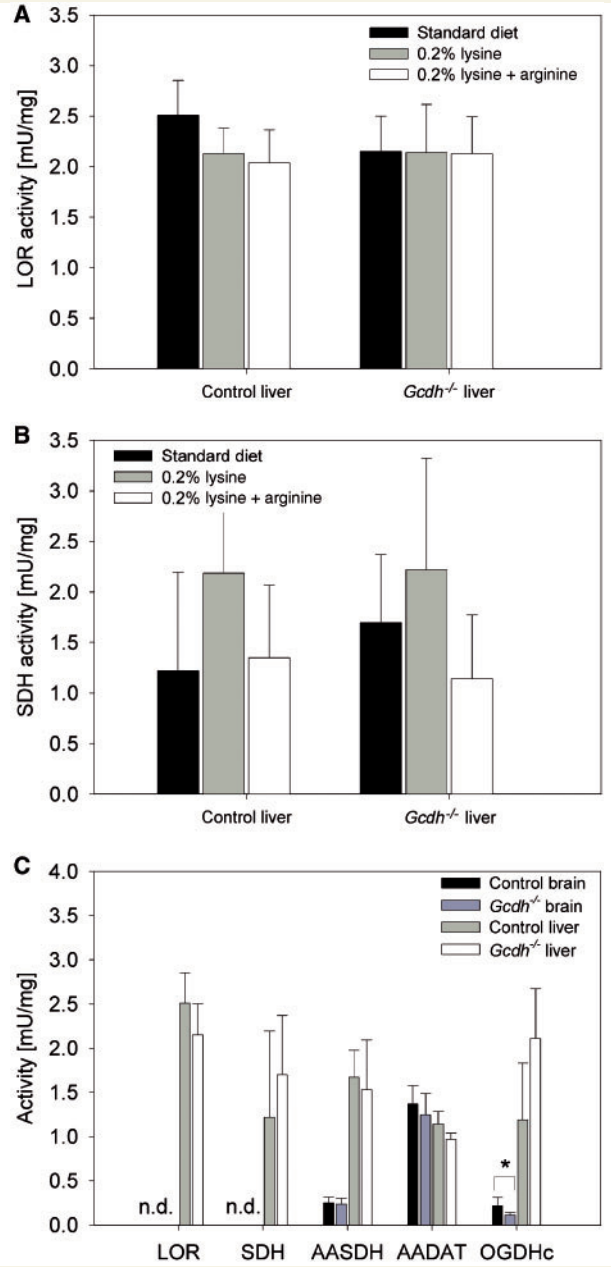

Figure 6 Mitochondrial saccharopine pathway in brain and liver. We investigated whether dietary treatment modulates the activity of the bifunctional enzyme AASS containing LOR and saccharopine dehydrogenase activity. Since there was no biochemical difference between animals receiving 2 and 3% L-arginine, these groups were pooled. Hepatic activity of LOR (**A**) and saccharopine dehydrogenase (SDH) (**B**) enzymes was not affected by dietary treatment in control and *Gcdh*$^{-/-}$ mice. We could not detect LOR or saccharopine dehydrogenase activity in brain tissue, whereas the subsequent enzymes of L-lysine oxidation, AASDH, AADAT and OGDHc, were detectable in brain and liver of control and *Gcdh*$^{-/-}$ mice (**C**). Cerebral OGDHc activity was decreased in *Gcdh*$^{-/-}$ mice compared to control mice. Data are expressed as mean ± SD in mU/mg protein ($n = 5$ mice per group; $^*P < 0.05$, Student's *t*-test; n.d. = not detectable).

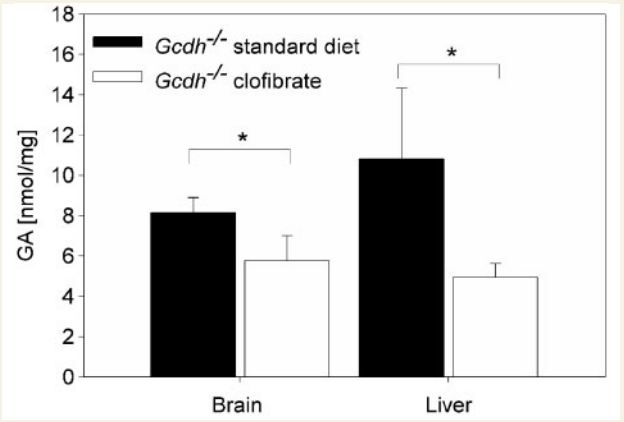

Figure 7 Clofibrate treatment. Mice were fed with the peroxisome proliferator-activated receptor-alpha agonist clofibrate (0.5%). Clofibrate treatment induced a decrease of glutaric acid (GA) concentrations in brain and liver of $Gcdh^{-/-}$ mice. Data are expressed as mean ± SD in nmol/mg protein ($n = 6$ mice per group; $^*P < 0.05$, Student's t-test).

Since the very low cerebral AASS (LOR/saccharopine dehydrogenase) activity is contrasted by relatively higher AASDH and AADAT activities (Fig. 6C), we wondered whether the pipecolate pathway is the major route of L-lysine oxidation in the brain. Pipecolate oxidase is the key enzyme of this pathway (Ijlst *et al.*, 2000). Determination of pipecolate oxidase activity in mitochondria- and peroxisome-enriched fractions of brain homogenates demonstrated a discrepant localization of L- and D-pipecolate oxidase activity. As reported in humans, L-pipecolate oxidase activity was detected in peroxisome-enriched fractions (0.04 ± 0.006 mU/mg protein), whereas D-pipecolate oxidase activity was found in mitochondria-enriched fractions (0.01 ± 0.005 U/mg) and mitochondrial membrane fractions (0.01 ± 0.002 mU/mg protein). D-Pipecolate oxidase activity was only detectable in undisrupted mitochondria or purified mitochondrial membranes (after sonication) indicating localization to the mitochondrial membrane. L- and D-Pipecolate oxidase activities were only detectable in a horseradish peroxidase-coupled system, suggesting oxygen as electron acceptor in both reactions.

