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Transport and distribution of 3-hydroxyglutaric acid before and during induced encephalopathic crises in a mouse model of glutaric aciduria type 1

Britta Keyser [a], Markus Glatzel [b], Franziska Stellmer [a], Bastian Kortmann [a], Zoltan Lukacs [a], Stefan Kölker [c], Sven W. Sauer [c], Nicole Muschol [a], Wilhelm Herdering [d], Joachim Thiem [e], Stephen I. Goodman [f], David M. Koeller [g], Kurt Ullrich [a], Thomas Braulke [a], Chris Mühlhausen [a,*]

[a] Children's Hospital, Department of Biochemistry, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany
[b] Department of Neuropathology, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany
[c] Department of General Pediatrics, Division of Inherited Metabolic Diseases, University Children's Hospital Heidelberg, 69120 Heidelberg, Germany
[d] Institute of Inorganic and Applied Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany
[e] Institute of Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany
[f] University of Colorado Health Sciences Center at Fitzsimons, Department of Pediatrics, 12605 E 16th Avenue, Denver, CO 80220, USA
[g] Oregon Health and Science University, Department of Pediatrics, CDRC, 707 SW Gaines Road, Portland, OR 97239, USA

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ABSTRACT

Glutaric aciduria type 1 (GA1) is caused by the deficiency of glutaryl-CoA dehydrogenase (GCDH). Affected patients are prone to the development of encephalopathic crises during an early time window with destruction of striatal neurons and a subsequent irreversible movement disorder. 3-Hydroxyglutaric acid (3OHGA) accumulates in tissues and body fluids of GA1 patients and has been shown to mediate toxic effects on neuronal as well as endothelial cells. Injection of (^{3}H)-labeled into 6 week-old Gcdh$^{-/-}$ mice, a model of GA1, revealed a low recovery in kidney, liver, or brain tissue that did not differ from control mice. Significant amounts of 3OHGA were found to be excreted via the intestinal tract. Exposure of Gcdh$^{-/-}$ mice to a high protein diet led to an encephalopathic crisis, vacuolization in the brain, and death after 4–5 days. Under these conditions, high amounts of injected ^{3}H-3OHGA were found in kidneys of Gcdh$^{-/-}$ mice, whereas the radioactivity recovered in brain and blood was reduced. The data demonstrate that under conditions mimicking encephalopathic crises the blood–brain barrier appears to remain intact.

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1. Introduction

Glutaric aciduria type 1 (GA1, MIM 231670) is caused by defects of the mitochondrial matrix protein glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7), an enzyme involved in the degradation of the amino acids lysine, hydroxylysine and tryptophan. Mutations in the GCDH gene lead to the accumulation of glutaric acid (GA), 3-hydroxyglutaric acid (3OHGA), and in some patients also glutaconic acid in body fluids and tissues. GA1 patients are prone to the development of encephalopathic crises triggered by catabolic stress induced by fever, infections, vomiting and/or diarrhea. During these catabolic crises, concentrations of the metabolites show a further increase. The development of an encephalopathic crisis is accompanied by destruction of striatal neurons with a subsequent irreversible disabling movement disorder. Children affected by GA1 are at risk for such a crisis during a time window from 3 to 36 months of age. After completion of the third year of life an encephalopathic crisis occurs only sporadically [1,2]. Current therapeutic approaches focus on the restriction of lysine intake, stabilization of energy supply, replenishing the diminished carnitine pool, and implementation of intensified therapy during illness with acute catabolic states [3,4]. Newborn screening techniques have been implemented to identify GA1 patients presymptomatically [5]. Despite early treatment and intensified management, a proportion of GA1 patients still suffer encephalopathic crises [6,7].

Several underlying mechanisms of GA1 have been discussed. It has been assumed that 3OHGA is the GA1-specific metabolite crucial for the disease-specific symptoms because GA also accumulates in other organic acidurias [8]. Thus, several effects of 3OHGA have been described: excitotoxic effects in vitro, depletion of energy-releasing phosphates, and impairment of endothelial barrier properties in vitro and in vivo [8–12].

