Aminoaciduria, but normal thyroid hormone levels and signaling, in mice lacking the amino acid and thyroid hormone transporter *Slc7a8*

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The system L amino acid transporter 2 (LAT2) is composed of the subunits *Slc7a8/Lat2* and *Slc3a2/4F2hc*. This transporter is highly expressed along the basolateral membranes of absorptive epithelia in kidney and small intestine, but is also abundant in the brain. Lat2 is an energy-independent exchanger of neutral amino acids, and was shown to transport thyroid hormones. We report here that targeted inactivation of *Slc7a8* leads to increased urinary loss of small neutral amino acids. Development and growth of *Slc7a8*^{-/-} mice appears normal suggesting functional compensation of neutral amino acid transport by alternative transporters in kidney, intestine, and placenta. Movement coordination is slightly impaired in mutant mice, although cerebellar development and structure remained inconspicuous. Circulating thyroid hormones, thyrotropin, and thyroid hormone-responsive genes remained unchanged in *Slc7a8*^{-/-} mice, possibly because of functional compensation by the thyroid hormone transporter Mct8, which is co-expressed in many cell types. The reason for the mild neurological phenotype remains unresolved.

INTRODUCTION

Physiological studies have established that transport of amino acids across the plasma membrane requires the presence of distinct transport “systems”, because the zwitter-ionic compounds cannot cross the lipid bilayer by passive diffusion [1]. These “systems” display broad substrate specificities and are accordingly termed neutral, basic, acidic, iminoglycine and beta-amino systems [2]. Amino acid transport is not specific for intestinal and renal tubule epithelia, but is often studied in models of absorptive epithelia. Inherited disorders of amino acid transport are mostly severe and characterized by malabsorption syndromes and/or by aminoaciduria. These disorders often involve the failure of apical amino acid transport systems (e.g. Hartnup disorder) or, more rarely, the failure of basolateral transport systems, e.g. lysinuric acid intolerance [2].

The *system L-like neutral amino acid transporter 2* (LAT2)¹ is composed of two polypeptides. The heavy chain subunit CD98/4F2hc (*Slc3a2*) is a single-pass, highly glycosylated protein, which associates through a disulfide bridge with a non-glycosylated, 12-transmembrane spanning light chain subunit Lat2 (*Slc7a8*) [3]. The heavy chain is required for correct membrane localization of the complex [4]. Amino acid transport is mediated by the light chain subunit, which is a member of the major facilitator superfamily. The complex is highly expressed along the renal proximal tubule epithelium and correlates with neutral amino acid reabsorption capacity along the nephron [5, 6]. LAT2 bears broad substrate specificity, including all neutral amino acids except proline, and acts as an obligatory exchanger [5, 7]. Antisense and overexpression studies in Madin-Darby canine kidney cells (MDCK-1) and in a hamster proximal tubule-derived cell line (OK) have indicated moderate changes of intracellular amino acid concentrations, and suggested a role in cysteine export [8, 9]. The frog homolog of Lat1 was cloned as IU12, a developmentally regulated gene, mediating T₃ (3,5,5'-triiodo-L-thyronine) and T₄ (3,3',5,5'-triiodo-L-thyronine) uptake into *Xenopus* oocytes [10]. Human LAT2 (*SLC7A8*) and LAT1 (*SLC7A5*) also accept thyroid hormones in the *Xenopus* oocyte system [11]. Considering the large number of neutral amino acid transporters (*Slc16a9*, *Slc6a15*, *Slc1a5*, *Slc6a14*, *Slc16a10*, *Slc43*, *Slc38*, *Slc7a10*, *Slc7a8*, *Slc7a5*), it appears plausible that Lat2 and Lat1 have evolved to accommodate additional substrates like iodothyronines.

