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Tissue-Specific Thyroid Hormone Deprivation and Excess in Monocarboxylate Transporter (Mct) 8-Deficient Mice

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Mutations of the X-linked thyroid hormone (TH) transporter (monocarboxylate transporter, *MCT8*) produce in humans unusual abnormalities of thyroid function characterized by high serum T_3 and low T_4 and rT_3. The mechanism of these changes remains obscure and raises questions regarding the regulation of intracellular availability and metabolism of TH. To study the pathophysiology of MCT8 deficiency, we generated *Mct8* knockout mice. Male mice deficient in Mct8 (*Mct8*$^{-/y}$) replicate the thyroid abnormalities observed in affected men. TH deprivation and replacement with L-T_3 showed that suppression of TSH required higher serum levels T_3 in *Mct8*$^{-/y}$ than wild-type (WT) littermates, indicating hypothalamus and/or thyrotroph resistance to T_3. Furthermore, T_4 is required to maintain the high serum T_3 level because the latter was not different between the two genotypes during administration of T_3. *Mct8*$^{-/y}$ mice have 2.3-fold higher T_3 content in liver associated with 6.1- and 3.1-fold increase in deiodinase 1 mRNA and enzymatic activity, respectively. The relative T_3 excess in liver of *Mct8*$^{-/y}$ mice produced a decrease in serum cholesterol (79 ± 18 *vs.* 137 ± 38 mg/dl in WT) and an increase in alkaline phosphatase (107 ± 23 *vs.* 58 ± 3 U/liter in WT) levels. In contrast, T_3 content in cerebrum was 1.8-fold lower in *Mct8*$^{-/y}$ mice, associated with a 1.6- and 10.6-fold increase in D2 mRNA and enzymatic activity, respectively, as previously observed in TH-deprived WT mice. We conclude that cell-specific differences in intracellular TH content due to differences in contribution of the various TH transporters are responsible for the unusual clinical presentation of this defect, in contrast to TH deficiency. (*Endocrinology* 147: 4036–4043, 2006)

THE EFFECTS OF thyroid hormone (TH) are dependent on the quantity of the hormone that reaches peripheral tissues and the availability of unaltered TH receptors in cell nuclei. Several classes of TH membrane transporters with different kinetics and substrate preferences have been identified that belong to different families of solute carriers, including organic anions (organic anion transporting polypeptides, OATP), amino acids, and monocarboxylate transporters (MCT) (1–5). Their characteristics in terms of tissue distribution and kinetics, as well as the binding of other possible ligands, provide them with potentially distinctive roles in the fine tuning of organ-specific TH availability.

Until recently, the physiological role of TH membrane transporters has remained elusive. The identification of patients with mutations in the X-linked TH transporter, monocarboxylate transporter 8 (MCT8), has revealed the role played by this transmembrane carrier in the intracellular availability of TH. Hemizygous MCT8-deficient males present a syndrome with two components: a thyroid defect (increased total and free serum T_3 and decreased total and free T_4 and rT_3 concentrations) and severe psychomotor and developmental delay (generalized dystonia combined with spasticity, mental retardation, lack of verbal communication, poor head control and coordination) (6–10). As is the case for most X-linked diseases, males are more severely affected in terms of both the neurological and thyroid defects (6–10), whereas female carriers have only mild thyroid function test (TFT) abnormalities (6–10). In the last year, *MCT8* gene mutations have been found to be the cause of Allan-Herndon-Dudley syndrome (9, 10), an X-linked syndromic form of mental retardation first described in 1944 (11) (Online Mendelian Inheritance in Man access no. OMIM 309600).

Understanding the mechanism producing the TFT abnormalities caused by *MCT8* mutations in humans has proved challenging. The presentation is not typical of hypothyroidism (12, 13). Investigations in humans are limited by the accessibility of tissues and by the patients' disability, rendering a mouse model mandatory for the study of this syndrome. We therefore generated a mouse deficient in *Mct8* through homologous recombination in embryonic stem (ES) cells. The resulting *Mct8* knockout male (*Mct8*$^{-/y}$) mice replicate the human thyroid phenotype. Our findings from *in vivo* and *in vitro* studies in male *Mct8*$^{-/y}$ mice indicate that Mct8-dependent tissues, such as the cerebrum, are TH deficient in the absence of Mct8. Tissues expressing other TH transporters, such as the liver, are thyrotoxic. This tissue-specific TH availability allows for the coexistence of increased deiodinase 1 (D1) and increased deiodinase 2 (D2) enzymatic activities stimu-

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Abbreviations: BW, Body weight; D1, deiodinase 1; D2, deiodinase 2; DTA, diphtheria toxin A; ES, embryonic stem; gDNA, genomic DNA; LoI/PTU, low-iodine diet containing 0.15% propylthiouracil; MCT, monocarboxylate transporter; Neo^R, neomycin resistance; TFT, thyroid function test; TH, thyroid hormone; WT, wild type.

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lated by opposite states of intracellular TH availability, further aggravating the status of increased 5′-deiodination unbalanced by 5-deiodination. We conclude that these tissue-specific differences in intracellular TH content are responsible for the unusual clinical presentation of this defect.

Materials and Methods

Experimental animals

Procedures carried out in mice, described below, were approved by the University of Chicago Institutional Animal Care and Use Committee.