A Gcdh-deficient mouse displays a biochemical and histopathological phenotype resembling findings in GA1 patients but develops no encephalopathic crises [13]. However, exposure of young Gcdh$^{-/-}$ mice to a high protein and/or high lysine diet induces an increase of GA and 3OHGA in body fluids and tissues accompanied by acute striatal injury and neuronal loss, appearing as striking movement disorder, paralysis, seizures and death occurring within 2–8 days [12]. Furthermore,

* Corresponding author. Tel.: +49 40 42803 1966; fax: +49 40 42803 8504.
E-mail address: muehlhau@uke.uni-hamburg.de (C. Mühlhausen).

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Table 1
Tissue distribution of (^{3}H)-3OHGA in Gcdh$^{-/-}$ and control mice under basal conditions

Tissue	Recovery of radioactivity (%)			
	42 d		100 d	
	Wildtype	Gcdh$^{-/-}$	Wildtype	Gcdh$^{-/-}$
Kidney	2.5 (2.2–2.8)	2.95 (1.3–3.7)	2.55 (2.5–2.6)	4.5 (2.4–6.6)
Liver	4.35 (3.7–5)	1.45 (1.3–2)[a]	3.95 (3–4.9)	3.95 (3.5–4.4)
Brain	6.05 (4.8–7.3)	2.95 (1.2–4.1)[a]	4.75 (2.9–6.6)	4.25 (4.1–4.4)
Blood	51.5 (34–69)	53 (40–64)	49 (29–69)	44 (34–54)
Stomach/bowel/ faeces	7.55 (5.6–9.5)	14.5 (13.8–29)[a]	36.8 (11.6–62)	16.1 (10.2–22)

Radioactivity was determined in the indicated tissues (% of total recovered radioactivity exclusive of radioactivity excreted into urine) in wildtype and Gcdh$^{-/-}$ mice. Values represent median and range of 2-4 experiments per group. Significance was tested by Mann–Whitney-*U* tests.
[a] Different but not significant ($p=0.06$).

Table 2
GA and 3OHGA concentrations in 42 d old Gcdh$^{-/-}$ mice on normal diet and HPD

	GA		3OHGA	
	Gcdh$^{-/-}$ ND	Gcdh$^{-/-}$ HPD	Gcdh$^{-/-}$ ND	Gcdh$^{-/-}$ HPD
Plasma (μmol/l)	255±180	338±204	7.2±2.1	*10.5±1.8
Urine (mmol/mol creatinine)	33,926±6056	*50,934±9927	898±126	*1414±168

Concentrations of endogenous GA and 3OHGA were determined at day 4 of dietary treatment. ND: normal diet; HPD: high protein diet. Levels are given as means±SD of determinations in 4–5 independent animals per group. Significance (HPD vs. ND) was tested by unpaired two-tailed *t*-test. *$p<0.05$.

damage of striatal vessels during the diet-induced encephalopathic crisis has been described [12]. Disturbances in the integrity of intracerebral endothelial barriers have also been considered in GA1 patients [14]. Previous in vitro and in vivo studies have shown that in the presence of 3OHGA functional properties of endothelial cells are impaired and the integrity of vasculature and endothelial barriers are disrupted [11].

In the present report we used (^{3}H)-labeled 3OHGA to monitor the tissue distribution and excretion of 3OHGA in wildtype and Gcdh$^{-/-}$ mice in vivo. We also evaluated the effect of a diet-induced encephalopathic crisis on the tissue distribution and excretion of 3OHGA, and the integrity of the blood–brain barrier. We show that in 6 week-old Gcdh$^{-/-}$ mice circulating (^{3}H)-3OHGA enters the brain only to a very limited extent under both basal conditions and during a diet-induced encephalopathic crisis. In contrast the kidney levels of (^{3}H)-3OHGA are significantly elevated during a diet-induced crisis. We also report a significant role for the gut in the excretion of 3OHGA.

2. Materials and methods

2.1. Mice

Gcdh$^{-/-}$ mice and wild type littermate controls were generated from heterozygotes [13]. The genetic background in all mice groups used in this study was C57BL6/SJ129 hybrid. Genotypes were confirmed by PCR and measurement of glutarylcarnitine concentration in dried blood spots. Mice were housed in the animal facility of the University Medical Center with a 12-hour light-dark cycle and allowed water and food ad libitum. Animal care and experiments were carried out in accordance with institutional guidelines as approved by local authorities.