To further investigate the role of the peroxisomal pipecolate pathway in L-lysine oxidation, we fed mice with the peroxisome proliferator-activated receptor-alpha activator clofibrate (0.5%). Peroxisomal proliferation was strongly induced by clofibrate treatment, as indicated by increased catalase activity (278 ± 28% of untreated $Gcdh^{-/-}$ mice). Unexpectedly, clofibrate treatment caused a significant decrease in the glutaric acid concentration in the liver ($P = 0.011$) and—less pronounced—also in the brain ($P = 0.049$), whereas serum glutaric acid concentration remained unchanged (Fig. 7; Supplementary Table 3). In contrast, serum and tissue 3-hydroxyglutaric acid concentrations were not influenced by clofibrate treatment (data not shown).

The finding of clofibrate-induced decrease in hepatic and cerebral glutaric acid concentrations suggests an alternative peroxisomal breakdown of glutaric acid. It has been shown that glutaryl-CoA can be oxidized by the inducible peroxisomal acyl-CoA (palmitoyl-CoA) oxidase I (van Veldhoven *et al.*, 1992; Wanders *et al.*, 1993). Therefore, we assayed this enzyme in purified liver peroxisomes using the same buffer as described for the L-pipecolate oxidase assay with palmitoyl-CoA as substrate. Inducible acyl-CoA oxidase I activity was significantly increased in clofibrate-treated mice (untreated mice: 7.5 ± 2.5 mU/mg protein; clofibrate-treated mice: 14.6 ± 1.1 mU/mg protein). Accordingly, glutaryl-CoA oxidase activity was detectable after clofibrate treatment in liver peroxisomes (0.5 ± 0.6 mU/mg). These results highlight that clofibrate pre-treatment enhances alternative hepatic and cerebral peroxisomal breakdown of glutaric acid thereby lowering glutaric acid concentrations.

Medium-chain acyl-CoA dehydrogenase activity was 2.5-fold increased after clofibrate treatment in liver mitochondria of $Gcdh^{-/-}$ mice, whereas the cerebral medium-chain acyl-CoA dehydrogenase activity was unchanged. However, neither in brain nor in liver mitochondria did we find evidence that glutaryl-CoA is a substrate of any other mitochondrial flavin adenine dinucleotide-dependent acyl-CoA dehydrogenase than Gcdh (which is lacking in $Gcdh^{-/-}$ mice), virtually excluding alternative mitochondrial breakdown of glutaryl-CoA in $Gcdh^{-/-}$ mice (data not shown).

Discussion

The aim of the present study was to elucidate the biochemical effects of current dietary treatment for glutaric aciduria type I, to develop additional treatment strategies and to investigate tissue-specific differences of L-lysine oxidation in brain and liver.

Therapeutic modulation of L-lysine transport and metabolism

The blood–brain barrier has low permeability for dicarboxylic acids, preventing plasma glutaric acid and 3-hydroxyglutaric acid from entering the brain at a significant rate (Sauer *et al.*, 2006, 2010). As a consequence, accumulation of neurotoxic glutaric acid, 3-hydroxyglutaric acid and glutaryl-CoA in the brains of glutaric aciduria type I patients and $Gcdh^{-/-}$ mice is mostly the result of L-lysine oxidation in the brain compartment (Sauer *et al.*, 2006; Zinnanti *et al.*, 2007). It is thus probable that modulation of L-lysine intake changes the cerebral concentrations of neurotoxic dicarboxylic metabolites. This is supported by increased cerebral glutaric acid and 3-hydroxyglutaric acid concentrations and induction of a phenotype similar to an acute encephalopathic crisis in weanling $Gcdh^{-/-}$ mice by a high L-lysine diet (Zinnanti *et al.*, 2006, 2007). In contrast, reduction of L-lysine intake to age-dependent minimal requirements of patients is recommended for patients with glutaric aciduria type I (Kölker *et al.*, 2007*a*). Two studies have shown a neuroprotective effect of low L-lysine diet (Kölker *et al.*, 2006*a*; Heringer *et al.*, 2010), whereas another study has failed to demonstrate a beneficial effect of dietary treatment (Strauss *et al.*, 2007). A mechanistic understanding of low L-lysine diet has been hampered by the fact that cerebral glutaric acid and 3-hydroxyglutaric acid concentrations can only be determined by invasive methods and therefore, rarely available data of

brain concentrations are limited to a few post-mortem investigations and one brain biopsy (Goodman *et al.*, 1977; Bennett *et al.*, 1986; Funk *et al.*, 2005; Külkens *et al.*, 2005). In the present study we show that reduced dietary L-lysine supply causes a concentration-dependent decrease of cerebral glutaric acid but not of 3-hydroxyglutaric acid concentrations. This is in line with previous observations in patients with glutaric aciduria type I showing a major decrease in urinary excretion of glutaric acid but only minor or no changes of 3-hydroxyglutaric acid concentrations after the start of dietary treatment (Strauss *et al.*, 2003; Harting *et al.*, 2009).