Targeting construct

Isogenic genomic DNA (gDNA) from the E14Tg1A.4 129P2 ES male feederless ES cell line was used to amplify the homology arms by long-range PCR using a mixture of 1/10 Pfu Turbo polymerase (Stratagene, La Jolla, CA) and 9/10 LA Taq Polymerase (Takara, Kyoto, Japan). All PCR fragments were cloned into pGEM-T-easy (Promega, Madison, WI) and sequenced. Fragments were then cloned into pBluescript used as the backbone vector for the final targeting construct. The components of the final targeting vector are shown in Fig. 1A. The diphtheria toxin A (DTA) cassette was excised from the pPGKneoDTA vector (from Dr. Soriano) and the ACN cassette from plasmid pACN (Dr. Capecchi). The final construct was confirmed by restriction digestions and sequencing. Targeting construct linearized with *Sac*II (25 μg) was electroporated into the feeder-independent E14Tg2A.4 129P2-derived ES cells. After selection with G418 (200 μg/ml), 307 neomycin-resistant colonies were screened by Southern blot with outside probes, a 5′ probe in exon 2 and a 3′ probe in exon 5 of mouse *Mct8* (Fig. 1B).

Generation of the knockout mice

All three positive clones that were obtained were injected into C57Bl/6J blastocysts at the University of Chicago Transgenic Core Facility. Fifteen male chimeras were produced, and germline transmission was obtained for all male chimeras tested. The excision of the ACN cassette and the deletion of exon 3 in the germline of the male chimeras generated the null *Mct8* allele, $Mct8^{-}$ (Fig. 1C). $Mct8^{-/y}$ males were generated in F_2 C57Bl/6J backcross in expected Mendelian ratios (see *Results*). Genotyping of mice was performed using a common forward primer and wild-type (WT) and null allele-specific reverse primers (Fig. 1D) (sequences available upon request). For most analyses presented here, we have assessed F_2 C57Bl/6J:129Sv mixed genetic background mice. Inbred 129Sv $Mct8^{-/y}$ mice were obtained as pups of the F_1 $Mct8^{-/+}$ females generated by breeding the transmitting male chimeras with 129Sv WT females (stock no. 002448; The Jackson Laboratory, Bar Harbor, ME).

Treatments

Mice were kept under a 12-h light cycle and provided food and water *ad libitum*. For the lower L-T_3 dose used in the TSH suppression, test mice were fed a low-iodine diet containing 0.15% propylthiouracil (LoI/PTU) (Harlan Teklad Co., Madison, WI) for 14 d, and 0.8 μg L-T_3/100 g body weight (BW)·d was injected ip in the last 4 d. LoI/PTU was used to suppress the endogenous T_3 production during the administration of the physiological (low dose) of L-T_3. The high T_3 dose of 5 μg L-T_3/100 g

