The importance of plasma membrane transporters for the transfer of thyroid hormones from the extracellular milieu to the interior of the cell is now widely recognized. For many years it was thought that thyroid hormones enter the target cells by passive or facilitated diffusion. However mutations in a specific T4 and T3 transporter, the monocarboxylate transporter 8 (MCT8, SLC16A2), were found in patients with a severe neurodevelopmental defect and abnormal levels of iodothyronines in blood, consisting of decreased T4 and rT3 and increased T3 (1, 2). These and subsequent findings revealed the physiological role of transporters in thyroid hormone action and their relevance to the brain (3–5).

The generation of Mct8 knockout (KO) mice demonstrated that absence of Mct8 impairs brain thyroid hormone uptake and metabolism, possibly due to a primary decreased uptake and degradation of T3 in target neurons (6, 7). As a consequence, T3 concentrations increase in serum, with stimulation of Dio1 expression in liver and other tissues. It is postulated that the increased Dio1 activity increases conversion of T4 to T3, thereby decreasing T4 and further increasing T3 in serum. On the other hand, circulating rT3 is also decreased, which might be due to increased degradation by Dio1 and/or decreased formation from T4 by inner ring deiodination.

However, whereas the absence of Mct8 in mice reproduces the endocrine changes characteristic for humans with MCT8 gene mutations, the mutant mice do not show signs of neurological impairment, which contrasts with the observations in humans. It is logical to think that the neurological syndrome is due to impaired T3 action in neurons as a consequence of restricted uptake. However, no histological changes suggestive of cerebral hypothyroidism in the mutant mice have been found, and only a moderately decreased expression of thyroid hormone

Abbreviations: EGL, External germinal layer; Gpd2, α-glycerol phosphate dehydrogenase; Hr, hairless; KO, knockout; MCT, monocarboxylate transporter; Nrgn, neuregulin; Oatp, organic anion-transporting polypeptide; P, postnatal day; Wt, wild type.
regulated genes such as neurogranin (Nrgn, also known as RC3) could be related to the decreased T₃ uptake (6, 7). At least in part, this could be interpreted as if the mice brains were in a state of locally compensated hypothyroidism because Dio2 activity is increased in the brain due to the decreased concentration of circulating T₄ (6, 7).

Early studies on Mct8 gene expression in rodents indicated that the gene is expressed predominantly in the choroid plexuses and in neurons (8). Recent studies have shown that Mct8 is also expressed in the blood-brain barrier (9). Other thyroid hormone transporters are expressed in the blood-brain barrier, such as organic anion transporters and L-type amino acid transporters (10, 11). In the absence of Mct8, the restriction to T₃ transport through the blood-brain barrier or through the neuronal plasma membrane would depend on the presence of alternative transporters.

In this work, we studied the relevance of Mct8 gene expression in neurons for T₃ action. We have analyzed the relative effects of low doses of T₄ and T₃ on two T₃ target genes, expressed in the striatum (Nrgn) and cerebellum [Hairless (Hr)]. We found that in male Mct8KO mice, when compared with wild-type (Wt) mice, these genes are less responsive to T₃ than T₄, indicating a restricted entry of plasma T₃ but not T₄, derived from T₃. On the other hand, the action of T₃ in primary cultures of cerebellar granular cells was little affected in the absence of Mct8. The data suggest that the critical restriction to T₃ transport in the absence of Mct8 is located at the blood-brain barrier rather than at the plasma membrane of individual neurons.

Materials and Methods

Animals

Protocols for animal handling were approved by the local institutional Animal Care Committee, and followed the rules of the European Union. Animals were housed in temperature- (22 ± 2 C) and light (12 h light, 12 h dark) controlled conditions and had free access to food and water. Mct8KO mice were originally produced by Dumitrescu et al. (6) by homologous recombination. Experiments were carried out on Wt (Mct8⁺/⁺) and KO (Mct8⁻/⁻) male litter mates derived from the third and fourth back crossing of heterozygous females (Mct8⁺/-) with Wt (Mct8⁺/⁺) males of the C57BL/6 strain. The genotype was confirmed by PCR of tail DNA (38 cycles at 55 C annealing temperature) using the following primers: forward common, 5′-H11032-CTCCCA; reverse knockout specific, 3′-H11032-GCAAAGGACATGACATGCGG; reverse common, 3′-H11032-ACAA-TGGTCCAGCTCAAAAGG; reverse knockout specific, 3′-CTCCCAAGCCTGATTTCAT-5′. Using this procedure the Wt allele generated a 476-bp product and the null allele a 239-bp PCR product.

