Neurobiology of Disease

Neuronal 3′,3,5-Triiodothyronine (T_3) Uptake and Behavioral Phenotype of Mice Deficient in *Mct8*, the Neuronal T_3 Transporter Mutated in Allan–Herndon–Dudley Syndrome

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Abstract

Thyroid hormone transport into cells requires plasma membrane transport proteins. Mutations in one of these, monocarboxylate transporter 8 (MCT8), have been identified as underlying cause for the Allan–Herndon–Dudley syndrome, an X-linked mental retardation in which the patients also present with abnormally high 3′,3,5-triiodothyronine (T_3) plasma levels. Mice deficient in *Mct8* replicate the thyroid hormone abnormalities observed in the human condition. However, no neurological deficits have been described in mice lacking *Mct8*. Therefore, we subjected *Mct8*-deficient mice to a comprehensive immunohistochemical, neurological, and behavioral screen. Several behavioral abnormalities were found in the mutants. Interestingly, some of these behavioral changes are compatible with hypothyroidism, whereas others rather indicate hyperthyroidism. We thus hypothesized that neurons exclusively dependent on *Mct8* are in a hypothyroid state, whereas neurons expressing other T_3 transporters become hyperthyroid, if they are exposed directly to the high plasma T_3. The majority of T_3 uptake in primary cortical neurons is mediated by Mct8, but pharmacological inhibition suggested functional expression of additional T_3 transporter classes. mRNAs encoding six T_3 transporters, including L-type amino acid transporters (LATs), were coexpressed with *Mct8* in isolated neurons. We then demonstrated *Lat2* expression in cultured neurons and throughout murine brain development. In contrast, *LAT2* is expressed in microglia in the developing human brain during gestation, but not in neurons. We suggest that lack of functional complementation by alternative thyroid hormone transporters in developing human neurons precipitates the devastating neurodevelopmental phenotype in *MCT8*-deficient patients, whereas *Mct8*-deficient mouse neurons are functionally complemented by other transporters, for possibly *Lat2*.

Introduction

Thyroid hormone signaling is critical for normal vertebrate development and controls the metabolic activity of many tissues throughout life. 3′,3,5-Triiodothyronine (T_3) is the active hormone that binds to nuclear thyroid hormone receptors. Many processes during human brain development are T_3 dependent (Zoeller et al., 2002; Bernal, 2005b) and the human embryo in the first weeks of gestation is dependent on sufficient maternal thyroid hormone supply. Therefore, even a transient decrease of maternal plasma T_4 levels to hypothyroid levels during gestation can result in neurodevelopmental deficits in the human (Haddow et al., 1999; Bernal, 2007; Gilbert et al., 2007). Unlike other nuclear receptors, both the T_3-liganded and the apo-receptors exert specific activating or repressing activities on target genes (Wallis et al., 2008).

For nuclear receptor binding, cellular entry of thyroid hormones is a prerequisite. It is now definite that carrier proteins mediate T_3 and T_4 transport across the plasma membrane (Hennemann et al., 2001; Visser et al., 2008). Recently, we and others demonstrated that mutations in one transporter, monocarboxylate transporter 8 (*MCT8*) (*SLC16A2*), underlie a severe form of X-linked mental retardation, the previously clinically described Allan–Herndon–Dudley syndrome (Dumitrescu et al., 2004; Friesema et al., 2004; Biebermann et al., 2005; Schwartz et al., 2005). Patients present with severe hypotonia during the first months after birth and develop a

permanent severe motor and mental retardation. Most patients never attain speech or manage to walk independently. Despite abnormally high plasma T_3 levels, some tissues are apparently resistant to their action.