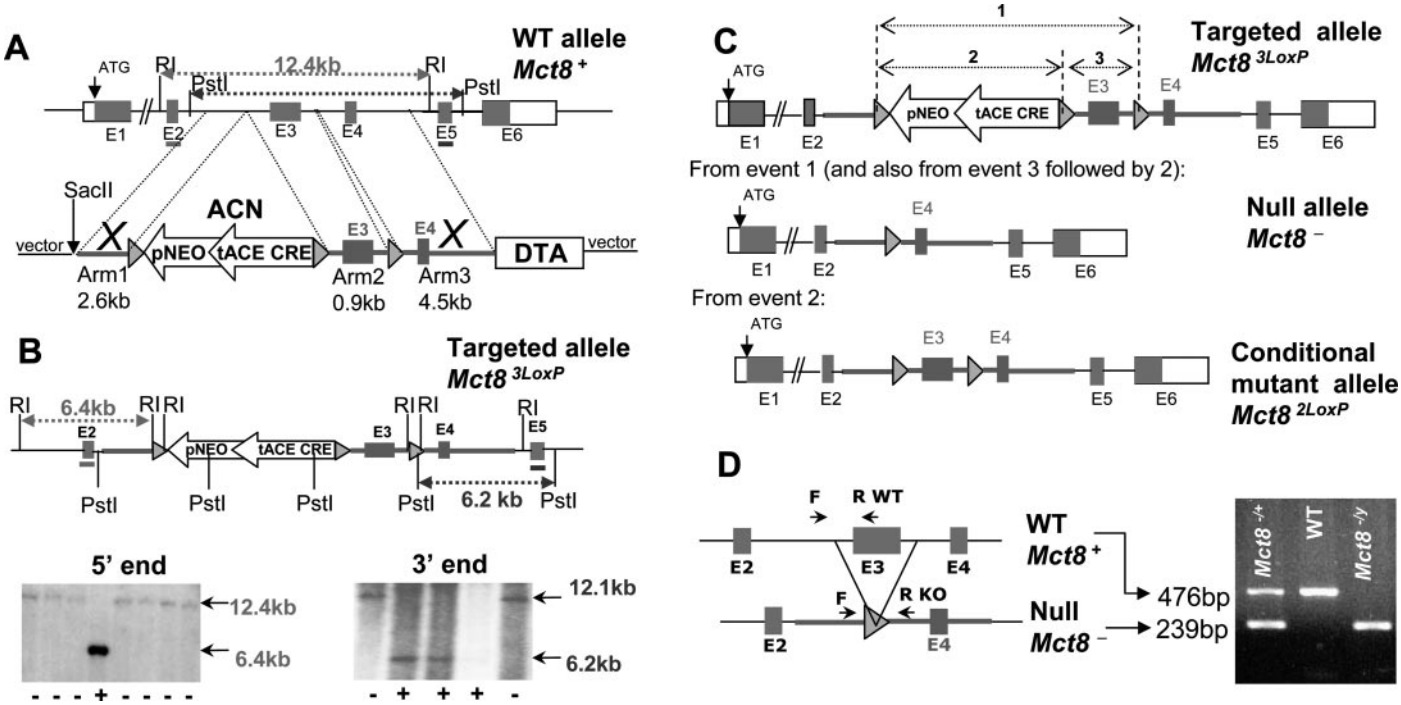

FIG. 1. Generation of $Mct8^{-/y}$ mice. A, Structure of *Mct8* gene and details of the targeting vector. *Sac*II is the unique restriction enzyme site used for the linearization of the vector before electroporation. The homology arms, the ACN and DTA cassettes and the three LoxP sites (*triangles*) are shown. Large Xs, Arms for which homologous recombination was selected. B, Schematic representation of the Southern blot screening. Probes are depicted as *bars* below exons 2 and 5. The locations of the *Pst*I and *Eco*RI (RI) restriction sites and the sizes of the blotted fragments are indicated. $Mct8^{+}$ is the WT allele, and $Mct8^{3LoxP}$ is the targeted allele in ES cells. The *gel* on the *left* shows the 5′ end screening on *Eco*RI-digested gDNA from ES clones. The endogenous band is 12.4 kbp (−), whereas a successfully targeted clone (+) has a 6.4-kbp band. The 3′ end Southern blot on *Pst*I-digested gDNA of the 5′ correctly targeted ES colonies is shown on the right. The endogenous band is 12.1 kbp (−), whereas the successfully targeted clones (+) have a 6.2-kbp band. C, Possible Cre recombination events in the germline of male chimeras. *Numbered arrows*, Possible recombination events on the targeted allele present in the germline of male chimeras, $Mct8^{3LoxP}$. D, Strategy of genotyping. The location of the three primers used is indicated by *arrows*: F, forward common; R WT, reverse WT specific (476-bp PCR product); and R KO, reverse knockout specific (239-bp PCR product). Genotyping gel shows heterozygous female ($Mct8^{-/+}$), hemizygous null ($Mct8^{-/y}$), and WT mice.

BW·d used in the TSH suppression test and in the time course of T_3 concentrations was administered for 4 d to animals receiving regular diet. Five WT and five *Mct8*$^{-/y}$ male littermates were used for each group of treatment. Mice were anesthetized 16 h after the last L-T_3 injection and, after obtaining a blood sample, were perfused to remove blood from organs and tissues were collected. For the time course experiment with high L-T_3 dose, blood was drawn at 3, 6, 16, and 20 h after the last injection.

Serum measurements

Serum total T_4 and T_3 concentration were measured by coated tube RIAs (Diagnostic Products, Los Angeles, CA) adapted for mouse serum using 25 and 50 μl serum, respectively. Total rT_3 was measured in 50 μl serum by RIA using reagents from Adaltis Italia (Rino, Italy). TSH was measured in 50 μl serum using a sensitive, heterologous, disequilibrium, double-antibody precipitation RIA (14). Cholesterol and alkaline phosphatase were measured each on 10 μl serum using a clinical chemistry autoanalyzer.

Tissue T_3 content

Before tissue collection, mice were perfused with heparinated PBS through a needle placed in the left ventricle. Tissues were rapidly collected on dry ice and stored at −80 C. T_3 was extracted from brain and liver using a method previously described (15, 16), and T_3 content was measured by RIA. Recovery was monitored in every batch of extraction by addition of the corresponding labeled iodothyronines to the tissues before homogenization.

Gene expression

Total RNA was extracted using phenol/guanidine isothiocyanate (TRIZOL, Invitrogen, Carlsbad, CA), and 2 μg total RNA was reverse transcribed using Superscript III RNase H Reverse Transcriptase Kit (Invitrogen) in the presence of 100 ng random hexamers. Reactions for the quantitation of mRNAs were performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), using SYBR Green I as detector dye. The oligonucleotide primers were designed to cross introns. Primers used for *Gsta2*, *Dio1*, *Dio2*, and *Dio3* are available upon request. Amplification of housekeeping gene RNA polymerase II was used as internal control (17).

D2 and D1 enzymatic activities

D2 enzymatic activity was performed as described (18) with the following modifications: 100 μg tissue homogenates in 100 μl reaction mixture containing 0.1 M phosphate buffer (pH 7), 1 mM EDTA, 20 mM dithiothreitol, 1 mM PTU, 100,000 cpm [^{125}I]-T_4, and 2 nM unlabeled T_4 were incubated at 37 C for 1 h. Saturating levels of unlabeled T_3 (1 μM) were added to the reaction mixture to inhibit the D3 enzyme. D1 enzymatic activity in liver was measured using [^{125}I]-T_4 as previously described (19) modified as follows: 20 μg tissue homogenates in 100 μl reaction mixture containing 0.1 M phosphate buffer (pH 7), 1 mM EDTA, 10 mM dithiothreitol, 100,000 cpm [^{125}I]-T_4, and 1 μM unlabeled T_4 were incubated at 37 C for 30 min. The enzymatic activities expressed in femtomoles (for D2) and picomoles (for D1) per hour and milligrams of protein were corrected for nonenzymatic deiodination observed in the tissue-free controls.

Statistic analysis was performed using ANOVA. Data are represented as mean ± SD. $P > 0.05$ was considered not to be significant.