Induction of hypothyroidism and thyroid hormone treatments

After crossing with Wt male mice, Mct8⁻/⁻ pregnant dams were given either drinking water or a solution containing 0.02% 1-methyl-2-mercapto-imidazol (Sigma Chemical Co., St. Louis, MO) plus 1% KClO₄ ad libitum. These anthyroid drugs were given from gestational day 17, and throughout the lactating period, until the end of the experiment on postnatal day (P) 21. The pups were genotyped on P11 to select for Mct8⁺/⁺ and Mct8⁻/⁻ mice from the same litters. For simplicity, these animals will be referred to as Wt and KO mice, respectively, throughout this paper. The hypothyroid pups were then divided into three groups receiving no hormonal treatment, 20 ng T₃ per gram body weight, or 3 ng T₄ per gram body weight respectively. The hormones were administered in PBS containing 0.1% BSA, as single ip injections from P16 to P20. The following groups were thus prepared: euthyroid (n = 7) and hypothyroid (n = 6) Wt mice; euthyroid (n = 8) and hypothyroid (n = 6) KO mice; hypothyroid Wt mice treated with either T₄ (n = 5) or T₃ (n = 6), and hypothyroid KO mice treated with either T₄ (n = 6) or T₃ (n = 6). The pups were killed by decapitation 24 h after the last T₄ or T₃ injection, on P21. The striatum and cerebellum were rapidly dissected out, frozen on dry ice, and kept at −80 °C until RNA isolation.

Histological methods

Examination of stained sections of the cerebellum and in situ mRNA hybridization analysis were performed on pups perfused with paraformaldehyde under anesthesia. Methods for perfusion, sectioning, staining, and in situ hybridization have been previously described in detail (12, 13).

PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was prepared from 250 ng RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). For quantitative PCR, a cDNA aliquot corresponding to 5 ng of the starting RNA was used, with Taqman Assay-on-Demand primers and the Taqman universal PCR master mix, No Amp EraseUNG (Applied Biosystems), on a 7900HT fast real-time PCR system (Applied Biosystems). The PCR program consisted in a hot start of 95 C for 10 min, followed by 40 cycles of 15 sec at 95 C and 1 min at 60 C. PCRs were performed in triplicates, using the 18s gene as internal standard and the 2-cycle threshold method for analysis. For quantitative assays, a standard curve was generated after amplification of known amounts of specific templates for each gene including 18s to calculate the number of mRNA copies in each sample.

Primary granular cell cultures

All media were purchased from Invitrogen. The cerebella were dissected from P6-P7 Wt and Mct8KO mice in Hanks’ balanced sodium salt solution, without Ca²⁺ and Mg²⁺, supplemented with 1 mM Na pyruvate and 10 mM HEPES (pH 7.4). The tissue was disaggregated by passing through a 0.9-mm syringe, rinsed in Hanks’ balanced sodium salt solution/pyruvate/HEPES and resuspended in serum-free culture medium (neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, 10 U/ml penicillin, and 10 U/ml streptomycin) before seeding on poly-L-ornithine (Sigma)-coated 12-well multiwells (Sigma; 2.5 × 10⁵ cells/well). After 4 d, the granular cells were incubated for 24 h in the absence or presence of T₃ (Sigma) (from 0.2 to 5 nM) in the same medium containing 0.1% newborn calf serum deprived of thyroid hormones. Astrocyte contamination of the cultures was 3% as determined by immunofluorescence. Cells plated on glass coverslips were fixed with 4% paraformaldehyde for 5 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min and then with methanol at −20 °C for 2 min. After blocking with 5% nonimmune serum (Vector Laboratories, Burlingame, CA), the cells were doubly stained by overnight incubation at 4 °C with the following combination of primary antibodies diluted 1:2000: rabbit polyclonal antifibrillary acidic protein (Daiko, Glostrup, Denmark) for astrocytes and mouse monoclonal anti-NeuN (Chemicon, Temecula, CA) for neurons. Nuclei were labeled with the nuclear stain 4′, 6-diamidino-2-phenylindole dihydrochloride.