Furthermore, we demonstrate that L-arginine supplementation in combination with L-lysine reduction additively reduces cerebral glutaric acid and 3-hydroxyglutaric acid, supporting the idea that L-arginine competes with L-lysine for transport into the brain at system y^+ of the blood–brain barrier. This is in line with a previous study showing that homoarginine, a homologue of L-arginine, also decreases cerebral glutaric acid concentrations (Zinnanti *et al.*, 2007). Notably, many patients with glutaric aciduria type I receive L-lysine-free amino acid supplements (Kölker *et al.*, 2007*a*, *b*; Heringer *et al.*, 2010) that also contain L-arginine (Supplementary Table 4). However, unlike in patients with urea cycle defects, selective L-arginine supplementation has not yet been used for the treatment of patients with glutaric aciduria type I (Coman *et al.*, 2008). Interestingly, L-arginine supplementation also reduced hepatic glutaric acid and 3-hydroxyglutaric acid concentrations. Since system y^+ (SLC7A1-3) is only expressed at the blood–brain barrier but not in the liver (Smith, 2000; Hawkins *et al.*, 2006), additional transport systems for cationic amino acids must contribute to this effect. Two mitochondrial transporters mediate dibasic amino acid transport from the cytosol into the mitochondrial matrix, human mitochondrial ornithine carriers 1 and—less active—2 (Fiermonte *et al.*, 2003). Mitochondrial L-lysine uptake is thought to be the rate-limiting step in L-lysine oxidation via the saccharopine pathway (Blemings *et al.*, 1998; Benevenga and Blemings, 2007) and L-lysine influx via human mitochondrial ornithine carrier 1 is inhibited by L-arginine and L-ornithine, but less effectively by their D-isomers or homoarginine (Fiermonte *et al.*, 2003). Accordingly, our study shows that mitochondrial ^{14}C-L-lysine uptake in brain and liver of *Gcdh*$^{-/-}$ and control mice was strongly reduced by L-arginine. We therefore assume that the competition between L-lysine and L-arginine at human mitochondrial ornithine carrier 1 contributes to reduced glutaric acid and 3-hydroxyglutaric acid levels, especially in the liver.

L-Carnitine is also recommended for the treatment of patients with glutaric aciduria type I (Kölker *et al.*, 2007*a*). L-Carnitine supplementation is thought to improve the outcome of patients with glutaric aciduria type I by: (i) amplification of the physiological detoxification of glutaryl-CoA by increased formation of glutarylcarnitine formation thereby replenishing the intracellular free CoA pool and (ii) prevention of secondary L-carnitine depletion (Seccombe *et al.*, 1986; Hoffmann *et al.*, 1996; Kölker *et al.*, 2006*a*, 2007*a*; Sauer *et al.*, 2006). Both effects could be shown in *Gcdh*$^{-/-}$ mice after carnitine supplementation. We found no evidence that L-carnitine also reduces cerebral or hepatic accumulation of glutaric acid and 3-hydroxyglutaric acid.

New insights in cerebral L-lysine metabolism

Cerebral L-lysine oxidation is poorly understood yet. Based on studies in bacteria, the first steps of mammalian L-lysine oxidation were originally believed to be catalyzed by the pipecolate pathway. Later, discovery of the bifunctional enzyme AASS in mammalian mitochondria (Blemings *et al.*, 1994; Sacksteder *et al.*, 2000) and identification of mutations of this enzyme in familial hyperlysinaemia suggested the mitochondrial saccharopine pathway to be the primary route of L-lysine oxidation in most tissues. Several studies in mice and rats, however, indicate that cerebral L-lysine oxidation differs from other organs in that the peroxisomal pipecolate pathway is the major route (Chang, 1976, 1978; Giacobini *et al.*, 1980; Rao *et al.*, 1993; Ijlst *et al.*, 2000).

Since our study aimed to therapeutically modulate cerebral L-lysine oxidation, we investigated the activity of involved enzymes. AASS activity is known to be upregulated by high protein diet (Blemings *et al.*, 1998; Sacksteder *et al.*, 2000), glucagon (Scislowski *et al.*, 1994) and starvation (Papes *et al.*, 1999). In our study we show that reduction of L-lysine intake to minimal dietary requirements did not upregulate AASS. This is an important finding since upregulated AASS would counteract the dietary aim of reducing L-lysine oxidation. In contrast to the liver, we did not detect AASS in brain mitochondria. The lack of cerebral AASS virtually excludes a significant role of the saccharopine pathway of L-lysine oxidation in the brain. This may explain why inherited deficiency of AASS causing familial hyperlysinaemia does not produce a neurological phenotype in affected individuals (Sacksteder *et al.*, 2000). Furthermore, our finding of a lack of cerebral AASS necessitates a thorough revision of the proposed mechanism underlying cerebral injury in weanling *Gcdh*$^{-/-}$ mice due to high-lysine diet. It was speculated that increased cerebral L-lysine influx enhances saccharopine formation and thus 2-oxoglutarate depletion (Zinnanti *et al.*, 2007). However, based on our results, enhanced saccharopine formation is likely to occur in liver but not in brain.

The absence of detectable AASS activity in brain mitochondria is in contrast to relatively high activities of the following enzymatic steps, i.e. AASDH, AADAT and OGDHc. Therefore we tested whether the pipecolate pathway of L-lysine degradation is active in brain. L-Pipecolate oxidase is the key enzyme of this pathway (Ijlst *et al.*, 2000) and is characterized by a species-dependent subcellular localization. Mitochondrial L-pipecolate oxidase activity was demonstrated in liver and brain of rabbit, guinea pig, pig, dog and sheep (Mihalik and Rhead, 1991), peroxisomal L-pipecolate oxidase activity was found in liver of monkey and man (Mihalik and Rhead, 1989; Singh *et al.*, 1989; Mihalik *et al.*, 1991; Rao and Chang, 1992) and in rats it was detected in mitochondria and peroxisomes (Rao *et al.*, 1993). Surprisingly, little is known about the murine enzyme. Our study provides evidence for L-pipecolate oxidase localized in peroxisomes of mouse brain. In context with the lack of AASS activity, our data suggest that cerebral L-lysine oxidation is initiated by the pipecolate pathway.