Results

Targeting of Mct8

Based on the location of the *Mct8* gene on the X-chromosome and the severe and often incapacitating neurological defect in human males with MCT8 deficiency (6–10), we chose a Cre conditional knockout strategy of targeting in a male ES cell line. This provides flexibility to generate full knockout or tissue-specific knockout mice. Exon 3 was targeted for deletion because it encodes more than four transmembrane domains of the transporter, and its deletion changes the frame of the remaining putative transcript. A DTA cassette was used for negative selection against nonhomologous recombination (20) and neomycin resistance (Neo^R) for positive selection. Instead of the standard Neo^R cassette, the self-excising ACN cassette (21) was used, containing the Neo^R gene under the regulation of the mouse RNA Pol II promoter, together with Cre recombinase under the control of the testis-specific promoter of angiotensin-converting enzyme (Fig. 1A). The orientation of the genes contained in the cassette was reverse to the gene to be targeted to minimize the effect of the strong promoters on the locus. Neo^R colonies were screened by Southern blot with probes outside of the homology arms (Fig. 1B). Male E14Tg1A.4 129P2 ES cells were used for targeting. Of a total of 307 Neo^R clones, three were identified that were correctly targeted by homologous recombination, a targeting efficiency of 1/102 Neo^R.

Generation of Mct8$^{-/y}$ *mice*

The three independently targeted clones in a 129P2/OlaHsd-derived ES cell line were injected into C57Bl/6J blastocysts. Fifteen high-percentage male chimeras were generated, and germline transmission was tested by mating with C57Bl/6J WT females. Expression of the Cre recombinase contained in the targeted *Mct8* locus is activated in the testis of transmitting male chimeras (Fig. 1C) and recombines the three LoxP sites resulting in several possible alleles, including the null allele *Mct8*$^{-}$. Here, we present the data regarding the null allele. Because the *Mct8* locus is X-linked, there is no male to male inheritance; therefore, the transmission of the targeted allele was tested by genotyping only the female agouti pups born in the first generation (F_1). F_2 progeny were produced (Fig. 1D) by backcrossing the F_1 heterozygous null females to WT C57Bl/6J males. Transmission of the null allele occurred in correct Mendelian ratios for an X-linked locus: of 120 pups from 14 litters, 26 (21.7%) were male null *Mct8*$^{-/y}$, 33 (27.5%) were heterozygous females *Mct8*$^{-/+}$, and 61 (50.8%) were WT (37 males, 24 females).

Thyroid phenotype of Mct8$^{-/y}$ *mice*

Mct8$^{-/y}$ mice are viable, fertile, and grow normally (7.4 ± 0.9 *vs.* 8 ± 1 g in WT at weaning and 21.2 ± 2.4 *vs.* 23.7 ± 2.5 g in WT at 6 wk of age). No obvious motor abnormalities were observed; mice ambulate and eat normally. Male *Mct8*$^{-/y}$ mice replicate the characteristic human thyroid phenotype, with significantly higher serum T_3 and lower T_4 and rT_3 than their male WT littermates (Table 1). Carrier *Mct8*$^{-/+}$ females have some of the TFT abnormalities, higher T_3 levels compared with WT female littermates (117.6 ± 17.9 *vs.* 91.8 ± 8.1 ng/dl in WT, $P < 0.02$), lower rT_3 (27.9 ± 10.3 *vs.* 41.7 ± 5.1 ng/dl in WT, $P < 0.03$) but similar T_4 (4.3 ± 0.9 *vs.* 4.8 ± 0.3 μg/dl in WT, not significant).

Mice were obtained from male chimeras derived from each of the three independently targeted ES cell clones. F_2 male mice from each ES cells clone show significant TFT abnormalities of similar magnitude (Table 1). Thus, the observed changes are due to the induced mutation rather than an ES

TABLE 1. Thyroid function tests in male mice generated from three independently targeted ES cell clones in mixed C57Bl/6J:129Sv strain, in 129Sv, and females from F_2 crosses

	Males: C57Bl/6J:129Sv mixed genetic background						Males: 129Sv: 8A5 ES clone		Females C57Bl/6J:129Sv: 8A5 ES clone	
	4D8 ES clone		8A5 ES clone		10C6 ES clone					
	WT (n = 10)	*Mct8*$^{-/y}$ (n = 13)	WT (n = 8)	*Mct8*$^{-/y}$ (n = 10)	WT (n = 6)	*Mct8*$^{-/y}$ (n = 7)	WT (n = 5)	*Mct8*$^{-/y}$ (n = 5)	*Mct8*$^{-/+}$ (n = 9)	*Mct8*$^{-/-}$ (n = 5)
T_3 (ng/dl)	88 ± 13	127 ± 21[c]	89 ± 9	135 ± 15[c]	97 ± 11	131 ± 23[a]	110 ± 7	167 ± 20[b]	94 ± 10	172 ± 29[b]
T_4 (μg/dl)	3.9 ± 0.7	1.3 ± 0.5[c]	4.1 ± 0.7	1.3 ± 0.3[c]	3.7 ± 0.4	1.6 ± 0.2[c]	2.9 ± 0.3	1.9 ± 0.3[a]	4.2 ± 0.3	3.2 ± 0.4[b]
rT_3 (ng/dl)	17 ± 6.9	<2.5[c]	13.9 ± 2.2	<2.5[c]	14.7 ± 3.4	<2.5[c]	17.8 ± 7.2	2.9 ± 0.9[b]	28.5 ± 14.4	4.7 ± 2.7[b]
TSH (mU/liter)	ND	ND	30 ± 15.8	33.6 ± 25.2	ND	ND	24.8 ± 10.4	44.3 ± 30.1	ND	ND

ND, Not determined.
[a] $P < 0.01$.
[b] $P < 0.001$.
[c] $P < 0.0001$.

clone-specific abnormality. Inbred mutant male mice (129) were generated (Table 1), and their TFT pattern was similar to that in mice of mixed genetic background. This indicates that the consequences of Mct8 deficiency on thyroid physiology are stronger than putative modifiers of thyroid function due to different genetic backgrounds.