T₃, as the most active form of iodothyronines, acts on nuclear receptors. Initially it was assumed that the hydrophobicity of thyroid hormones would allow passive diffusion through the lipid bilayer, despite the known zwitter-ionic character of the molecules [12]. The concept of facilitated diffusion of thyroid hormones was only widely adopted after the identification of patients with mutations in the most specific thyroid hormone transporter MCT8 (monocarboxylate transporter 8, *SLC16A2*) [13-15]. Only then it was discovered that mutations in *MCT8* are the cause for Allan-Herndon-Dudley syndrome (OMIM 300523), a severe X-linked mental retardation syndrome first described in 1944 [16]. Mice deficient in *Mct8* replicate the endocrine phenotype of *MCT8*-deficient patients, but do not suffer from severe mental retardation. Since T₃ uptake into primary neurons does not completely depend on *Mct8*, but can be competed with the Lat-specific substrate BCH (2-Aminobicyclo-(2.2.1)-heptane-2-carboxylic acid), we have suggested that co-expression of Lat2 in neurons complements inactivation of *Mct8* in mice [17]. Later, we could demonstrate that both *Mct8* and Lat2 mediate thyroid hormone uptake into cultured astrocytes [18]. In this report, we describe for the first time the impact of *Slc7a8*-deficiency on amino acid and thyroid hormone metabolism in mice.

EXPERIMENTAL PROCEDURES

Mice

Slc7a8-deficient mice on a C57Bl/6 genetic background were obtained from Jackson Laboratory (Bar Harbor, Maine, USA). To produce the targeted allele, a β -galactosidase/neomycin phosphotransferase II-*poly A*-signal cassette (geo) was inserted into exon2 of *Slc7a8* replacing part of the coding sequence of transmembrane helices 1 and 2. The following primers were used for PCR genotyping: 5'-CAAATGCCAGCTGTCCTGACCTCAC-3', 5'-CAGACTTAGGGATGGTGACGCCTAG-3' and 5'-GGGTGGGATTAGATAAATGCCTGCTCT-3'.

Mice were fed ad libitum with standard rodent breeding chow (Snniff, Soest, Germany) and were sacrificed around noon. Animals were kept in accordance with local regulations (Landesamt für Gesundheit und Soziales Berlin #T458/09), under standard conditions (12 h light/dark cycle) in a specific pathogen-free environment in the central animal facility of the Charité, Berlin. Animals analysed were always littermates from heterozygous intercrosses in order to avoid potential genetic drift between wild-type and mutant colonies.

Western Blot

Frozen mouse organs were powdered under liquid nitrogen, and cytosolic and membrane extracts for analysis were prepared as described elsewhere [18]. 80µg protein was separated on 12.5% polyacrylamide gels. After transfer onto nitrocellulose membranes, membranes were incubated with primary antibodies (Mct8, 1:500 (ATLAS, Stockholm, Sweden); Lat2 1:200 (ImmunoGlobe, Himmelstadt, Germany); β -actin, 1:2000 (Rockland, Gilbertsville, PA, USA) over night at 4 °C, and afterwards with HRP-conjugated secondary antibody. Signals were detected using Amersham ECL plus (GE Healthcare, Freiburg, Germany) and Kodak Hyperfilm plus as described [17].

Immunohistochemistry

Tissues were fixed in 4 % paraformaldehyde over night and stained with Dako Cytomation Systems (DAKO, Ort, Denmark) according to the manufacturer's instructions. The primary Lat2 antibody (1:250) was incubated over night. Nuclei were counterstained with hematoxylin. Pictures were taken at a Zeiss Axioscope 2 mot plus equipped with an AxioCam MRe5 and Axiovision software. Alternatively, Cy2- and Cy3-labelled secondary antibodies (Jackson ImmunoResearch) were used to stain calbindin (1:000; Swant, Bellinzona, Switzerland) and parvalbumin (1:000; Swant), and confocal images were taken at a Leica instrument at the Neuroscience Research Center core facility.

qPCR

Total RNA of powdered mouse tissue was isolated according to the TRIzol protocol (Invitrogen, Darmstadt, Germany). cDNA was synthesized using the iScript cDNA synthesis kit (Biorad, München, Germany) according to the manufacturer's protocol. qPCR was performed using SYBR Green from Abgene (Thermo Scientific, Epsom, UK) on an iCycler (Biorad, München, Germany). Primers used for qPCR detection of thyroid hormone transporters were described before [17].

Hormone measurements

Thyroid stimulating hormone (TSH), total serum T₄, and T₃ were measured by immunoassays as described before [19]. 8-12 male animals were analyzed per genotype.