It has been demonstrated that *Mct8* mRNA is expressed during brain development and thereafter in mouse neurons and other cells (Heuer et al., 2005). Since the neurological phenotype of *MCT8*-deficient patients is more severe than the phenotype of patients with primary congenital hypothyroidism, it was suggested that impaired transport of maternal thyroid hormones into neurons during the first weeks of gestation may lead to neurodevelopmental defects (Bernal, 2005a). To create a model for *Mct8* deficiency, two independent transgenic mouse lines were generated lacking functional MCT8 (Dumitrescu et al., 2006; Trajkovic et al., 2007). These mice closely mimic the human endocrinological phenotype. Surprisingly, however, *Mct8*-deficient mice do not exhibit an overt neurological impairment or locomotion deficits. Therefore, we subjected *Mct8*-deficient mice to an extensive battery of histochemical, neurological, and behavioral tests. Only subtle behavioral changes are observed in *Mct8*-deficient mice, but indicate a complex phenotype reflecting characteristics of euthyroid and dysthyroid states. We demonstrate that Mct8 accounts for most, but not all, T_3 uptake in primary cortical neurons, and identify mRNAs encoding several other T_3 transporters in murine neurons, including *Lat2*. Then, we explore the possibility whether *Lat2* may compensate for the lack of *Mct8* in the mouse, but not in the human brain. Unlike in the rodent, *LAT2* is not coexpressed with *MCT8* in human fetal neurons. Thus, we suggest that species-specific and cell type-specific differences account for the phenotypic differences between patients and mice lacking functional MCT8.

Materials and Methods

Mice.

Mct8-deficient mice on a C57BL/6 genetic background (>F_8) were obtained from Deltagen. *Mct8* was inactivated by insertion of a bacterial *lacZ-neomycin phosphotransferase-II* gene into exon 2 (Trajkovic et al., 2007). Mice were kept under standard conditions (12 h light/dark cycle) in a specific pathogen-free environment in the central animal facility of the Charité, Berlin, according to local regulations. For phenotypic assessment in the German Mouse Clinic, 15 mice each of the following genotypes were transferred to their animal house and maintained as described previously: *Mct8* $^{+/+}$, *Mct8* $^{+/-}$, *Mct8* $^{-/-}$, and *Mct8* $^{-/y}$ (Gailus-Durner et al., 2005). For all phenotypic assessments, *Mct8*-deficient and littermate control mice were used. The dataset will be available on the EuroPhenome webpage (http://www.europhenome.org/).

Antibodies and reagents.

All reagents were of the highest chemical purity available and supplied by Sigma-Aldrich, unless otherwise indicated. Antibodies against MCT8 were raised in rabbits against an N-terminal peptide comprising amino acids 55–158 (counted from first start codon in human). Antibodies directed against the C terminus of murine LAT2 were raised in rabbits (ImmunoGlobe). Antibodies against neurochemical markers were as follows: NeuN, somatostatin-14, neuropeptide Y, GFAP, $p75^{NTR}$, ChAT, MAP2, Tau (all Millipore Bioscience Research Reagents), GFAP (Dako), parvalbumin, calbindin, calretinin, GAD67 (all from Swant). Secondary antibodies and peroxidase reagents were either from Vector Laboratories (Vectastain and MOM kit; Linaris) or from Dako. Cy2- and Cy3-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories and of preabsorbed quality. Human multiple tissue Western blot custom manufactured from crude membrane fractions was purchased from BioChain (BioCat).

Western blot.

Mouse organs were frozen on dry ice directly after dissection, powdered under liquid nitrogen, and cytosolic and membrane extracts were prepared as described previously (Renko et al., 2008). Neurons were cultured as described in six-well plates and scraped in ice-cold PBS. After collection of cells by centrifugation, a crude membrane fraction was prepared as described previously. Protein content was determined by the Bradford method (Bio-Rad). A total of 40 μg of protein was separated in 10% polyacrylamide gels and transblotted to nitrocellulose membrane, and even transfer was confirmed with Ponceau staining. Then the membrane was blocked with 5% milk powder and incubated overnight with antiserum (1:1000). After washing, HRP-conjugated secondary anti-rabbit antibody was applied and bands were detected using chemiluminescence (Bio-Rad) and x-ray films (Kodak). As additional loading control, blots were probed with mouse anti-transferrin receptor (TfR) antibody (Invitrogen) or rabbit anti-β-actin (Rockland).