2.2. (^{3}H)-3OHGA distribution in vivo

(^{3}H)-3OHGA was synthesized as described previously [15]. Mice at an age of 42 and 100 days fed for 4 days with standard or high protein (HPD) diet were injected intravenously with (^{3}H)-3OHGA (10^7 cpm in 50 μl phosphate buffered saline pH 7.4). During incubation time, urine and faeces were collected. After 2 or 6 h mice were anesthetized, intracardiac blood and intravesicular urine were sampled, and animals were instantly perfused with 0.9% NaCl solution. Organs (brain, liver and kidneys) were removed and solubilized in Tissue Solubilizer according to the manufacturer's instructions (NCS-II, Amersham Buchler, Germany). Additionally, stomach and bowel were removed and dissected. Faeces were collected and all three samples processed separately as described above. To prevent effects of tissue chromogens on scintillation counting, samples were decolourized by addition of small aliquots of 30% hydrogen peroxide (H_2O_2), and counted in Rotiszint (Roth, Karlsruhe, Germany).

2.3. Injection and detection of fluorescence-labeled dextrans

Forty-two day-old wildtype and Gcdh$^{-/-}$ mice fed on normal diet or HPD were injected with 3 kDa biotin-, 10 kDa biotin-, 10 kDa Alexa Fluor 594- or 70 kDa fluorescein-labeled dextran (Catalog-Nos. D7135, D1956, D22913, and D1822, respectively; Molecular Probes, Eugene, OR, USA; 5 μg/g body weight). After 1 h of incubation, animals were anaesthetized, perfused with 4% paraformaldehyde in phosphate buffered saline (pH 7.4), and organs were paraffin-embedded and cut in 3 μm sections. Fluorescence-labeled dextrans were visualized using a Leica DMIRE 2 confocal fluorescence microscope applying the respective excitation wavelengths. Biotin-labeled samples were treated with streptABComplex/HRP duet (Dako, Copenhagen, Denmark) according to the manufacturer's instructions and visualized using a Zeiss Axiovert S100 microscope.

2.4. Histology and immunohistochemistry

For histologic examination, sections of organs of 42 d old wildtype and Gcdh$^{-/-}$ mice fed on normal diet or HPD were deparaffinized, hydrated and either stained with HE or treated with appropriate antigen retrieval regimens. Immunohistochemical stainings for glial fibrillary acidic protein (GFAP) were carried out using standard protocols [16]. For quantification of vacuoles, 5 representative regions of cortex, hippocampus and striatum were assessed. Quantification was performed by counting numbers of vacuoles per visual field (283×214 μm).

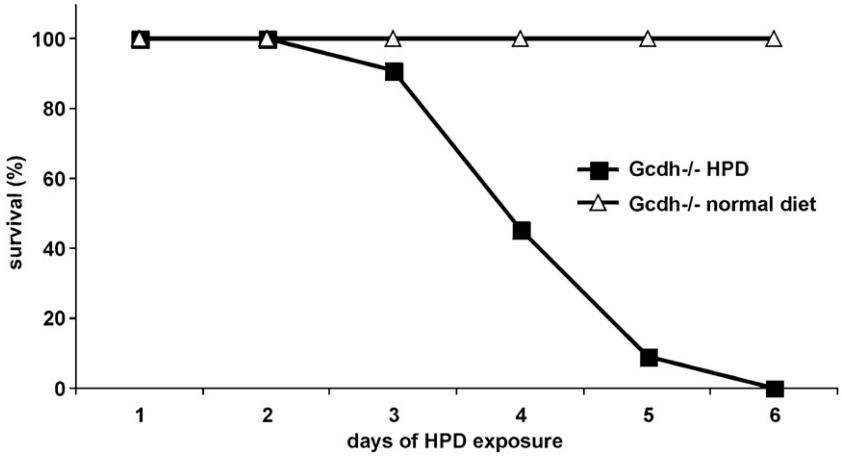

Fig. 1. Survival of Gcdh$^{-/-}$ mice during diet-induced encephalopathic crises. Surviving percentage of Gcdh$^{-/-}$ mice fed with HPD (black boxes, $n=11$) and normal diet (white triangles, $n=15$). HPD treatment was started at 38 d of age, designated as day 1 of HPD exposure. 55% of Gcdh$^{-/-}$ mice on HPD had died at day 4 of HPD exposure, 91% at day 5.