Homozygous null female (*Mct8*$^{-/-}$) mice were born from F_2 intercrosses of male *Mct8*$^{-/y}$ and female *Mct8*$^{-/+}$ mice. No WT females are born from this type of cross; thus, the comparison was done between *Mct8*$^{-/+}$ and *Mct8*$^{-/-}$ female littermates. Their thyroid phenotype (Table 1) had the same characteristics as the phenotype of males *Mct8*$^{-/y}$, indicating no gender-specific differences in the thyroid phenotype providing a proof of principle for the consequences of complete Mct8 loss. A corresponding human female has not been reported because the known males with MCT8 defect have not reproduced. For further investigations, we used the male *Mct8*$^{-/y}$ mice with their WT littermates for consistency of comparisons.

A T_3 suppression test was used to investigate pituitary sensitivity to T_3. This identical test has been used previously to test the sensitivity to T_3 in mice deficient in the TH receptors β and α (22, 23). Serum TSH levels in 6-wk-old F_2 male *Mct8*$^{-/y}$ untreated mice were not different compared with their WT littermates (Table 1). By 16 wk, differences in baseline serum TSH concentrations (at the limit of detection, <20 mU/liter in WT and 51.5 ± 16.2 mU/liter in Mct8$^{-/y}$) became significant at $P < 0.02$. When fed a LoI/PTU diet, TSH increased similarly in both genotypes (6907 ± 3062 mU/liter in WT *vs.* 4937 ± 1754 mU/liter in *Mct8*$^{-/y}$ mice) and serum T_3 levels decreased to the same level (58 ± 14 and 53 ± 10 ng/dl in WT and *Mct8*$^{-/y}$ mice, respectively). These values are not significantly different, and they are not graphed in Fig. 2. Administration of 0.8 μg/100 g BW·d L-T_3, just sufficient to correct hypothyroidism induced by the LoI/PTU diet, failed to suppress the serum TSH in the *Mct8*$^{-/y}$ mice to the levels observed in the WT controls despite similar T_3 serum levels (Fig. 2). A 6-fold higher L-T_3 dose (5 μg/100 g BW·d) was able to achieve full TSH suppression in both genotypes (Fig. 2). These results show a relative pituitary resistance to T_3 in the *Mct8*$^{-/y}$ mice and in addition demonstrate that T_4 is responsible for maintaining the high serum T_3 levels in Mct8 deficiency because both T_3 treatment protocols result in undetectable (<0.25 μg/dl) serum T_4 levels and similar serum T_3 levels.

Tissue uptake of circulating T_3

The different tissue distribution and kinetics of the known TH transporters provide them with distinctive roles in the fine tuning of organ-specific TH availability. We measured the serum T_3 levels and tissue T_3 content (Fig. 3) in 16-wk-old *Mct8*$^{-/y}$ and WT littermates, at baseline and after L-T_3 treatment (5 μg/100 g BW·d), for 4 d to suppress the endogenous production, thus allowing the comparison of the distribution of equivalent amounts of T_3 in mice that under basal condition have different concentrations. Serum was collected at 3, 6, 16, and 20 h after the last (fourth) L-T_3 injection. Tissues were frozen after intracardiac perfusion with heparinated PBS. We chose to study the liver and brain because in the liver, several classes of TH transporters are known to be expressed, some of which are liver specific (24); therefore, functional redundancy is possible, whereas in brain, only two TH transporters are known to be expressed, Mct8 and Oatp1c1 (24). Their expression pattern does not overlap; therefore, no high degree of redundancy is expected.

The serum T_3 followed a similar pattern of disappearance in both genotypes ($P = 0.6$) (Fig. 3A). In the liver of WT and

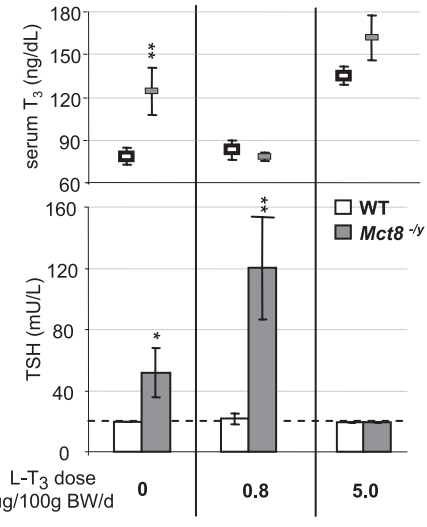

FIG. 2. Serum TSH and T_3 concentrations in untreated controls and after the administration of two doses of L-T_3 (*, $P < 0.02$; **, $P < 0.01$). *Empty boxes*, WT; *filled boxes*, Mct8$^{-/y}$. Animals given the low (physiological) dose of L-T_3 also received LoI/PTU diet to suppress endogenous T_3 (see *Materials and Methods*).