To upregulate the peroxisomal L-pipecolate oxidation, we administered the peroxisome proliferator-activated receptor-alpha

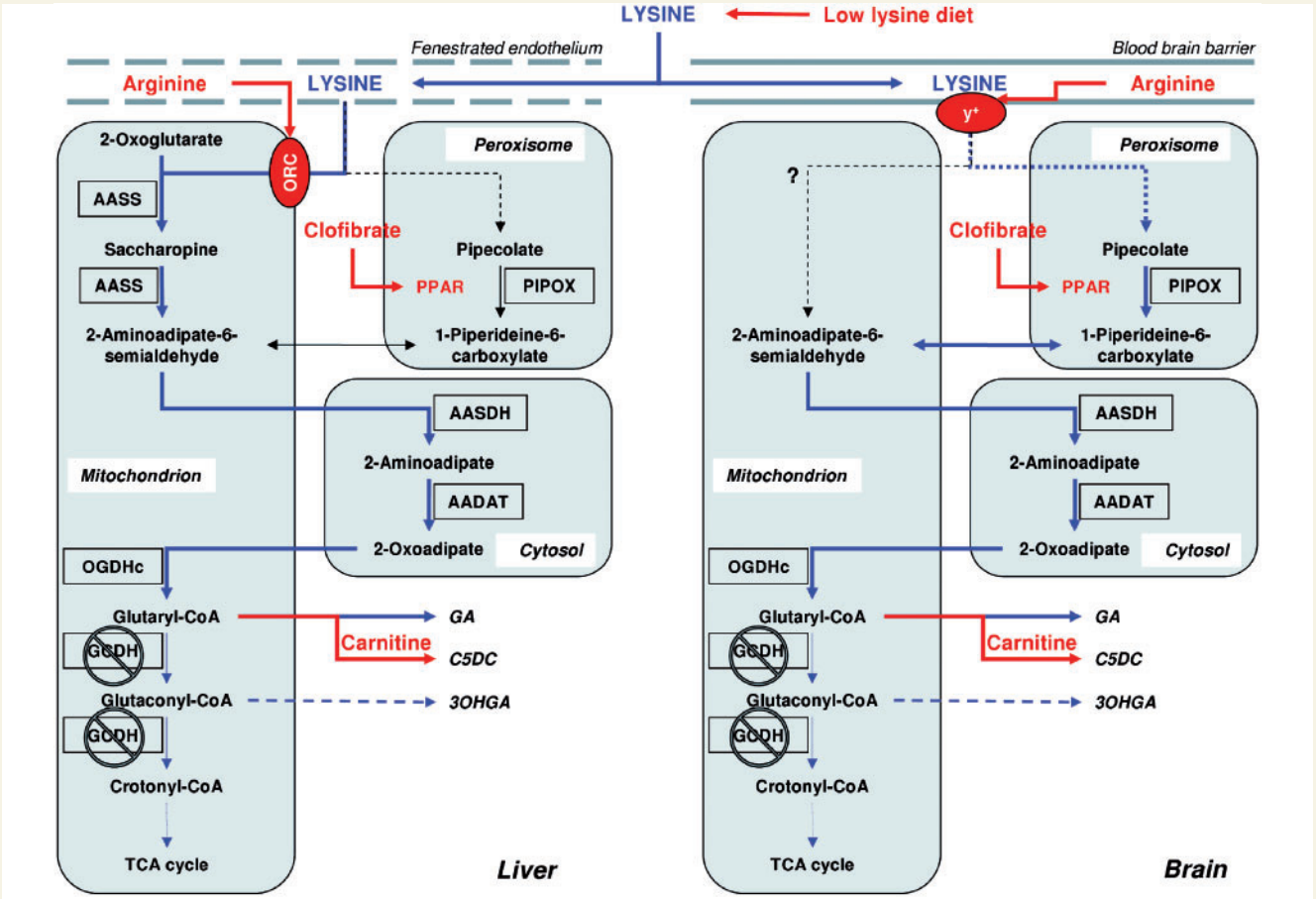

Figure 8 Discrepant cerebral and hepatic L-lysine oxidation pathways and proposed mechanisms of their therapeutic modulation. Based on our findings in *Gcdh*$^{-/-}$ mice, we propose that L-lysine oxidation differs in liver (*left*) and brain (*right*). The mitochondrial saccharopine pathway is the major route in the liver, whereas the peroxisomal pipecolate pathway is the major route in the brain. After mitochondrial (saccharopine pathway) or peroxisomal (pipecolate pathway) initiation of L-lysine oxidation, both branches converge in 2-aminoadipic-6-semialdehyde and then follow the same distal route. Cerebral L-lysine oxidation is best described by a three-compartment model (peroxisome, cytosol, mitochondrion), whereas in the liver L-lysine oxidation is merely a two-compartment model (mitochondrion, cytosol). However, the peroxisomal compartment can be activated by clofibrate treatment. In *Gcdh*$^{-/-}$ mice, L-lysine oxidation and the formation of neurotoxic metabolites can be modulated by: (i) reduction of the precursor amino acid L-lysine by low L-lysine diet; (ii) competition between L-arginine and L-lysine at the mitochondrial membrane and the blood–brain barrier and (iii) enhanced formation of glutarylcarnitine from glutaryl-CoA by L-carnitine supplementation. Furthermore, clofibrate pre-treatment reduces glutaric acid concentration. The underlying mechanism of this effect is still unknown, however, upregulation of peroxisomal glutaryl-CoA oxidation via acyl-CoA thioesterases and inducible acyl-CoA oxidase I is a putative explanation. Blue lines highlight the proposed major pathways of L-lysine breakdown in brain and liver, therapeutic means and proposed targets are highlighted in red. 3OHGA = 3-hydroxyglutaric acid; C5DC = glutarylcarnitine; GA = glutaric acid; ORC = human mitochondrial ornithine carriers 1 and 2; PIPOX = pipecolate oxidase; PPAR = peroxisome proliferator-activated receptor-alpha; TCA cycle = tricarboxylic acid cycle; y^+ = system y^+ of the blood–brain barrier.