Histology.

Mice were perfused with 0.1 M phosphate buffer (PB), pH 7.4, followed by 4% paraformaldehyde in PB and postfixed overnight at 4°C in the same fixative. After rinsing in PB, brains were either embedded in paraffin and cut on a microtome or embedded in agarose and cut at 100 μm on a vibratome; alternatively, the brains were cryoprotected in 30% sucrose in PB and cut on a cryostat at 30 μm. Blocking was performed with sera from the species from which the secondary antibodies were derived. Stainings were performed using the rabbit polyclonal antibodies against LAT2 (dilution, 1:250) and MCT8 (dilution, 1:250). Secondary antibodies and a specific streptavidin–biotin peroxidase amplification kit were from Vector Laboratories or Dako. Micrographs were taken at a Zeiss Axioscope 2mot plus equipped with an AxioCam MRc5 and Axiovision software. Alternatively, confocal images were taken at a Leica instrument at the Neuroscience Research Center core facility.

Primary cell cultures.

At embryonic day 15, brains were removed from embryos and cortical hemispheres were dissected free of meninges and other brain regions. Neurons were cultured on poly-L-lysine and collagen-treated glass coverslips or cell culture multiwell plates in Neurobasal medium (NBM) supplemented with serum-free B27 (Invitrogen) at densities of 1.5×10^5 cells/cm^2 (uptake assays) and 7.5×10^4 cells/cm^2 (stainings). Since B27 contains T_3, we used a self-made serum-free culture supplement in those experiments in which thyroid hormone content was varied. This supplement can replace B27 for culture times up to 3 weeks (S. Roth and U. Schweizer, unpublished observations). After 7–14 d *in vitro*, RNA was prepared from neocortical neuron cultures, protein was prepared for Western blotting, or T_3 uptake assays were performed. As judged by GFAP and NeuN staining, neuronal cultures were >90% pure. Primary fibroblasts were prepared from embryonic tissue after removal of head, heart, liver, and gastrointestinal tract and cultured on tissue culture plastic in DMEM-F12 (Invitrogen). Fibroblasts cultured in 24-well plates at passages 3–4 were used in radioactive uptake assays at 80% confluence.

T_3 uptake assay.

^{125}I-T_3 solution (PerkinElmer) was liberated from iodide ions by affinity chromatography and finally resuspended in NBM for neuronal uptake assays or DMEM:F12 for MDCK1 or fibroblast uptake assays. The complete medium of neurons or fibroblasts was removed and cells were incubated for indicated times in 300 μl of prewarmed medium containing 10 nM ^{125}I-T_3. MDCK1 cells stably expressing MCT8 from pcDNA3.1 (Invitrogen) were incubated for 4 min in 500 μl of prewarmed DMEM:F12 containing radioactive T_3. For inhibitor studies, probenecid (Prob) [1 mM as used by Sugiyama et al. (2003)] was dissolved in 0.1 M NaOH, rebuffered to pH 7.4, and dissolved in tracer medium. All other inhibitor substances were directly dissolved in medium containing ^{125}I-T_3. 2-Aminobicyclo-(2.2.1)-heptane-2-carboxylic acid (BCH) was used at 1 mM [three times K_i (Morimoto et al., 2008)]. Inhibitors were added to the cells together with ^{125}I-T_3. At the end of the incubation, cells were placed on ice, medium was aspirated, and the cells were washed with ice-cold PBS. Cells were lysed in 500 μl of 40 mM NaOH, and the radioactivity of the lysate was measured in a gamma counter. For experiments involving transfected MDCK1 cells, background was defined as radioactivity associated with cells transfected with empty pcDNA3.1 vector.

Human fetal tissue.