Statistical calculations

Differences between means were obtained by two-way ANOVA, with the two factors being genotype and thyroidal state. As post hoc test, we used the Bonferroni tests using the Graph-Pad Prism software (http://www.graphpad.com/prism/).
In this study we examined the relative effects of low doses of T4, and T3, administered to hypothyroid Wt and Mct8-deficient mice. The goal of this study was to evaluate the relative role of Mct8 in the transport of T4 and T3 in the brain in vivo by determining the effects of the hormones on cerebellar structure and the expression of thyroid hormone-regulated genes. Preliminary studies using morphological techniques failed to reveal consistent differences between age-matched, Wt, and KO mice during development that could be related to deficient thyroid hormone transport into the brain. Although not shown in this paper, we examined the laminar structure of the cerebral cortex, myelin protein expression, maturation of glial cells and different classes of interneurons and Purkinje cells, and number of interneuron precursors in the cerebellum and found no consistent deficits in the KO mice. These observations agree with previous studies reporting no obvious phenotype of cerebral hypothyroidism in these mice (6, 7).

The next question we addressed was whether the absence of the Mct8 transporter impaired the biological activities of exogenous T4 and T3 selectively. Wt and KO mice were made hypothyroid by administration of antithyroid drugs and received 20 ng/g T4 or 3 ng/g T3 daily for 5 d before the animals were killed on P21. This dosage schedule was sufficient to completely correct the delayed migration of granular cells in the cerebellum of hypothyroid Wt mice, as shown in Fig. 1. Cerebellar sections from each group of mice were stained and examined by optical microscopy. Migration of granular cells was already completed in the euthyroid Wt animals by P21 so that the external germinal layer (EGL) was absent. As expected, the EGL was still present in the hypothyroid Wt mice at this age. Both T4 and T3 treatments were equally able to prevent the effects of hypothyroidism in the Wt mice.

In the absence of Mct8, the structure of the cerebellar cortex in the untreated KO mice was identical with that of the Wt mice, with no EGL remaining, illustrating the lack of morphological developmental abnormalities. As in the hypothyroid Wt mice, the EGL was still present in the hypothyroid KO P21 mice. T4 treatment prevented the effects of hypothyroidism. However, in contrast to the effect on Wt mice, T3 treatment did not correct the migration abnormality.

To examine the effects of T4, and T3 on gene expression, two well-known thyroid hormone target genes, Nrgn in the striatum, and Hr in the cerebellum, were examined by real-time PCR. The results are shown in Fig. 2. Interestingly and despite the lack of morphological impairment, Hr expression was decreased in the cerebellum of untreated KO mice with respect to the Wt mice, with levels similar to those present in WT hypothyroid mice. Induction of hypothyroidism decreased Hr expression further in
the KO mice. T₄ treatment significantly increased Hr expression in the hypothyroid Wt mice to levels that were similar to the untreated Wt mice. The effect of T₄ treatment in the hypothyroid KO mice was not different from the hypothyroid Wt mice. The response to T₃ was similar to that of T₄ in the Wt mice but was significantly different when the hypothyroid Wt and KO mice were compared. T₃ was without effect in the hypothyroid KO mice.

Nrgn expression was also lower in the untreated KO than the Wt mice and decreased further with hypothyroidism. In contrast to Hr, the hypothyroid Wt and KO mice had similar Nrgn mRNA levels. Although neither T₄ nor T₃ treatment were able to fully normalize Nrgn expression, again there was a significant difference between the responses to T₃ between the Wt and the KO mice but no difference in the responses to T₄.

The liver mRNA Gpd2 (encoding mitochondrial α-glycerophosphate dehydrogenase) was increased in the untreated KO mice relative to the untreated Wt mice. Hypothyroidism decreased Gpd2 expression in both genotypes (P < 0.001). T₄ and T₃ significantly increased the Gpd2 mRNA level compared with hypothyroid mice (P < 0.05). This increase was of similar magnitude in both genotypes and with both hormones.

To correlate the effects of thyroid hormones on Hr and Nrgn gene expression with Mct8 gene expression, we performed in situ mRNA hybridization (Fig. 3). The Mct8 gene was heavily expressed in the choroid plexus (Fig. 3, A–C) and the ependymal lining of the third ventricle (Fig. 3B). Other sites of expression were the upper layers of the cerebral cortex, especially the cingulate, visceral, and piriform cortices; the pyramidal and granular layers of the hippocampus; and the amygdala (Fig. 3B). In the cerebellum, besides expression in the choroid plexus, Mct8 mRNA had low but detectable abundance in the cerebellar cortex. In the striatum Mct8 was poorly expressed (Fig. 3A). Figure 3D shows Nrgn mRNA, which is abundantly present after a lateral-medial gradient, contrasting with the poor expression of Mct8. The effect of Mct8 gene deletion (Fig. 3E) did not affect the Nrgn mRNA signal gradient, in contrast to the effect of hypothyroidism (not shown, but see Ref. 14), which results in a total suppression of the gradient. Interestingly, Dio2 mRNA distribution in the striatum (Fig. 3F) also followed a similar gradient, with no changes in the pattern of distribution in the Mct8 KO mice (not shown). The lack of correlation between the sites of expression of Mct8 with that of the T₃-target genes, Nrgn and Hr, indicate that Mct8 might be playing a minor role in thyroid hormone transport through the plasma membrane of cerebellar granular cells and striatal neurons in vivo.