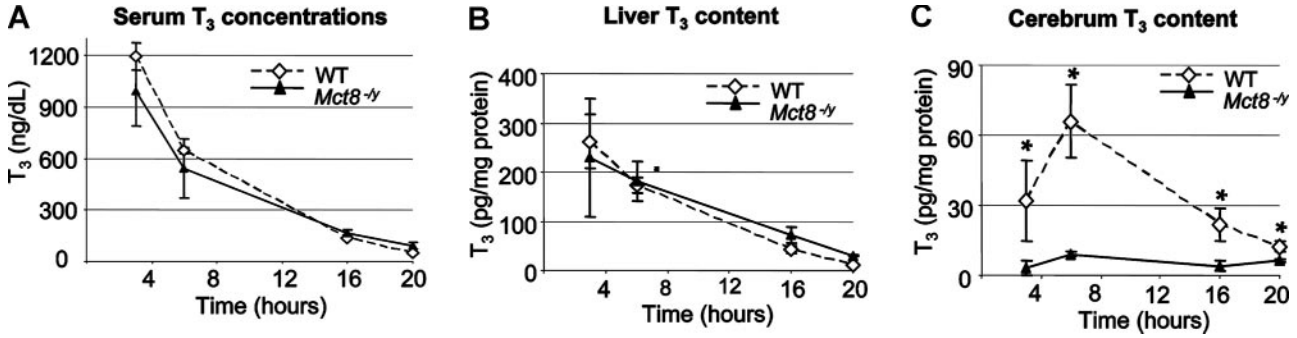

FIG. 3. Time course of T_3 concentrations after L-T_3 treatment. In serum (A; P value of the time course in Mct8$^{-/y}$ *vs.* WT is 0.6), liver (B; P value of the time course in Mct8$^{-/y}$ *vs.* WT is 0.9), and in cerebrum (C; P value of the time course in Mct8$^{-/y}$ *vs.* WT is <0.001, *) at different time points after the last injection of a 4-d treatment with 5 μg/100 g BW·d.

Mct8$^{-/y}$ mice, changes in T_3 concentrations paralleled those observed in serum over the same period of time ($P = 0.9$) (Fig. 3B). In the cerebrum of WT mice, T_3 content was highest at 6 h, whereas T_3 content in the *Mct8*$^{-/y}$ mice were 2 to 10 times lower at all times ($P < 0.001$) (Fig. 3C). These results indicate that the liver is not dependent on Mct8, despite its relatively high expression (1–5), and that other liver TH transporters ensure hepatic T_3 transport resulting in levels that parallel serum. In contrast, lack of Mct8 results in brain-specific deficiency in TH.

Tissue-specific TH excess and deprivation in Mct8$^{-/y}$ *mice*

We further investigated the TH status in liver and brain of Mct8-deficient mice. In liver, the baseline T_3 content was 2.3 times higher in *Mct8*$^{-/y}$ than in their WT littermates (Fig. 4A), thus reflecting the higher T_3 serum levels in these mice.

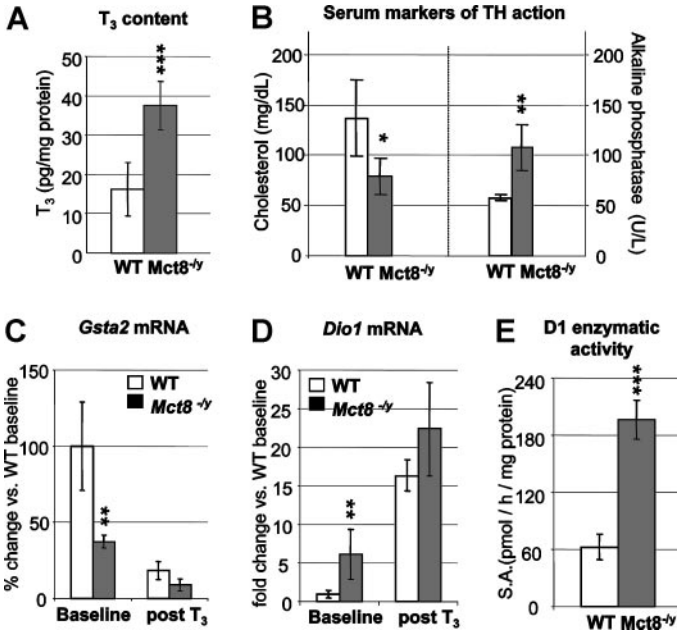

FIG. 4. Consequences of Mct8 deficiency in liver. A, Baseline tissue T_3 content. B, Baseline serum cholesterol and alkaline phosphatase. *Dio1* (C) and *Gsta2* (D) mRNA at baseline and after L-T_3 treatment (5 μg/100 g BW·d); differences between baseline and after L-T_3 treatment within genotypes were significant ($P < 0.001$). Data are expressed as percent change compared with baseline WT, being 100%. E, Baseline D1-specific enzymatic activity (S.A.). *, $P < 0.02$; **, $P < 0.01$; ***, $P < 0.001$.