2.5. Determination of GA, 3OHGA and glutarylcarnitine

GA and 3OHGA in plasma and urine were quantified by GC/MS using stable isotope standards as described previously [17]. Glutarylcarnitine concentrations in mouse bile were determined by electrospray MS/MS spectrometry [18].

2.6. Data analysis

Significance was tested as indicated in the figure legends and accepted at $p \leq 0.05$. Calculations were performed using Microsoft Excel 2003 and SPSS 12.0 software (SPSS Inc., Chicago, IL, USA).

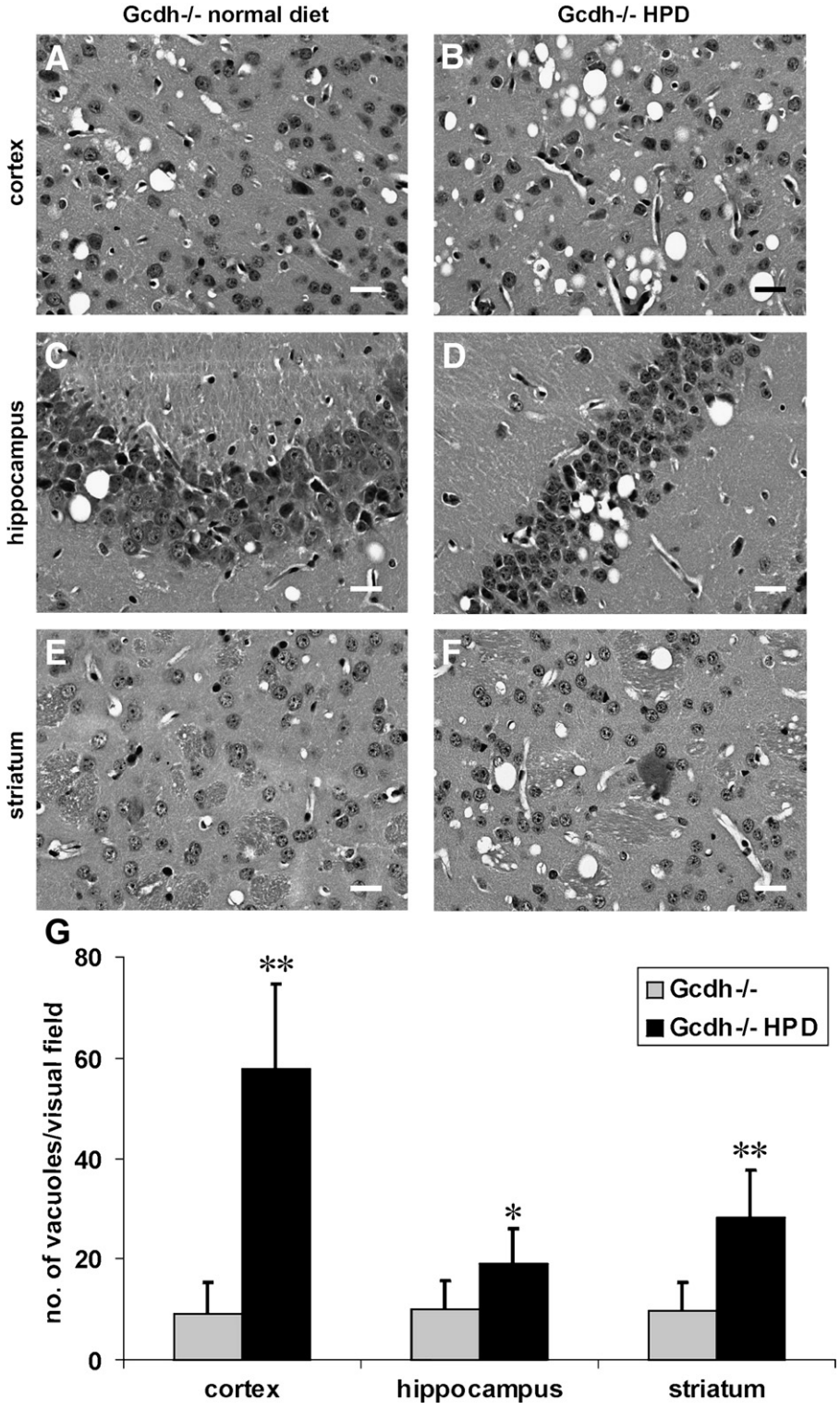

Fig. 2. Increased vacuolization in Gcdh$^{-/-}$ brain tissue during encephalopathic crises. HE-stained brains of 42 d old Gcdh$^{-/-}$ mice fed for 4 days with a normal diet (A, C, E), or a HPD (B, D, F). Cortex (A, B), hippocampus (C, D) and striatum (E, F) show vacuolization under basal conditions, further increasing under conditions of a diet-induced encephalopathic crisis. G: Quantification of number of vacuoles per visual field (283×214 µm) in HE-stained brain slices shows a significant increase in the number of vacuoles in brains of Gcdh$^{-/-}$ mice on a HPD. For each group (Gcdh$^{-/-}$ on normal diet vs. Gcdh$^{-/-}$ on HPD), 2 animals were examined; 5 visual fields were counted per animal and region, respectively. Level of significance was determined using unpaired, two-tailed *t*-tests and accepted at $p<0.05$. *$p<0.05$; **$p<0.001$. Bars: 25 µm.

3. Results

3.1. Tissue distribution of (^{3}H)-3OHGA in Gcdh$^{-/-}$ mice under basal conditions