Fetal brain tissues were obtained from the autopsy material archive of the Institute of Neuropathology, Charité. Local ethics committee approval for research purposes was obtained. Brains were fixed for at least 2 weeks in buffered 4% formalin before being sliced and tissue blocks embedded in paraffin. A thorough neuropathological examination and diagnosis followed. A total of 14 cases was submitted to immunohistochemical staining. The gestational age ranged from 13 to 40 weeks, and the selected cases showed no major pathology. The hippocampus was chosen for the immunohistochemical analysis, which was performed using the rabbit polyclonal antibodies against LAT2 (dilution, 1:50) and MCT8 (dilution, 1:250). Counterstaining was done according to standard protocols. The respective secondary antibody and a specific streptavidin–biotin peroxidase amplification kit were applied (Ventana). A small fragment of kidney tissue was used as a positive control on each slide.

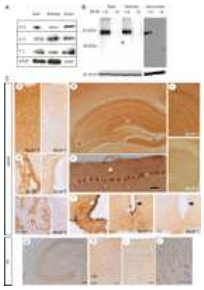
Results

Specific pattern of thyroid hormone transporter Mct8 immunoreactivity in the brain

Using a new and very specific antiserum directed against MCT8, we show Mct8 protein expression in the murine neocortex during development from embryonic day 15 (E15) to the adult (Fig. 1 *A*). Specificity of the antiserum for Mct8 was demonstrated by the absence of signal in Western blot performed with membrane protein from *Mct8* $^{-/y}$ mice (Fig. 1 *B*). Moreover, specific immunohistochemical staining was absent in sections from *Mct8*-deficient mice (Fig. 1 *C*). Previously, *in situ* hybridization had demonstrated *Mct8* expression in the adult mouse brain (e.g., in dentate granule cells) (Heuer et al., 2005). Here, we show that the distribution of Mct8 protein is more complex than expected from the mRNA expression pattern. Specifically, we detected a strong signal in granule cell dendrites extending into the molecular layer (ml), whereas granule cell bodies (gc) and axons in the stratum lucidum (sl) remained mostly unstained (Fig. 1 *Cb*). In the cerebellar cortex, not only Purkinje cells (pc) but also bona fide stellate (arrow) and Golgi (asterisk) interneurons were stained (Fig. 1 *Ce*). Tanycytes also express *Mct8* mRNA (Heuer et al., 2005). Here, we demonstrate that Mct8-positive processes line the wall of the third ventricle (black arrows), wrap hypothalamic blood vessels, and extend to the median eminence (Fig. 1 *Cf*, white arrow). From at least E15 to the adult, there is strong Mct8 expression in the choroid plexus on the apical membrane (Fig. 1 *Cd*). Since Mct8 protein is widely distributed to dendrites, individual Mct8-expressing cells are not easily recognized in the adult cerebral cortex (Fig. 1 *Ca*), whereas at postnatal day 5 (P5) single pyramidal cells expressing Mct8 immunoreactivity were easily distinguishable (Fig. 1 *Cg–j*).

Figure 1.

Developmental and cell type-specific expression of MCT8 immunoreactivity in the mouse brain. ***A*** , Western blots demonstrating the relative abundance of cerebrocortical MCT8 protein compared with liver and kidney at E15, E17, P2, and adult. ***B*** , Specificity of the MCT8 antiserum in Western blot. Molecular weight markers are indicated on the left. β-Actin was used as loading control. ***C*** , MCT8 immunoreactivity in *Mct8* $^{+/y}$ brain compared with *Mct8* $^{-/y}$ controls. ***a*** , Cerebral cortex. ***b*** , Hippocampus. ***c*** , Dentate gyrus with hilus. ***d*** , Choroid plexus. The bottom panel is from embryonic day 15. ***e*** , Cerebellar cortex. Scale bar,



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50 μm. ***f*** , Hypothalamus. ***g*** , Hippocampus at P5. Scale bar, 100 μm. ***h*** , CA3 region magnified from ***g*** . s.p., Stratum pyramidale; s.o., stratum oriens. ***i*** , Somatosensory cortex at P5. ***j*** , Magnified view of ***i*** . Scale bars: ***h–j*** , 50 μm.