Enzymatic activity measurements

Type I-deiodinase activity was determined as described before [19]. 10µg protein of liver and 15µg of kidney homogenate were used, as well as 0.1µM unlabeled rT₃. 3-4 male animals were analyzed per genotype. The measurement was repeated 2-4 times with similar results.

Amino acid analysis

Urine was collected in metabolic cages over a period of 24 hours. Urinary and serum amino acids were determined in the Interdisciplinary Endocrinological Special Laboratory of Charité-Universitätsmedizin Berlin according to a routine high-pressure-liquid-chromatography method after derivatization and fluorescence detection. Norleucine was added as an internal standard. While serum amino acid values agree well with reported data from other laboratories, absolute numbers of urinary amino acid concentrations may represent an overestimate, which may be caused by crowding of chromatograms. However, methods and results vary in the literature.

RESULTS

Targeted inactivation of Slc7a8 in mice

Lat2 is considered an important neutral amino acid transporter in kidney and intestine [2], but its physiological role in the intact animal is not known. Insertion of a *geo* cassette (see methods) into exon 2 of *Slc7a8* disrupted the protein (Fig 1A). Homozygous mutant animals (*Slc7a8*^{-/-}) were born at the expected Mendelian frequency and were identified by PCR genotyping (Fig. 1B). *Slc7a8*^{-/-} mice are indistinguishable from their wild-type or *Slc7a8*^{+/-} littermates with respect to body weight and length (Fig. 1C). Lat2 protein is expressed in kidney, brain, pituitary, testis, spleen (Fig. 1D), and small intestine (Fig. 1E). The Lat2 signal along the gastrointestinal tract was entirely abolished in *Slc7a8*^{-/-} tissue (Fig. 1E, F). Immunohistochemistry suggests that the faint signal observed in liver is derived from Kupffer cells (not shown). The protein of 38kDa detected in liver and brain is unrelated to Lat2, as it remains in *Slc7a8*^{-/-} tissue. Expression of Lat2 in kidney is restricted to the basolateral membrane of proximal tubules (Fig.2A).

Aminoaciduria in Slc7a8-deficient mice

Mutation of the related transporter *SLC7A7* leads to lysinuric protein intolerance in patients [20] and a similar phenotype in *Slc7a7*^{-/-} mice [21]. We therefore asked whether inactivation of *Slc7a8* would similarly affect urinary amino acid excretion and reflect the substrate spectrum of Lat2 *in vivo*. Mice were placed in metabolic cages for 24 hours and urine was collected. Urinary amino acid analysis revealed aminoaciduria in *Slc7a8*^{-/-} mice (Fig. 2B). Glycine, serine, threonine, glutamine, leucine, and valine were increased in urine from mutant animals. Interestingly, some classical L-type amino acid transporter

substrates like isoleucine or aromatic amino acids were not affected. We therefore speculated that other amino acid transporters compensate partially for the loss of Lat2 transporters. Expression of several candidate transport proteins was compared by qPCR (Fig.2C). While *Slc7a8* mRNA was virtually undetectable, a slight increase in *Slc16a10* (a transporter for aromatic amino acids) was observed, which was, however, only significant at the 5% level upon one-sided *Student's t*-test. Amino acid profiles in serum were also altered in *Slc7a8*^{-/-} animals. Several small neutral amino acids were increased in serum partly resembling the pattern in urine. The most striking difference was an increase of lysine serum levels in the mutants (Fig. 2D).

Serum thyroid hormone levels are unaltered in Slc7a8-deficient mice

Lat2 is clearly capable of thyroid hormone transport *in vivo* [18] and is expressed in tissues responsive to thyroid hormones, including brain, pituitary, and kidney. Inactivation of Lat2 may thus alter tissue responses to thyroid hormone or impact on circulating thyroid hormone levels. We therefore determined circulating levels of thyroid hormones T₃ and T₄, as well as TSH in a group of male animals (Fig 3). Neither of the hormones was altered in *Slc7a8*^{-/-} mice.