The tissue distribution of (^{3}H)-3OHGA was determined 2 and 6 h after intravenous injection in 100 d old mice under basal conditions. The majority of radioactivity (90–95%) was found in urine. Due to lower variability in radioactivity recovered from tissues and blood, all further experiments were performed using an incubation period of 6 h after injection of (^{3}H)-3OHGA.

To examine tissue distribution of (^{3}H)-3OHGA under basal conditions, (^{3}H)-3OHGA was intravenously injected into 42 and 100 d old wildtype and Gcdh$^{-/-}$ mice. Six hours after injection, animals were sacrificed and radioactivity was determined in blood, urine and tissues (kidney, liver, brain, stomach, bowel, faeces) by scintillation counting. When the radioactivity found in urine was subtracted, approximately 2–6% of (^{3}H)-3OHGA were recovered in kidney, liver or brain tissue (Table 1). The increased level of endogenous 3OHGA in plasma of 42 d old Gcdh$^{-/-}$ mice (Table 2) competing with (^{3}H)-3OHGA for putative transporter binding sites might explain the reduced ($p=0.06$) content of radioactivity in liver and brain tissue (Table 1). About half of the radioactivity was still in the circulation. Surprisingly, a significant amount of (^{3}H)-3OHGA was detected in stomach, bowel and faeces. Although this percentage was variable, Gcdh$^{-/-}$ mice at an age of 42 d appear to excrete more (^{3}H)-3OHGA into the gut and via faeces than control mice. With the exception of stomach, bowel and faeces, the tissue and blood distribution of (^{3}H)-3OHGA in Gcdh$^{-/-}$ mice did not differ significantly from control mice, nor between 42 and 100 d old mice (Table 1). The percentage of (^{3}H)-3OHGA excreted via stomach, bowel and faeces of wildtype mice appears to increase with age, whereas no changes were observed between young and older Gcdh$^{-/-}$ mice.