agonist clofibrate to *Gcdh*$^{-/-}$ mice assuming that this treatment should increase glutaric acid production. Surprisingly, clofibrate treatment decreased glutaric acid concentrations. This finding suggests concomitant upregulation of a peroxisomal glutaryl-CoA degradation pathway by clofibrate. The notion of peroxisomal glutaryl-CoA oxidation is underlined by the existence of the peroxisomal acyl-CoA thioesterases 4 and 8 with chain-length specificity for medium-chain dicarboxyl-CoA esters, such as succinyl-CoA and glutaryl-CoA (Sacksteder *et al.*, 1999; Westin *et al.*, 2005). Furthermore, glutaryl-CoA can be oxidized by the inducible acyl-CoA oxidase I (van Veldhoven *et al.*, 1992; Wanders *et al.*, 1993), an enzyme that was strongly induced after clofibrate treatment in *Gcdh*$^{-/-}$ mice. Upregulation of an alternative peroxisomal glutaryl-CoA catabolism may provide a novel therapeutic option that should be further studied.

The enzymatic steps leading to pipecolate formation in mammalian cells are still unknown. In this context, a recent study by Struys and Jakobs (2010) on L-lysine oxidation in fibroblasts of patients with pyridoxine-dependent epilepsy suggested that pipecolate is formed via an unspecific mechanism from 2-aminoadipate semialdehyde and does not directly derive from L-lysine, suggesting that L-pipecolate oxidase is a metabolite repair

enzyme. However, these experiments have been performed in fibroblasts and do not necessarily reflect the cerebral and hepatic L-lysine oxidation. Furthermore, the proposed mechanism does not explain why hyperpipecolataemia is found in patients with inherited AASS deficiency (Dancis and Hutzler, 1986). In this condition, 2-aminoadipate semialdehyde cannot be formed due to the lack of AASS. In addition, a study by Murthy and colleagues (1999) provides evidence for the enzymatic formation of delta-1-piperidine-2-carboxylate, the precursor of pipecolate, in mouse brain.

In conclusion, we demonstrate for the first time that low L-lysine diet decreases cerebral and hepatic concentrations of glutaric acid and glutarylcarnitine, and that L-arginine supplementation amplifies this effect due to competition with L-lysine at dibasic amino acid transporters, including system y^+ at the blood–brain barrier and mitochondrial L-ornithine carriers 1 (and 2). Supplementary Table 4 shows how these results could be translated to improved dietary treatment for patients with glutaric aciduria type I, which remains to be tested in clinical trials. Furthermore, we provide evidence that the peroxisomal pipecolate pathway but not the mitochondrial saccharopine pathway initiates L-lysine oxidation in the brain. In addition, our study indicates that glutaric acid can be degraded in peroxisomes and that this pathway is enhanced by peroxisome proliferator-activated receptor-alpha activation, suggesting novel approaches to treatment may be possible via pharmacologic manipulation of alternative metabolic pathways. A synopsis of discrepant cerebral and hepatic L-lysine oxidation pathways as well as the proposed mechanisms of their therapeutic modulation is shown in Fig. 8.

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Supplementary material

Supplementary material is available at *Brain* online.

References

Bahr O, Mader I, Zschocke J, Dichgans J, Schulz JB. Adult onset glutaric aciduria type I presenting with a leukoencephalopathy. Neurology 2002; 59: 1802–4.

Baric I, Wagner L, Feyh P, Liesert M, Buckel W, Hoffmann GF. Sensitivity and specificity of free and total glutaric acid and 3-hydroxyglutaric acid measurements by stable-isotope dilution assays for the diagnosis of glutaric aciduria type I. J Inherit Metab Dis 1999; 22: 867–82.

Benevenga NJ, Blemings KP. Unique aspects of lysine nutrition and metabolism. J Nutr 2007; 137: 1610–5.

Bennett JP, Logan WJ, Snyder SH. Amino acids as central nervous transmitters: the influence of ions, amino acid analogues and ontogeny on transport systems for L-glutamic and L-aspartic acids and glycine into central nervous synaptosomes of the rat. J Neurochem 1973; 21: 1533–50.

Bennett MJ, Marlow N, Pollitt RJ, Wales JK. Glutaric aciduria type 1: biochemical investigations and postmortem findings. Eur J Pediatr 1986; 145: 403–5.

Bijarnia S, Wiley V, Carpenter K, Christodoulou J, Ellaway CJ, Wilcken B. Glutaric aciduria type I: outcome following detection by newborn screening. J Inherit Metab Dis 2008; 31: 503–7.

Bjugstad KB, Goodman SI, Freed CR. Age at symptom onset predicts severity of motor impairment and clinical outcome of glutaric acidemia type 1. J Pediatr 2000; 137: 681–6.

Blemings KP, Crenshaw TD, Benevenga NJ. Mitochondrial lysine uptake limits hepatic lysine oxidation in rats fed diets containing 5, 20 or 60% casein. J Nutr 1998; 128: 2427–34.