Thyroid hormone-dependent gene activation

It is still possible that minor changes in thyroid hormone uptake into some tissues are not reflected by alterations in circulating hormone levels, but can be revealed on the level of target gene expression. We have therefore measured the activity of type I-deiodinase (Dio1), a T₃-responsive gene [22], in liver and kidney from *Slc7a8*^{-/-} mice and wild-type littermates (Fig. 4A, B). Again, no differences were discovered. Impaired function of the thyroid axis is often reflected by changes in gene expression of *Dio2* and *Tsh* in the pituitary. Again, no differences were observed by qPCR analysis suggesting that the thyroid hormone axis functions normally in *Slc7a8*-deficient mice.

Brain development and impaired movement coordination

We then probed thyroid hormone signaling in the cerebral cortex and cerebellum by qPCR. *Neurogranin* (*RC3*), *hairless* (*Hr*), and *Dio3* are known thyroid hormone-responsive genes in the brain. None of these genes was differentially expressed in *Slc7a8*-mutant cerebellum (Fig. 5A-C) and cerebral cortex (see supplemental figure 1). Gross histology did not reveal any apparent changes in brain size, structure, and expression of neurochemical markers in somatosensory cortex and hippocampus (see supplemental figure 2). In particular, a reduction of parvalbumin expression in cortical interneurons is known to occur in mice, which have experienced postnatal hypothyroidism [23] or are expressing a dominant negative thyroid hormone receptor $\alpha 1$ [24]. Parvalbumin expression is unchanged in *Slc7a8*^{-/-} cerebral cortex (supplemental figure 2). In contrast, movement coordination was slightly impaired in *Slc7a8*^{-/-} mice as revealed in the rotarod assay (Fig. 5D). Cerebellar histogenesis was normal as judged by Nissl staining (Fig. 5E,F) arguing against developmental hypothyroidism. In addition, immunohistochemical staining with markers for Purkinje and stellate cells was normal in *Slc7a8*- mutants (Fig. 5G,H).

DISCUSSION

The phenotype of *Slc7a8*^{-/-} mice is remarkably mild. In contrast, mutations of related transporters *Slc7a9* and *Slc7a7* in mice cause cystinuria [25] and lysinuric protein intolerance [21]. Based on the *Slc7a8*/Lat2 substrate spectrum, Bröer has proposed *SLC7A8* as a candidate gene for isolated cystinuria (OMIM 238200) [2]. While we have detected aminoaciduria in the mutants, their phenotype does not support this idea – in contrast, cysteine excretion is diminished in our mutants.

Lat2 is not exclusively, but most strongly expressed in absorptive epithelia, in kidney proximal tubules, small intestine, and placenta. Accordingly, aminoaciduria is observed in *Slc7a8*^{-/-} mice. Interestingly, only a subset of known Lat2 substrate amino acids is affected, i.e. excluding aromatic amino acids. This finding suggests that (i) aromatic amino acid transport is fully maintained by an aromatic amino acid transporter present in kidney tubular cells, and (ii) that remaining neutral amino acid transport is partially rescued by an alternative transporter. In the kidney, it has been proposed that Lat2 cooperates with two other transport activities expressed on the basolateral membrane [26]. A unidirectional exporter is required to drive amino acid export from the proximal tubule epithelium, because Lat2, as an obligate exchanger, cannot create a net flux over the membrane. The role of Lat2 is thus to extend the substrate spectrum for export, if the substrate spectrum of the uniporter is more limited [2, 27]. A good candidate for such a uniporter is Tat1/Mct10/*Slc16a10*, an aromatic amino acid transporter. Compatible with this notion, we have found moderate induction of *Slc16a10* mRNA in kidneys of *Slc7a8* mutant animals. Influx of exported aromatic amino acids may then allow the export of the whole spectrum of Lat2 substrate amino acids. Some of these amino acids are then used in turn as co-substrates for *Slc7a7*, the γ^+ Lat1 transporter of basic amino acids mutated in lysinuric protein intolerance. Functional interference of *Slc7a8* with basic amino acid transport is supported by increased lysine serum levels in *Slc7a8* mutants. However, the mechanism cannot be inferred from presently available data. Intestinal amino acid absorption has not been tested in these mice, since it is apparently not limiting considering normal growth and weight gain of mutants. Moreover, Lat2 has been implicated in amino acid transport across the placenta [28]. Normal frequency of *Slc7a8* mutants among offspring and normal growth together argue in favor of alternative placental transporters, although we have not formally investigated this question by, e.g. weighing *Slc7a8*^{-/-} mouse embryos or newborn offspring from *Slc7a8*^{-/-} mothers. This finding is in sharp contrast to the fetal and growth phenotype of *Slc7a7*^{-/-} mice [21]. It appears likely that other neutral amino acid transporters can compensate for many *Slc7a8* functions *in vivo*. Limiting the dietary availability of essential Lat2 substrate amino acids may help reveal a phenotype compensated in our experiments by affluent amounts of amino acids in our breeding diet.