3.2. Tissue distribution of (^{3}H)-3OHGA during an encephalopathic crisis

To examine characteristics of Gcdh$^{-/-}$ mice under conditions of an encephalopathic crisis, we studied animals exposed to a high protein diet (HPD) [12]. In an attempt to model the time window of vulnerability seen in human GA1 patients [2], and the age-dependence of diet-induced encephalopathic crises in Gcdh$^{-/-}$ mice [12], these investigations were performed with 42 d old mice. Under our conditions, ≥90% of 38 d old Gcdh$^{-/-}$ mice died after 4–5 days of HPD treatment (Fig. 1). Onset of symptoms (reduced body temperature, reduced and dystonic movements) was observed 12–24 h prior to death of affected mice. HPD exposure did not affect either age-matched wildtype control or heterozygous Gcdh$^{+/-}$ mice (data not shown). In comparison to Gcdh$^{-/-}$ mice fed with a normal diet, Gcdh$^{-/-}$ mice on HPD showed a significant increase of metabolites in plasma (3OHGA: 10.5±1.8 vs. 7.2±2.1 µmol/l) and urine (GA: 50,934±9,927 vs. 33,926±6,056 mmol/mol creatinine; 3OHGA: 1,414±168 vs. 898±126 mmol/mol creatinine) (Table 2). Determination of glutarylcarnitine revealed significantly increased levels in bile of Gcdh$^{-/-}$ mice compared to heterozygote and wildtype animals (Supplementary Fig. 1).

Examination of brains after transcardiac perfusion revealed extravasation of blood into the subarachnoid space as well as intraventricular blood surrounding the choroid plexus in 42 d old Gcdh$^{-/-}$ mice maintained on HPD (Supplementary Fig. 2D–H). Histologic examination showed no evidence of erythrocyte degradation or hematoma formation, indicating that the blood was the result of an acute subarachnoid haemorrhage, and not a chronic process (Supplementary Fig. 2G–H). There was no evidence of intracerebral bleeding in Gcdh$^{-/-}$ mice on a normal diet, or in wildtype controls on either diet (Supplementary Fig. 2A–C). Exposure to the HPD also significantly increased the number of vacuoles in the cortex, striatum and hippocampus of 42 d old Gcdh$^{-/-}$ mice (6-, 3- and 2-fold, respectively; Fig. 2). No vacuoles were observed in brains of wildtype mice fed with normal diet or HPD (not shown). GFAP-specific staining of brain slices was similar in wildtype and Gcdh$^{-/-}$ mice, on both diets, indicating that the HPD did not result in gliosis (Supplementary Fig. 3). To determine whether organs other than the brain were affected by the HPD, we examined the kidney and bowel of wildtype and Gcdh$^{-/-}$ mice on a normal diet or after 4 days of the HPD. During 4 days of the HPD water intake as well as the volume of urine in Gcdh$^{-/-}$ mice were similar and slightly increased, respectively, compared to Gcdh$^{-/-}$ mice on normal diet. HE-stained sections of the kidney revealed neither glomerular, nor tubular abnormalities in any of the mice. Similarly, the appearance of the intestinal brush border was similar in all of the mice (Supplementary Fig. 4).

To investigate whether endothelial barrier functions are altered under conditions of an encephalopathic crisis, we compared the tissue