Blemings KP, Crenshaw TD, Swick RW, Benevenga NJ. Lysine-alpha-ketoglutarate Reductase and Saccharopine Dehydrogenase Are Located Only in the Mitochondrial Matrix in Rat Liver. J Nutr 1994; 124: 1215–21.

Bunik VI, Pavlova OG. Inactivation of alpha-ketoglutarate dehydrogenase during its enzymatic reaction. Biochemistry (Mosc) 1997; 62: 973–82.

Chang YF. Pipecolic acid pathway: the major lysine metabolic route in the rat brain. Biochem Biophys Res Commun 1976; 69: 174–80.

Chang YF. Lysine metabolism in the rat brain: blood-brain barrier transport, formation of pipecolic acid and human hyperpipecolatemia. J Neurochem 1978; 30: 355–60.

Chang YF, Ghosh P, Rao VV. L-pipecolic acid metabolism in human liver: L-alpha-aminoadipate delta-semialdehyde oxidoreductase. Biochim Biophys Acta 1990; 1038: 300–5.

Christensen E, Ribes A, Merinero B, Zschocke J. Correlation of genotype and phenotype in glutaryl-CoA dehydrogenase deficiency. J Inherit Metab Dis 2004; 27: 861–8.

Coman D, Yaplito-Lee J, Boneh A. New indications and controversies in arginine therapy. Clin Nutr 2008; 27: 489–96.

Dancis J, Hutzler J. The significance of hyperpipecolatemia in Zellweger syndrome. Am J Hum Genet 1986; 38: 707–11.

Djouadi F, Bastin J. PPARs as therapeutic targets for correction of inborn mitochondrial fatty acid oxidation disorders. J Inherit Metab Dis 2008; 31: 217–25.

Dwyer TM, Rao KS, Goodman SI, Frerman FE. Proton Abstraction Reaction, Steady-State Kinetics, and Oxidation Reduction Potential of Human Glutaryl-CoA Dehydrogenase. Biochemistry 2000; 39: 11488–99.

Fiermonte G, Dolce V, David L, Santorelli FM, Dionisi-Vici C, Palmieri F, et al. The mitochondrial ornithine transporter bacterial expression, renconstitution, functional characterization and tissue distribution of two human isoforms. J Biol Chem 2003; 278: 32778–83.

Funk CB, Prasad AN, Frosk P, Sauer S, Kolker S, Greenberg CR, et al. Neuropathological, biochemical and molecular findings in a glutaric acidemia type 1 cohort. Brain 2005; 128: 711–22.

Giacobini E, Nomura Y, Schmidt-Glenewinkel T. Pipecolic acid: origin, biosynthesis and metabolism in the brain. Cell Mol Biol Incl Cyto Enzymol 1980; 26: 135–46.

Goodman SI, Markey SP, Moe PG, Miles BS, Teng CC. Glutaric aciduria; a "new" disorder of amino acid metabolism. Biochem Med 1975; 12: 12–21.

Goodman SI, Norenberg MD, Shikes RH, Breslich DJ, Moe PG. Glutaric aciduria: biochemical and morphologic considerations. J Pediatr 1977; 90: 746–50.

Goodman SI, de Stein X, Schlesinger S, Christensen E, Schwartz M, Greenberg CR, et al. Glutaryl-CoA dehydrogenase mutations in glutaric acidemia (type I): review and report of thirty novel mutations. Hum Mutat 1998; 12: 141–4.

Greenberg CR, Duncan AM, Gregory CA, Singal R, Goodman SI. Brief Report: assignment of Human Glutaryl-CoA Dehydrogenase Gene (GCDH) to the Short Arm of Chromosome 19 (19p13. 2) by in Situ Hybridization and Somatic Cell Hybrid Analysis. Genomics 1994; 21: 289–90.

Harting I, Neumaier-Probst E, Seitz A, Maier EM, Assmann B, Baric I, et al. Dynamic changes of striatal and extrastriatal abnormalities in glutaric aciduria type I. Brain 2009; 132: 1764–82.

Hawkins RA, O'Kane RL, Simpson LA, Viña JR. Structure of the blood-brain barrier and its role in the transport of amino acids. J Nutr 2006; 136: 218–26.

Helenius A, Simons K. The binding of detergents to lipophilic and hydrophilic proteins. J Biol Chem 1972; 247: 3656–61.

Heringer J, Boy SPN, Ensenauer R, Assmann B, Zschocke J, Harting I, et al. Use of guidelines improves the neurological outcome in glutaric aciduria type I. Ann Neurol 2010; in press.

Hirashima M, Hayakawa T, Koike M. Mammalian alpha-Keto Acid Dehydrogenase Complexes. II. An improved procedure for the preparation 2-oxoglutarate dehydrogenase complex from pig heart muscle. J Biol Chem 1967; 242: 902–7.

Hoffmann GF, Athanassopoulos S, Burlina AB, Duran M, de Klerk JB, Lehnert W, et al. Clinical course, early diagnosis, treatment, and prevention of disease in glutaryl-CoA dehydrogenase deficiency. Neuropediatrics 1996; 27: 115–23.

Hu CA, Donald SP, Yu J, Lin WW, Liu Z, Steel G, et al. Overexpression of proline oxidase induces proline-dependent and mitochondria-mediated apoptosis. Mol Cell Biochem 2007; 295: 85–92.

Ijlst L, de Kromme I, Oostheim W, Wanders RJ. Molecular cloning and expression of human L-pipecolate oxidase. Biochem Biophys Res Commun 2000; 270: 1101–5.

Koeller DM, Woontner M, Crnic LS, Kleinschmidt-DeMasters B, Stephens J, Hunt EL, et al. Biochemical, pathologic and behavioral analysis of a mouse model of glutaric acidemia type I. Hum Mol Genet 2002; 11: 347–57.