The high abundance of Lat2 in brain suggests some role for the protein in the brain. This notion is supported by the moderate impairment of movement coordination in *Slc7a8* mutants revealed in the

rotarod task. Bröer et al. proposed a possible role of Lat2 in alanine uptake into astrocytes [29]. Alanine was suggested as the source of ammonia in the cerebral glutamine/glutamate cycle [29], which also involves a net transfer of ammonia to neurons. In this cycle, astrocytes take up extracellular glutamate released by excitatory neurons and convert it into glutamine, which is then provided back again to neurons as a source of glutamate [30]. Inactivation of Lat2 may thus slow down the glutamine/glutamate cycle by reducing the flux of alanine. Alternatively, or in addition, Lat2 may participate in astroglial glutamine export or neuronal glutamine import, although it is currently assumed that these activities are covered by systems A and N [31]. The exceptionally high concentration of glutamine in cerebrospinal fluid would make Lat2 a good candidate for glutamine uptake into neurons. It is beyond the scope of this study to distinguish the relative roles of systems L, N, and A in the glutamine/glutamate cycle. A dedicated neurochemical study would be needed to address this issue.

Lat2 accepts thyroid hormones *in vitro* [10, 11, 32] and *in vivo* [18]. T₃ and T₄ uptake into primary *Slc7a8*^{-/-} astrocytes is significantly reduced, to a level identical to uptake in the presence of BCH, a specific Lat inhibitor [18]. We have analyzed thyroid hormones and thyroid hormone-dependent gene expression in organs from *Slc7a8*^{-/-} mice, because we had proposed a possible compensation of thyroid hormone transport by Lat2 in *Mct8/Slc16a2*-deficient mice [17]. Plasma T₃ levels are increased and T₄ levels decreased in *Mct8*^{-/-} mice [33, 34]. Both *Mct8* and Lat2 are expressed in kidney, brain, and pituitary. Our findings of normal T₃, T₄, and TSH levels in *Slc7a8*^{-/-} mice suggest that cells determining these hormone levels depend on *Mct8*, but not Lat2. Thus, a feature of these critical cells may be lack of co-expression of *Mct8* and Lat2. Since urinary loss of thyroid hormones in *Mct8*-deficient mice may reduce T₄ levels [35], we have attempted to determine urinary thyroid hormone loss in *Slc7a8*^{-/-} mice by our liquid chromatography-tandem mass spectrometry method [36]. While T₄ was clearly detectable, the signal was close to background and thus could not be quantified reliably. Normal activity of renal Dio1 is in contrast to elevated activity in *Mct8*^{-/-} kidney. This finding suggests that intracellular T₃ signaling is not changed in *Slc7a8*^{-/-} kidney.

Normal thyroid hormone levels in the mutants are in agreement with normal (thyroid hormone-dependent) cerebral and cerebellar development in *Slc7a8* mutants. Moreover, T₃-dependent gene expression is not changed. We conclude that there is no indication at the moment for insufficient thyroid hormone transport into brain cells. This could be a consequence of co-expression in neurons and astrocytes of *Mct8* and Lat2 [17, 18] and would be consistent with a lack of overt neurological phenotype in *Mct8*-deficient mice that contrasts the devastating neurological disorder in patients lacking functional MCT8. It will be interesting to test, by compound inactivation of both genes, the hypothesis that Lat2 and *Mct8* compensate for each other in thyroid hormone transport into brain cells.