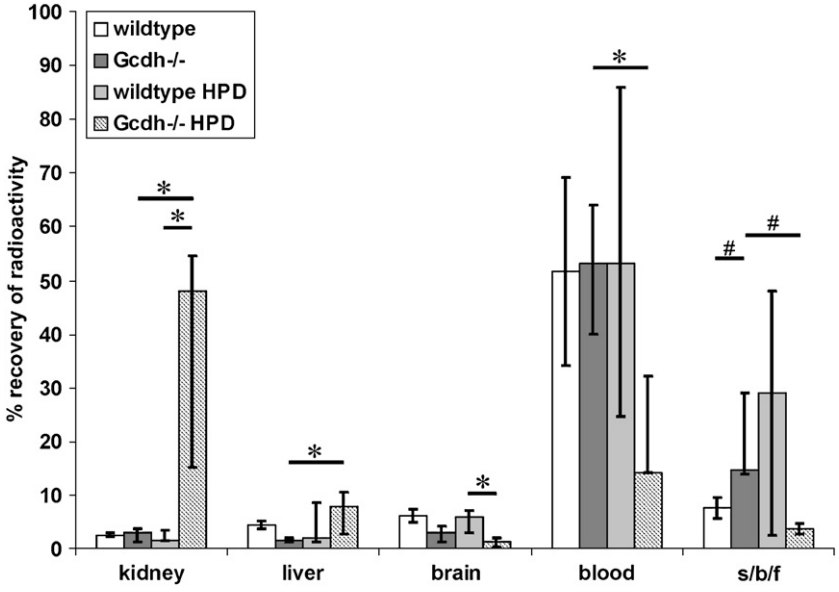

Fig. 3. Tissue distribution of intravenously injected (^{3}H)-3OHGA. Percentage of recovered radioactivity 6 h after intravenous injection of (^{3}H)-3OHGA exclusive of radioactivity excreted via urine. Bars represent median±range, data are derived from 4 independent experiments per group. There is a significant accumulation of (^{3}H)-3OHGA in kidney and liver tissue of HPD-treated Gcdh$^{-/-}$ mice compared to Gcdh$^{-/-}$ mice on normal diet, accompanied by a reduction of radioactivity in blood. In contrast, intracerebral (^{3}H)-3OHGA was reduced in Gcdh$^{-/-}$ mice on HPD compared to controls. On a normal diet, Gcdh$^{-/-}$ mice had increased excretion of (^{3}H)-3OHGA via faeces (s/b/f: stomach, bowel, faeces) in comparison with wildtype mice. This was reversed in HPD-treated mice. Levels of significance were tested using Mann–Whitney-*U* tests. *$p<0.05$. #$p=0.06$.

distribution of (^{3}H)-3OHGA in 42 d old Gcdh$^{-/-}$ and wildtype mice on a normal diet and after four days of the HPD. In wildtype animals the HPD had no effect on the tissue distribution of (^{3}H)-3OHGA (Fig. 3). In contrast, a striking effect of the HPD was observed in the kidneys of Gcdh$^{-/-}$ mice, which demonstrated a 16- to 32-fold increase of perfusion-resistant radioactivity. An increased accumulation of (^{3}H)-3OHGA was also seen in the liver (5-fold). Significantly reduced levels of (^{3}H)-3OHGA were observed in the blood (~4-fold) and brains (5-fold) of HPD-fed Gcdh$^{-/-}$ mice compared to HPD-treated wildtype animals. The capability to excrete (^{3}H)-3OHGA into the stomach, bowel and faeces found in Gcdh$^{-/-}$ mice on the normal diet was not observed in HPD-fed Gcdh$^{-/-}$ mice.

We also evaluated endothelial barrier function via intravenous injection of fluorescent-labeled dextrans (3, 10 and 70 kDa) into 42 d old wildtype and Gcdh$^{-/-}$ mice on either normal diet or the HPD. One hour after injection none of the examined animals showed any extravasation of the labeled dextrans in the brain or in the kidney (data not shown). These results, and the data on brain levels of (^{3}H)-3OHGA, indicate that under conditions mimicking an encephalopathic crisis the blood–brain barrier appears to remain intact, preventing the influx of (^{3}H)-3OHGA into the brain from the systemic circulation.