Serum cholesterol and alkaline phosphatase, markers of TH action on liver (25), showed changes compatible with a thyrotoxic state in the liver of *Mct8*$^{-/y}$ mice compared with their WT littermates (Fig. 4B). The significantly higher T_3 content in the liver of *Mct8*$^{-/y}$ mice affected also the expression of TH target genes, glutathione S transferase α 2 (*Gsta2*) (Fig. 4C) and D1 (*Dio1*) (Fig. 4D), normally down-regulated and up-regulated, respectively, by TH (23, 26). At baseline, the expression of *Dio1* mRNA was 6.1 times higher (Fig. 4D), with D1 enzymatic activity being 3.1 times higher (Fig. 4E) in the liver of *Mct8*$^{-/y}$ than in WT mice. After L-T_3 treatment, *Dio1* mRNA levels were comparable in both genotypes in agreement with the equalization of T_3 content (Fig. 3B). Similarly, basal *Gsta2* mRNA levels were significantly lower in *Mct8*$^{-/y}$ mice and become equally reduced in *Mct8*$^{-/y}$ and WT mice after treatment with the TSH-suppressive dose of L-T_3. These results demonstrate a relatively thyrotoxic state at baseline in the liver of *Mct8*$^{-/y}$ mice, reflecting the higher serum concentration of T_3, indicating that in these mice, the transfer of T_3 from blood to liver is mediated through TH transmembrane carriers other than Mct8 (24).

In the cerebrum of *Mct8*$^{-/y}$ mice, T_3 content was 1.8 times lower than in WT controls (Fig. 5A). This tissue is heterogeneous in cell types and genes regulated by TH, and their developmentally regulated expression is restricted to discrete population of cells (27, 28). Therefore, it is not appropriate to measure in whole-organ mRNAs of TH-regulated genes with limited cell expression. We measured instead *Dio2* expression and activity to assess the overall thyroid status. The lower T_3 content was accompanied by a 1.6-fold higher mRNA expression of *Dio2* in the *Mct8*$^{-/y}$ mice (Fig. 5B), whereas the D2 enzymatic activity was increased by 10.6-fold (Fig. 5D), likely due to posttranscriptional regulation of the D2 protein (29, 30). We also measured *Dio3* mRNA at baseline and after T_3 treatment. Although *Dio3* has been shown to respond to T_3 (29, 30), the mechanism for its positive regulation is not known. Baseline *Dio3* expression was not significantly different between the two genotypes (Fig. 5C), and T_3 treatment increased *Dio3* mRNA in WT mice but did not change the expression in *Mct8*$^{-/y}$ mice, in agreement with the impaired T_3 uptake demonstrated in the cerebrum (Fig. 3C). These results demonstrate the Mct8 dependency of the brain for adequate T_3 levels and uncover the increase in D2 activity, likely a consequence of the low T_4 levels and a compensatory mechanism to protect a minimum neces-

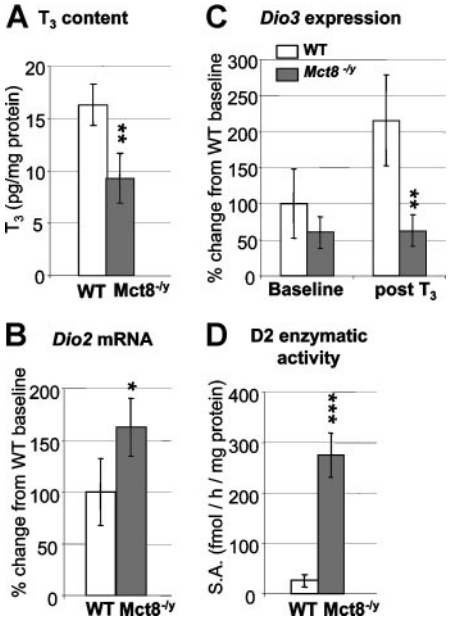

FIG. 5. Consequences of Mct8 deficiency in cerebrum. A, Baseline tissue T_3 content. B, *Dio2* mRNA at baseline. C, *Dio3* mRNA at baseline and after L-T3 treatment (5 μg/100 g BW·d). Data are expressed as percent change compared with baseline WT, being 100%. D, Baseline D2-specific enzymatic activity (S.A.). *, $P < 0.02$; **, $P < 0.01$; ***, $P < 0.001$.

sary local supply of T_3 as it has been demonstrated in rat hypothyroid brain (31).

Discussion

Description of the phenotype caused by mutations in the X-linked TH transporter *MCT8* in humans has raised many questions regarding the regulation of membrane transport and cellular availability of TH. The combination of unusual TFT (high T_3, low T_4 and rT_3) and severe psychomotor defect in patients with *MCT8* gene defects is not typical of TH deficiency. Studies that we performed in cultured skin fibroblasts from such patients have demonstrated impaired uptake of both T_3 and T_4 and increased D2 enzymatic activity (32). These results only partially explain the thyroid phenotype. Understanding the pathophysiology of the defect requires the study of the entire organism. We therefore generated Mct8-deficient mice. Exon 3 of mouse *Mct8* was targeted for deletion using a Cre conditional knockout strategy because this exon encodes four transmembrane domains, and its deletion is likely to cause a null mutation. This design also allows for the generation of tissue-specific *Mct8* knockout animals as an alternative approach for future experiments.

The thyroid phenotype of Mct8 null ($Mct8^{-/y}$) mice replicates the phenotype observed in humans. The characteristic unusual TFT have been found in male $Mct8^{-/y}$ mice originating from all three independently targeted ES cell clones on both C57Bl/6J:129Sv mixed and 129Sv genetic background. Furthermore, female $Mct8^{-/-}$ born from F_2 intercrosses have the same pattern of TFT as the males. The consistency of the thyroid phenotype in different genetic backgrounds indicates that the consequences of Mct8 defect on thyroid physiology are stronger than putative modifiers of thyroid function in different strains.