Gene disruption of Mct8 does not alter brain histogenesis

In search of structural or molecular alterations in *Mct8*-deficient mouse brains, we undertook an immunohistochemical study of *Mct8*-deficient brains using a panel of common informative markers (NeuN, parvalbumin, somatostatin 14, calbindin, calretinin, neuropeptide Y, $p75^{NTR}$, choline acetyl transferase, microtubule associated protein 2, glutamic acid decarboxylase 67). Brain regions inspected were cerebral cortex (M1, S1BF, Pir), hippocampus, basal forebrain, amygdala, hypothalamus, and vermal cerebellar cortex. As shown in Figure 2, we did not observe a cortical layering defect in the barrel field cortex or changes in the number or distribution of parvalbumin+ interneurons, both changes that can be observed when thyroid hormone signaling is reduced during development (Lavado-Autric et al., 2003; Ausó et al., 2004; Gilbert et al., 2007; Wallis et al., 2008). Similarly, we did not observe changes in the number or distribution of the other major classes of cortical interneurons, somatostatin+ and calretinin+ cells, or in the distribution of any other marker tested. Likewise, no changes were apparent in the structure or neurochemical organization of the hippocampus (Fig. 2 *B*).

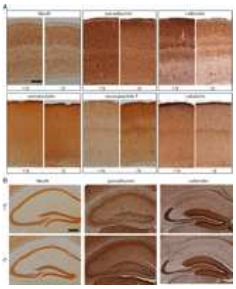

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Figure 2.

Immunohistochemical analysis of *Mct8*-deficient mouse brains. ***A*** , Cerebral cortex (S1BF). No differences were observed in the staining patterns for NeuN, parvalbumin, somatostatin, calbindin, neuropeptide Y, and calretinin. Scale bar, 200 μm. ***B*** , Dorsal hippocampus. No differences were observed in the staining patterns for NeuN, parvalbumin, and calbindin. Markers tested, but not shown are MAP2, ChAT, $p75^{NTR}$, GAD67, GFAP, NPY, and AChE activity.

Behavioral phenotype of mice deficient for the thyroid hormone transporter Mct8

Since we and others did not observe a clear behavioral phenotype that may have guided our analysis to specific neural circuits or transmitter systems, we subjected *Mct8*-deficient mice to the comprehensive neurological and behavioral screens at the German Mouse Clinic. In the neurological screen, the mice were tested according to the SHIRPA protocol, a battery of 24 independent assessments. As shown in the supplemental tables (available at www.jneurosci.org as supplemental material), the SHIRPA screen detected no differences between *Mct8*-deficient mice and their control littermates including normal reflexes (supplemental Tables S1–S4, available at www.jneurosci.org as supplemental material). The mice were also subjected to the modified hole-board test to reveal more subtle behavioral alterations. Unlike the situation in affected humans deficient for *MCT8*, *Mct8*-knock-out mice did not display altered locomotion (Fig. 3 *A*). Distances traveled and velocities were not different from control littermates. In addition, there was no difference in grip strength between *Mct8*-deficient mice and control littermates (control vs *Mct8*-deficient: males, 139 ± 7, vs 134 ± 6; females, 103 ± 1, vs 103 ± 2)—in sharp contrast to the severe hypotonia observed very early in patients with *MCT8* mutations. Finally, the rotarod assay did not reveal any significant differences in movement coordination between *Mct8*-deficient mice and controls. However, *Mct8*-deficient mice exhibited decreased anxiety-related behavior (Fig. 3 *B*). The mutant mice entered the board significantly more often, spent more time on the board, and kept a larger mean distance to the wall of the arena. This is the opposite finding of TRα deficient mice, which exhibit increased anxiety (Guadaño-Ferraz et al., 2003; Venero et al., 2005). A notable finding was shortened latency in the hot plate test (Table 1). Such a shortened latency may indicate hyperalgesia and was described previously in hyperthyroid