4. Discussion

After intravenous injection of (^{3}H)-3OHGA in control mice small amounts of radioactivity were recovered from the tissues studied, whereas the majority was excreted into the urine. The tissue distribution pattern was similar in 42 and 100 d old control mice. In young Gcdh$^{-/-}$ mice, however, the distribution of the (^{3}H)-labeled metabolite was changed in comparison to wildtype animals. The amount of (^{3}H)-3OHGA was reduced in brain tissue suggesting that the steep gradient of 3OHGA between brain tissue and plasma [18] reduces the influx of (^{3}H)-3OHGA into the brain. Alternatively, competitive binding of endogenously produced organic acids (Table 2) with injected (^{3}H)-3OHGA may explain the data in Gcdh$^{-/-}$ mice. Moreover, alterations in the transport capacity of endothelial barriers for (^{3}H)-3OHGA are likely. We have recently identified the first transporter for 3OHGA, the sodium-dependent dicarboxylate transporter 3 (NaC3; Slc13a3), which is localized in basolateral membranes of proximal tubule cells as well as in astroglial cells [15,19]. The expression of NaC3 is upregulated in kidneys of Gcdh$^{-/-}$ mice. The transporters at the apical membranes of endothelial cells responsible for the influx of 3OHGA into the brain and how they are regulated are presently unknown. Interestingly, a significant proportion of injected (^{3}H)-3OHGA was found in the stomach, bowel and faeces of wildtype mice, and an even higher amount in Gcdh$^{-/-}$ mice, indicating the existence of additional pathways to secrete 3OHGA into the gut. The level of glutarylcarnitine is significantly elevated in the bile of Gcdh$^{-/-}$ mice (Supplementary Fig. 1), suggesting that biliary excretion is one of the mechanisms responsible for the accumulation of labeled 3OHGA in the gut. The clinical significance of this faecal route of excretion, and its correlation with the phenomenon of high and low urine excretors among GA1 patients is unknown. These subgroups are defined by the concentrations of GA and 3OHGA they excrete into the urine, which correlates with residual GCDH activity and with distinct alleles, e.g. p.R227P, but not with clinical phenotype [17,20,21].

A high protein diet induced an encephalopathic crisis in Gcdh$^{-/-}$ mice that was lethal to 42 day-old animals within 5 days (Fig. 1). This was associated with accumulation of GA and 3OHGA in serum and urine (Table 2), a significant increase in the numbers of vacuoles in specific regions of the brain (Fig. 2), and subarachnoid haemorrhages (Supplementary Fig. 2). In contrast to findings in 28 d old Gcdh$^{-/-}$ mice exposed to a high lysine diet for 3 days [12], no evidence of blood–brain barrier breakdown was observed under conditions used in this study. The influx of (^{3}H)-3OHGA into the brain was actually reduced, rather than increased, after 4 days exposure to a high protein diet (Fig. 3). These data are also surprising because it was found that 2 mM 3OHGA impairs both the VEGF-induced endothelial tube formation in vitro as well as chick chorioallantoic membrane (CAM) integrity in vivo [11]. Differences between the concentrations of 3OHGA and VEGF used in tube formation and CAM assays, and the actual serum 3OHGA level in Gcdh$^{-/-}$ mice might explain the discrepancies. On the other hand, a strong accumulation of injected (^{3}H)-3OHGA was found in the kidney of Gcdh$^{-/-}$ mice after 4 days exposure to a high protein diet (Fig. 3). The renal clearance of 3OHGA is substantially greater than the GFR indicating that the renal clearance occurs mainly via tubular secretion [22]. In proximal tubule cells the transport of dicarboxylates such as GA and 3OHGA is mediated by organic anion transporters (OAT) and NaC3 [15,23]. Recently, OAT1 and OAT4 were identified as high affinity transporters for GA and 3OHGA [24], which were found to be localized at the basolateral and apical membrane, respectively, of human proximal tubule cells. Therefore, the data suggest that during metabolic crises in Gcdh$^{-/-}$ mice, injected (^{3}H)-3OHGA is translocated from the blood across the basolateral membrane of renal proximal tubule cells via OAT1 and NaC3. However, the secretion of (^{3}H)-3OHGA at the apical side into the urine appears to be impaired, resulting in an accumulation of the substrate in kidneys. OAT4 has been detected only in human but so far not in mice [23]. The 3OHGA transporter at the apical membrane of proximal tubular cells in mice, most likely an OAT4-like transporter, and the regulated expression of transporters during metabolic crisis remain to be investigated.

We have shown that the influx of (^{3}H)-3OHGA from the circulation into the brain tissue of wildtype mice is low, confirming data with deuterium-labeled 3OHGA [18]. Additionally, our present study provides evidence that in Gcdh$^{-/-}$ mice both under basal conditions and during encephalopathic crises the permeability of the blood–brain barrier for 3OHGA and fluorescence-labeled dextrans was not affected, suggesting that organic acids produced by brain cells are responsible for neuropathological alterations rather than metabolites formed in peripheral organs. Finally, we report that significant amounts of 3OHGA can be excreted via the intestinal tract, which might present a novel target for therapeutic interventions in GA1 patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbadis.2008.02.008.

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