The fact that the neurological manifestations present in male humans with *MCT8* defects are not replicated in the mouse model might be due to species-specific regulation of intracellular TH availability and TH demands for normal function of central nervous system. The absence of a neurologic phenotype in mice that are hypothyroid at the brain level is not totally unexpected because other instances of neurologic symptoms associated with hypothyroidism, such as the spastic neurologic cretinism associated with iodine deficiency in humans, are not replicated in mouse models of iodine deficiency (33). It is possible that subtle abnormalities are present in the brains of Mct8-deficient mice, and detailed studies focused on investigation of the consequences of Mct8 defect on central nervous system are needed to elucidate this aspect.

At 6 wk, TSH levels were similar in male WT and $Mct8^{-/y}$ mice, but by 16 wk, the TSH levels in $Mct8^{-/y}$ mice slightly increased. Pituitary sensitivity was tested using a standardized test of TSH suppression for mice (22, 23). L-T_3 levels sufficient to correct TH deficiency in WT mice were unable to equally suppress the TSH in $Mct8^{-/y}$ mice, therefore establishing the reduced ability of the circulating T_3 to reach the thyrotrophs and/or hypothalamus. Six-fold higher doses of L-T_3 were able to suppress serum TSH levels in the $Mct8^{-/y}$ mice, indicating a relative central resistance likely due to impaired uptake of L-T_3. The fact that similar serum T_3 levels are achieved in both genotypes when the endogenous T_4 production is suppressed indicates that T_4 is responsible for maintaining the high serum T_3 levels characteristic for Mct8 deficiency.

To assess T_3 uptake and its tissue availability, we examined the time course of T_3 disappearance from serum and tissues after administration for 4 d of a daily supraphysiological dose of L-T_3. The goal of this protocol was to suppress the endogenous production of TH, thus allowing the comparison of the responses to the equivalent amount of T_3 in mice that under basal conditions have different concentrations of TH. Serum T_3 levels and liver T_3 content followed a similar pattern after the last L-T_3 injection in mice from both genotypes, indicating that degradation of T_3 is not reduced in this defect. T_3 uptake in the cerebrum of WT mice occurred later than in the liver, whereas the uptake in the cerebrum of in $Mct8^{-/y}$ mice was minimal, up to 10 times lower. These results demonstrate tissue-specific differences in TH uptake in Mct8-deficient mice dependent upon the redundancy in TH transport.

To study the consequences of Mct8-dependent TH uptake differences, we measured tissue T_3 content in the liver of 16-wk-old mice, perfused to remove blood-derived T_3, and measured the expression of genes positively and negatively regulated by TH, at baseline and after L-T_3 treatment. Liver T_3 content was significantly higher in the $Mct8^{-/y}$ mice at baseline, reflecting the circulating T_3 levels. The consequences of this T_3 level were confirmed by its effect on TH-responsive genes, *Dio1* (29) and *Gsta2* (26). The significant differences found between $Mct8^{-/y}$ and WT mice at baseline were abrogated by treatment with high-dose L-T_3 that equilibrated the serum T_3 levels. In addition, the baseline hepatic thyrotoxicosis resulted in decreased serum cholesterol and increased alkaline phosphatase in $Mct8^{-/y}$ mice compared with WT, similar to the lower serum cholesterol

levels and increased SHBG reported in humans with *MCT8* mutations (34) and mice given TH (25).

The TH deficiency in brain, as demonstrated by low baseline tissue T_3 content, is undoubtedly the consequence of reduced T_3 uptake in this tissue, which contributes to further augment the serum T_3 concentration. This hypothyroid state in cerebrum, also maintained by the low serum T_4, results in a 10.6-fold increase in D2 enzymatic activity by posttranscriptional mechanisms, such as increased half-life of the enzyme and decreased degradation (29, 30).

Our findings of coexistent TH excess and deficiency in Mct8 knockout mice can explain in part the mechanisms responsible for the TFT pattern observed in Mct8 deficiency. Several tissue-specific events come into play. Multiple TH transporters expressed in liver allow entry of serum T_3 producing hepatic thyrotoxicosis. Furthermore, the increased T_3 content in liver of *Mct8*$^{-/y}$ mice stimulates *Dio1* expression and D1 enzymatic activity. The latter increases the conversion of T_4 to T_3, which likely results in the consumption of T_4 and further increase in T_3. The increased D1 enzymatic activity in liver also stimulates the metabolism of rT_3. This, together with the decreased rT_3 production expected from 5-deiodination of T_4, play a role in the low serum rT_3 levels characteristic in Mct8 deficiency. The coexistence of increased D1 and D2 activity stimulated by opposite states of intracellular TH availability has an additive consumptive effect on T_4 levels. In cerebrum, the D2 activity functions to maintain minimal local levels of T_3 in the context of Mct8 deficiency, whereas the thyrotoxic environment in the liver results in increased D1 activity and due to the size effect of this organ contributes to the high serum T_3 levels.

The *in vivo* and *in vitro* studies carried out in these Mct8-deficient mice have provided insight into the pathophysiology of this defect. Deiodinases modulate the intracellular availability of active T_3 in a tissue-specific fashion. Due to the complex nature of deiodinase regulation and differences in tissue-specific TH availability, the overall increased 5′ deiodination is not counterbalanced by 5 deiodination. This is maintained through a cycle probably triggered by the impaired uptake of TH in Mct8-dependent tissues and the subsequent increased T_3 levels. This knockout mouse model represents a useful tool to study the pathophysiology of TFT abnormalities characteristic in MCT8 deficiency and demonstrates the complexity of this defect, with tissue-specific TH excess and deficiency depending on the redundancy in supply of TH through various classes of TH transporters.

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