mice (Edmondson et al., 1990) and rats (Bruno et al., 2005, 2006). Interestingly, both hyperalgesia and reduced anxiety-related behavior are more indicative of hyperthyroidism than hypothyroidism, which would be expected from the T_3 transporter deficiency. In contrast, reduced grooming and increased latency of grooming (Fig. 3 *C*) have been reported in hypothyroid rats (Negishi et al., 2005). Thus, certain brain regions may be exposed to the increased circulating T_3 levels present in *Mct8*-deficient mice, whereas other neuronal systems remain functionally hypothyroid in *Mct8* $^{-/y}$ mice. These findings and the lack of a histopathologically discernible hypothyroid phenotype in the brain, prompted us to investigate the possible presence of additional thyroid hormone transporters that may compensate for the lack of Mct8 in the mouse brain.

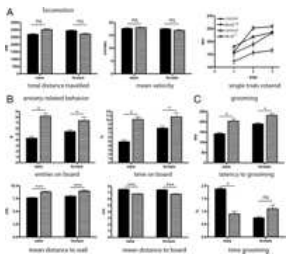

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Figure 3.

Behavioral analysis of *Mct8*-deficient mice. ***A*** , Locomotion as observed in the modified hole board test. Controls are represented by black columns, and *Mct8*-deficient mice are represented by shaded columns. Total distance traveled and mean velocity were not different between *Mct8*-deficient mice and littermate controls. For rotarod test, no difference related to *Mct8* genotype in the latency to fall from the rotating drum was observed. ***B*** , Anxiety-related behavior. In the modified hole board test, entries on board, time on board, mean distance to wall, and mean distance to board were assessed. All parameters suggest decreased anxiety-related behavior. ***C*** , Grooming behavior. In the modified hole board test, an increased latency to grooming was observed in mutants of both sexes, as well as reduced time spent grooming in male mutants. Error bars indicate SEM. *$p < 0.05$, ***$p < 0.001$.

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Table 1.

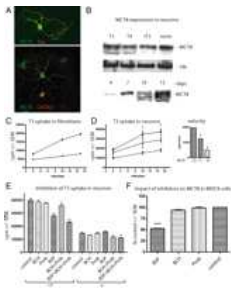
Hot plate test

Functional characterization of thyroid hormone transporters in cortical neurons

There is no systematic data on the expression of thyroid hormone transporters in mouse neurons, but previous physiological studies have characterized several T_3 transporters in brain cells (Chantoux et al., 1995; Hennemann et al., 2001). The relative importance of Mct8-mediated T_3 uptake in differentiated neurons has not been demonstrated previously. To investigate Mct8-mediated T_3 uptake in primary cortical neurons, we first determined expression of the protein and found almost all NeuN-positive cells stained with the Mct8 antibody (Fig. 4 *A*). Expression of Mct8 protein in neurons as estimated by Western blot did not depend on the presence of iodothyronines, but increased with time in culture (Fig. 4 *B*). To validate our T_3 uptake assay, we measured ^{125}I-T_3 uptake in primary cultured fibroblasts from *Mct8*-deficient embryos and their wild-type littermates (Fig. 4 *C*). Then, we tested primary neurons for T_3 uptake. Clearly, loss of *Mct8* significantly diminished the rate of T_3 uptake in a gene dose-dependent manner (Fig. 4 *D*). Still, we noted a moderate linear increase of ^{125}I-T_3 uptake even in *Mct8* $^{-/y}$ neurons. To establish whether other thyroid hormone transporters are functional in primary neurons, we pharmacologically characterized T_3 uptake in cortical neurons. To this end, we measured T_3 uptake in the presence of bromosulfophtalein (BSP) (a Mct8 inhibitor), BCH (a specific Lat inhibitor), and Prob (a broad-spectrum Oatp inhibitor) (Fig. 4 *E*). BSP significantly inhibited T_3 uptake in *Mct8* $^{+/y}$ neurons, but the reduction was only one-half the effect in *Mct8* $^{-/y}$ neurons, although BSP was used at five times its K_i value in cortical neurons (190 μM) (data not shown). Incomplete inhibition by BSP was also observed in *Mct8*-transfected MDCK1 cells (Fig. 4 *F*). BCH and Prob inhibition was reproducible, but small, and did not reach statistical significance. However, combination of BCH and Prob exhibited an additive effect and significantly reduced T_3 uptake in both *Mct8* $^{+/y}$ and *Mct8* $^{-/y}$ neurons. Moreover, inhibition by the combination of BSP, BCH, and Prob was larger than for BSP alone. Together, these data demonstrate the functional contribution of BCH- and Prob-sensitive T_3 transporters in cortical neurons (Fig. 4 *E*). Moreover, overexpressed Mct8 was insensitive to BCH and Prob excluding the possibility of inadvertent inhibition of Mct8 by the Lat and Oatp inhibitors (Fig. 4 *F*). These data strongly suggest that Lats and/or Oatps are functionally expressed in primary cortical neurons and may partially compensate for the loss of the major T_3 transporter, Mct8. The residual T_3 uptake capacity may thus suffice to prevent a major neurodevelopmental defect in the *Mct8*-deficient mouse.