To address this question more directly, we studied transporter expression and Hr induction by T₃ in primary cultures of neurons. Granular cells from newborn mice cerebella were cultured. To analyze the effect of Mct8 deficit on the effect of T₃ on Hr gene induction, T₃ was added to granular cells from Wt and KO mice, and Hr mRNA was measured by quantitative PCR. One representative experiment using different concentrations of T₃ is shown in Fig. 4. Starting at the lowest concentration used, 0.2 nM, all T₃ concentrations gave a significant stimulation of Hr expression (P < 0.001) in both the Wt and KO cells. There were no significant differences in the effect of T₃ in the KO mice as compared with the Wt except for the 1.25 nM T₃ concentration in this particular experiment.

We also examined the profile of transporter expression in the same cultures used to analyze the effect of T₃ on Hr. We measured the amounts of mRNA of organic anion-transporting polypeptide (Oatp)-2 (Slco1a4), Oatp14 (Slc1c1), and Mct8 (Table 1). Granular cells from Wt cells expressed predominantly Mct8 (591 ± 130 mRNA copies, relative to 18S RNA), which was undetectable in the KO cells. Oatp2 and Oatp14 were expressed at much lower levels (27.0 ± 9.1 and 7.4 ± 3.4, respec-

![FIG. 3. 35S in situ hybridization for Mct8 (A–C), Nrgn (D and E) and Dio2 (F) mRNAs. The slices are from coronal sections at the level of the caudate (A, B, and D–F) and sagittal section of the cerebellum (C). All slices are from P21 Wt mice except for E, which shows the typical Nrgn expression in a P21 KO mouse. The arrows in A–C show heavy Mct8 expression in the choroid plexus. The asterisks show the caudate nucleus in A, with low hybridization signal, and the faint but detectable hybridization in the cerebellar cortex in C. 3V, Third ventricle.](https://example.com/fig3)

![FIG. 4. Hr expression in primary cultures of granular cells from Wt or Mct8-deficient mice, as a function of T₃ added to the cultures. Differences in Hr expression between the cells without T₃ added and the 0.2 nM T₃ concentration were P < 0.001 (a). Differences between the Wt and KO cells at each T₃ concentration, by two-way ANOVA were not significant, except for the 0.25 nM T₃, with P < 0.05 (b).](https://example.com/fig4)
suggest that, in the doses used, T4 could reach the cerebellar KO mice. Except for the higher dose, which decreased Oatp14 mRNA in the KO cells used in this experiment are the same as for Hr mRNA quantification shown in Fig. 4. Two-way ANOVA using the data from all T3 concentrations revealed that there was no difference of genotype or treatment, except for the highest T3 dose that decreased Oatp14 mRNA in the KO cells.

Discussion

The main finding of the present work is that the brain of animals lacking the thyroid hormone transporter Mct8 do not readily respond to a low dose of T3, whereas the sensitivity to an equally low dose of T4 is similar to that of Wt animals. Five nanograms per gram T3 were previously shown to normalize circulating thyroid mice. In contrast, they were insufficient for Nrgn expression in the Wt hypothyroid mice. In the context of these findings, it was surprising that in isolated granular cells the Mct8 gene was by far the more abundantly expressed transporter. However, its absence in Mct8<sup>−/−</sup> cells caused only a minimal impairment of T3 action at the nuclear level, as evidenced by Hr gene expression, with a trend toward a lower effect at intermediate doses in the KO cells. Although expression of other transporters was much lower, it was enough to elicit almost identical responses to T3 in the absence as in the presence of Mct8. The presence of other transporters is also likely the cause for the similar effect of T4 in vivo in Wt and KO mice. The relative effects of T4 and T3 on the expression of the Nrgn gene in the striatum suggests a similar conclusion.

These results agree with the preferential accumulation of administered T4, relative to the restricted accumulation of administered T3 in the brain of Mct8 KO mice (6, 7). Brain T3 concentrations in the KO mice were about two thirds of normal. Given the restriction to T3 entry, most T3 in the brain of these animals must be derived from T4.