Kölker S, Koeller DM, Okun JG, Hoffmann GF. Pathomechanisms of neurodegeneration in glutaryl-CoA dehydrogenase deficiency. Ann Neurol 2004; 55: 7–12.

Kölker S, Garbade SF, Greenberg CR, Leonard JV, Saudubray JM, Ribes A, et al. Natural History, Outcome, and Treatment Efficacy in Children and Adults with Glutaryl-CoA Dehydrogenase Deficiency. Pediatr Res 2006a; 59: 840–7.

Kölker S, Sauer SW, Surtees RA, Leonard JV. The aetiology of neurological complications of organic acidaemiasa role for the blood-brain barrier. J Inherit Metab Dis 2006b; 29: 701–4.

Kölker S, Christensen E, Leonard JV, Greenberg CR, Burlina AB, Burlina AP, et al. Guideline for the diagnosis and management of glutaryl-CoA dehydrogenase deficiency (glutaric aciduria type I). J Inherit Metab Dis 2007a; 30: 5–22.

Kölker S, Garbade SF, Boy N, Maier EM, Meissner T, Mühlhausen C, et al. Decline of acute encephalopathic crises in children with glutaryl-CoA dehydrogenase deficiency identified by newborn screening in Germany. Pediatr Res 2007b; 62: 357–63.

Külkens S, Harting I, Sauer S, Zschocke J, Hoffmann GF, Gruber S, et al. Late-onset neurologic disease in glutaryl-CoA dehydrogenase deficiency. Neurology 2005; 64: 2142–4.

Kyllerman M, Skjeldal O, Christensen E, Hagberg G, Holme E, Lönnquist T, et al. Long-term follow-up, neurological outcome and survival rate in 28 Nordic patients with glutaric aciduria type 1. Eur J Paediatr Neurol 2004; 8: 121–9.

Leibel RL, Shih VE, Goodman SI, Bauman ML, McCabe ER, Zwerdling RG, et al. Glutaric acidemia: a metabolic disorder causing progressive choreoathetosis. Neurology 1980; 30: 1163–8.

Lindner M, Kölker S, Schulze A, Christensen E, Greenberg CR, Hoffmann GF. Neonatal screening for glutaryl-CoA dehydrogenase deficiency. J Inherit Metab Dis 2004; 27: 851–9.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265–75.

Majamaa K, Turpeenniemi-Hujanen TM, Latipää P, Günzler V, Hanauske-Abel HM, Hassinen IE, et al. Differences between collagen hydroxylases and 2-oxoglutarate dehydrogenase in their inhibition by structural analogues of 2-oxoglutarate. Biochem J 1985; 229: 127–33.

Mihalik SJ, McGuinness M, Watkins PA. Purification and characterization of peroxisomal L-pipecolic acid oxidase from monkey liver. J Biol Chem 1991; 266: 4822–30.

Mihalik SJ, Rhead WJ. L-pipecolic acid oxidation in the rabbit and cynomolgus monkey. Evidence for differing organellar locations and cofactor requirements in each species. J Biol Chem 1989; 264: 2509–17.

Mihalik SJ, Rhead WJ. Species variation in organellar location and activity of l-pipecolic acid oxidation in mammals. J Comp Physiol B 1991; 160: 671–5.

Murthy SN, Janardanasarma MK. Identification of L-amino acid/L-lysine alpha-amino oxidase in mouse brain. Mol Cell Biochem 1999; 197: 13–23.

Naughten ER, Mayne PD, Monavari AA, Goodman SI, Sulaiman G, Croke DT. Glutaric aciduria type I: outcome in the Republic of Ireland. J Inherit Metab Dis 2004; 27: 917–20.

O'Kane RL, Vina JR, Simpson I, Zaragoza R, Mokashi A, Hawkins RA. Cationic amino acid transport across the blood-brain barrier is mediated exclusively by system y+. Am J Physiol Endocrinol Metab 2006; 291: 412–9.

Okuno E, Tsujimoto M, Nakamura M, Kido R. 2-Aminoadipate-2-oxoglutarate aminotransferase isoenzymes in human liver: a plausible physiological role in lysine and tryptophan metabolism. Enzyme Protein 1993; 47: 136–48.

Papes F, Kemper EL, Cord-Neto G, Langone F, Arruda P. Lysine degradation through the saccharopine pathway in mammals: involvement of both bifunctional and monofunctional lysine-degrading enzymes in mouse. Biochem J 1999; 344: 555–63.

Porciúncula LO, Dal-Pizzol A, Coitinho AS, Emanuelli T, Souza DO, Wajner M. Inhibition of synaptosomal (3H) glutamate uptake and (3H) glutamate binding to plasma membranes from brain of young rats by glutaric acid in vitro. J Neurol Sci 2000; 173: 93–6.

Rao VV, Chang YF. Assay for L-pipecolate oxidate activity in human liver: detection of enzyme deficiency in hyperpipecolic acidaemia. Biochim Biophys Acta - Molecular Basis of Disease 1992; 1139: 189–95.

Rao VV, Tsai MJ, PAN X, Chang YF. L-pipecolic acid oxidation in rat: subcellular localization and developmental study. Biochim Biophys Acta - Protein structure and molecular enzymology 1993; 1164: 29–35.

Sacksteder KA, Biery BJ, Morrell JC, Goodman BK, Geisbrecht BV, Cox RP, et al. Identification of the alpha-aminoadipic semialdehyde synthase gene, which is defective in familial hyperlysinemia. Am J Hum Genet 2000; 66: 1736–43.