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Footnotes: The abbreviations used are: 4F2hc/CD98, heavy chain subunit of Lat2, gene symbol *Slc3a2*; Dio1, iodothyronine deiodinase 1; *Hr*, hairless gene; Lat2, system L-amino acid transporter 2, gene symbol *Slc7a8*; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Mct8, monocarboxylate transporter 8, gene symbol *Slc16a2*; Mct10, amino acid transporter Tat1, gene symbol *Slc16a10*; RC3, neurogranin; T₃, 3,3',5-triiodo-L-thyronine; T₄, 3,3',5,5'-tetraiodo-L-thyronine, thyroxine; TH thyroid hormone; TSH, thyroid stimulating hormone; *Tshb*, β -subunit of TSH, gene symbol.

TABLES AND FIGURE LEGENDS

FIGURE 1: Gene targeting of *Slc7a8* in mice. A, Sketch of the murine *Slc7a8* locus. Boxes designate exons. Exon 2 encoding parts of transmembrane helices 1 and 2 (white circles) was replaced with an in-frame β -galactosidase/neomycin-resistance gene fusion cassette (geo) leading to a functional *null* allele. B, PCR genotyping of mice using primers located as shown in A. The black circle denotes the wild-type allele, the white circle denotes the targeted allele. C, Comparison of body weight and body length between *Slc7a8*-deficient and wild-type mice did not show any significant differences. n=8-12 male mice, age 60-250 days. D, Expression of Lat2 in multiple tissues of wild-type mice. For Western blot, 80 μ g protein of membrane fraction was loaded per lane. β -actin served as control. Molecular weight markers are indicated on the left. E, Expression of Lat2 along the gastrointestinal tract comparing wild-type and *Slc7a8*^{-/-} mice. F, Comparative Western blot analysis of Lat2 expression in liver, kidney and brain. β -actin was used as a control. The specific band is labeled with an arrow. Mct8 protein expression is shown for comparison.

FIGURE 2: Amino acid analysis in *Slc7a8*^{-/-} mice. A, Immunohistochemical staining for Lat2 in kidney locates the protein in the basolateral membrane of proximal tubule epithelial cells. Counterstaining with hematoxylin. Scale bar 100 μ m. The signal is completely abrogated in *Slc7a8*^{-/-} tissue. B, Amino acid analysis in urine reveals neutral aminoaciduria in *Slc7a8*^{-/-} mice. n=5-6 male mice per genotype. C, Comparative qPCR analysis of amino acid and thyroid hormone transporters in the kidneys of wild-type

and *Slc7a8*^{-/-} mice. 18s rRNA was used for normalization. n=3-4 male mice per genotype. D, Amino acid analysis in serum. n=4-7 male mice per genotype. Statistical analysis was calculated with ANOVA. *p<0.05, **p<0.01, ***p<0.001.

FIGURE 3: Circulating thyroid hormone and thyrotropin levels remain normal in *Slc7a8*^{-/-} mice. A, Total T₃. B, Total T₄. C, TSH levels. Hormones were measured by immunoassays in sera from 8-12 male mice per genotype.

FIGURE 4: Thyroid hormone-responsive genes in liver, kidney, and pituitary are unchanged in *Slc7a8*^{-/-} mice. A, Activity of hepatic Dio1 was determined in 3-4 male mice per genotype. No significant difference was observed (Student's *t*-test). B, Activity of renal Dio1 was determined in 3-4 male mice per genotype. No significant difference was observed (Student's *t*-test). C, Gene expression of *Dio2* and *Tshb* in pituitary was assessed by qPCR in 5 male animals per genotype. 18S-rRNA served as a reference gene. No differences were observed.

FIGURE 5: Cerebellar phenotype of *Slc7a8*^{-/-} mice. Expression of genes responsive to thyroid hormone was assessed by qPCR in 3-4 cerebella per genotype. A, *RC3*, neurogranin. B, *Hr*, hairless, C, *Dio3*. No significant differences were observed by Student's *t*-test. D, Rotarod analysis as a measure of movement coordination revealed a slight impairment in *Slc7a8*^{-/-} mice as compared with controls. N=8-12 male mice per genotype. E,F Nissl staining of cerebellar sections does not reveal differences in histogenesis as usually observed in hypothyroid animals. G,H Immunohistochemical staining for calbindin (red), a Purkinje cell marker and parvalbumin (green), a marker for Purkinje cells and cerebellar interneurons. Scale bars 50µm.

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