The main site of Mct8 expression is the choroid plexus. Consequences of the absence of Mct8 in the choroid plexus are not known. Intrathecally administered T4 and T3 can access brain structures (16). However, most studies on the routes of thyroid hormone entry to the brain agree that the cerebrospinal fluid allows only limited access of thyroid hormone to the brain parenchyma, preferentially reaching cells located near the surface of the ventricles (17, 18). Therefore, the main access of thyroid hormone to the brain parenchyma is through the blood-brain barrier. In keeping with this concept, our data suggest that the restriction of T3 entry in Mct8-deficient mice is at the blood-brain barrier. Indeed Mct8 has been recently demonstrated in the membrane of the brain parenchyma capillaries (9). The presence of other transporters such as Oatp14 and Oatp2, with more affinity for T4 than T3, may explain the different sensitivities to T4 and T3. Whereas normal T4 uptake may preserve the compensated phenotype in mice, the lack of alternative transporters in the human blood-brain barrier would be the reason for the neurological impairment (9).

In conclusion, the data show that the main restriction to T3 action in the absence of Mct8 is at the level of the blood-brain barrier. The thyroid hormone transport role of Mct8 in the brain parenchyma from the circulation through the blood-brain barrier. Indeed Mct8 has been recently demonstrated in the membrane of the brain parenchyma capillaries (9). The presence of other transporters such as Oatp14 and Oatp2, with more affinity for T4 than T3, may explain the different sensitivities to T4 and T3. Whereas normal T4 uptake may preserve the compensated phenotype in mice, the lack of alternative transporters in the human blood-brain barrier would be the reason for the neurological impairment (9).

In conclusion, the data show that the main restriction to T3 action in the absence of Mct8 is at the level of the blood-brain barrier. The thyroid hormone transport role of Mct8 in the

### Table 1. Effect of T<sub>3</sub> treatment on transporter expression in cultured granular cells

<table>
<thead>
<tr>
<th>Transporter mRNA</th>
<th>Wt No T&lt;sub&gt;3&lt;/sub&gt;</th>
<th>KO Wt No T&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Oatp2</th>
<th>KO Oatp2</th>
<th>Slc16a2</th>
<th>KO Slc16a2</th>
<th>Mct8</th>
<th>KO Mct8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mct8</td>
<td>591 ± 130</td>
<td>KO 596 ± 48</td>
<td>Oatp2</td>
<td>270 ± 9.1</td>
<td>KO 293 ± 5.4</td>
<td>Oatp14</td>
<td>7.4 ± 3.4</td>
<td>KO 4.9 ± 0.9</td>
</tr>
<tr>
<td>Oatp2</td>
<td>280.0 ± 8.5</td>
<td>KO 23.9 ± 6.1</td>
<td>Slc16a2</td>
<td>6.8 ± 2.4</td>
<td>KO 2.8 ± 0.4</td>
<td>Slc10a4</td>
<td>Oatp14</td>
<td>KO 2.5 ± 0.3</td>
</tr>
<tr>
<td>Oatp14</td>
<td>591 ± 130</td>
<td>KO 596 ± 48</td>
<td>Mct8</td>
<td>591 ± 130</td>
<td>KO 596 ± 48</td>
<td>Mct8</td>
<td>591 ± 130</td>
<td>KO 596 ± 48</td>
</tr>
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</table>

Primary cultures of granular cells from the cerebella of Wt and Mct8 KO mice were incubated in the presence of 0, 0.2, 0.5, 1.25, 2.5, and 5.0 nM T<sub>3</sub> for 24h. Expression of Mct8 (Slc16a2), Oatp2 (Slc10a4), and Oatp14 (Slc1c1) was quantified by real-time PCR using TaqMan probes. Shown are the data (mean number of RNA copies relative to 18S RNA ± 1 SD) from cells incubated without added T<sub>3</sub> or in the presence of 2.5 and 5.0 nM only. Mct8 mRNA was not detected in the KO cells. The cells used in this experiment are the same as for Hr mRNA quantification shown in Fig. 4. Two-way ANOVA using the data from all T3 concentrations revealed that there was no difference of genotype or treatment, except for the highest T3 dose that decreased Oatp14 mRNA in the KO cells.

<sup>a</sup> P < 0.05.
plasma membrane of neurons, at least in the striatum and the cerebellum, seems to be minimal.

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Disclosure Summary: The authors have nothing to disclose.

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