Sacksteder KA, Morrell JC, Wanders RJ, Matalon R, Gould SJ. MCD encodes peroxisomal and cytoplasmic forms of malonyl-CoA decarboxylase and is mutated in malonyl-CoA decarboxylase deficiency. J Biol Chem 1999; 274: 24461–8.

Sauer SW, Okun JG, Schwab MA, Crnic LR, Hoffmann GF, Goodman SI, et al. Bioenergetics in glutaryl-coenzyme A dehydrogenase deficiency: a role for glutaryl-coenzyme A. J Biol Chem 2005; 280: 21830–6.

Sauer SW, Okun JG, Fricker G, Mahringer A, Muller I, Crnic LR, et al. Intracerebral accumulation of glutaric and 3-hydroxyglutaric acids secondary to limited flux across the blood-brain barrier constitute a biochemical risk factor for neurodegeneration in glutaryl-CoA dehydrogenase deficiency. J Neurochem 2006; 97: 899–910.

Sauer SW, Opp S, Mahringer A, Kamiński MM, Thiel C, Okun JG, et al. Glutaric aciduria type I and methylmalonic aciduria: simulation of cerebral import and export of accumulating neurotoxic dicarboxylic acids in in vitro models of the blood-brain barrier and the choroid plexus. Biochim Biophys Acta 2010; 1802: 552–60.

Scislowski PW, Foster AR, Fuller MF. Regulation of oxidative degradation of L-lysine in rat liver mitochondria. Biochem J 1994; 300: 887.

Seccombe DW, James L, Booth F. L-Carnitine treatment in glutaric aciduria type I. Neurology 1986; 36: 264–7.

Sherman EA, Strauss KA, Tortorelli S, Bennett MJ, Knerr I, Morton DH, et al. Genetic Mapping of Glutaric Aciduria, Type 3, to Chromosome 7 and Identification of Mutations in C7orf10. Am J Hum Genet 2008; 83: 604–9.

Singh H, Usher S, Poulos A. Mitochondrial and peroxisomal -oxidation of stearic and lignoceric acids by rat brain. J Neurochem 1989; 53: 1711–8.

Sadilkova K, Gospe SM Jr, Hahn SH. Simultaneous determination of alpha-aminoadipic semialdehyde, piperideine-6-carboxylate and pipecolic acid by LC-MS/MS for pyridoxine-dependent seizures and folinic acid-responsive seizures. J Neurosci Methods 2009; 184: 136–41.

Smith QR. Transport of glutamate and other amino acids at the blood-brain barrier. J Nutr 2000; 130: 1016–22.

Stellmer F, Keyser B, Burckhardt BC, Koepsell H, Streichert T, Glatzel M, et al. 3-Hydroxyglutaric acid is transported via the sodium-dependent dicarboxylate transporter NaDC3. J Mol Med 2007; 85: 763–70.

Stokke O, Goodman SI, Moe PG. Inhibition of brain glutamate decarboxylase by glutarate, glutaconate, and beta-hydroxyglutarate: explanation of the symptoms in glutaric aciduria? Clin Chim Acta 1976; 66: 411–5.

Strauss KA, Lazovic J, Wintermark M, Morton DH. Multimodal imaging of striatal degeneration in Amish patients with glutaryl-CoA dehydrogenase deficiency. Brain 2007; 130: 1905–20.

Strauss KA, Puffenberger EG, Robinson DL, Morton DH. Type I glutaric aciduria, part 1: natural history of 77 patients. Am J Med Genet 2003; 15: 38–52.

Struys EA, Jakobs C. Metabolism of lysine in alpha-aminoadipic semialdehyde dehydrogenase-deficient fibroblasts: evidence for an alternative pathway of pipecolic acid formation. FEBS Lett 2010; 584: 181–6.

van Veldhoven PP, Vanhove G, Assselberghs S, Eyssen HJ, Mannaerts GP. Substrate specificities of rat liver peroxisomal acyl-CoA oxidases: palmitoyl-CoA oxidase (inducible acyl-CoA oxidase), pristanoyl-CoA oxidase (non-inducible acyl-CoA oxidase), and trihydroxycoprostanoyl-CoA oxidase. J Biol Chem 1992; 267: 20065–74.

Wanders RJ, Denis SW, Dacremont G. Studies on the substrate specificity of the inducible and non-inducible acyl-CoA oxidases from rat kidney perxiosomes. J Biochem 1993; 113: 577–82.

Westin MA, Hunt MC, Alexson SE. The identification of a succinyl-CoA thioesterase suggests a novel pathway for succinate production in peroxisomes. J Biol Chem 2005; 280: 38125–32.

Woontner M, Crnic LS, Koeller DM. Analysis of the expression of murine glutaryl-CoA dehydrogenase: in vitro and in vivo studies. Mol Genet Metab 2000; 69: 116–22.

Yodoya E, Wada M, Shimada A, Katsukawa H, Okada N, Yamamoto A, et al. Functional and molecular identification of sodium-coupled dicarboxylate transporters in rat primary cultured cerebrocortical astrocytes and neurons. J Neurochem 2006; 97: 162–73.

Zinnanti WJ, Lazovic J, Housman C, LaNoue KO, Callaghan JP, Simpson I, et al. Mechanism of age-dependent susceptibility and novel treatment strategy in glutaric acidemia type I. J Clin Invest 2007; 117: 3258–70.

Zinnanti WJ, Lazovic J, Wolpert EB, Antonetti DA, Smith MB, Connor JR, et al. A diet-induced mouse model for glutaric aciduria type I. Brain 2006; 129: 899–910.

Zschocke J, Quak E, Guldberg P, Hoffmann GF. Mutation analysis in glutaric aciduria type I. Br Med J 2000; 37: 177–81.