Figure 4.

Functional characterization of T_3 transporters in primary cortical neurons. ***A*** , Primary cortical neurons were cultured for 7 d *in vitro* and immunostained for Mct8, the axonal marker Tau, and the interneuron marker



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GAD67. ***B*** , Western blot for Mct8 from primary cortical neurons cultured *in vitro* with the indicated iodothyronines at 10 nM. TfR served as control. ***C*** , Kinetic analysis of ^{125}I-T_3 uptake in mouse embryonic fibroblasts derived from control (■) and *Mct8* $^{-/y}$ (▼) mice. Cell-associated radioactivity (cpm) was measured in triplicate. Error bars (SEM) are smaller than the symbols. ***D*** , Kinetic analysis of ^{125}I-T_3 uptake in mouse primary cortical neurons derived from wild-type (■), *Mct8* $^{+/-}$ (▲), and *Mct8* $^{-/y}$ (▼) mice. Cell-associated radioactivity (cpm) was measured in triplicate from two to four independent animals. Error bars denote SEM. Inset, Initial rate kinetics of T_3 uptake in relation to *Mct8* genotype expressed as cpm per minute. Note that 75% of the T_3 uptake rate depends on Mct8. ***E*** , Pharmacological inhibition of neuronal T_3 uptake reveals Mct8-independent transport. T_3 uptake assays were performed in triplicate by addition of inhibitors (1 mM) together with T_3. ***F*** , BCH and Prob do not inhibit MCT8. Tracer was incubated on MDCK1 cells stably transfected with MCT8 for 4 min and background activity of empty vector-transfected MDCK1 cells was subtracted. **p* < 0.05; ***p* < 0.01; ****p* < 0.001 versus no inhibitor, one-way ANOVA followed by Dunnett's posttest.

Expression of thyroid hormone transporter mRNA in mouse neurons

Pharmacological inhibitors are useful tools for the functional characterization of T_3 uptake, but do not allow the unambiguous identification of specific transporters. To determine all T_3 transporters expressed in cortical neurons, we performed real-time PCR [quantitative PCR (qPCR)] for 12 described thyroid hormone transporters. mRNAs for seven transporters were detected in cultured neurons (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Among these were at lower levels *Mct10* (*Slc16a10*), the closest relative of *Mct8*, which is also able to efficiently transport T_3 (Friesema et al., 2008), Oatp-14 (*Slco1c1*), although it was mostly described in brain microcapillary endothelium (Sugiyama et al., 2003; Chu et al., 2008; Roberts et al., 2008b), and *Slco4a1* and *Slco4c1*, which are less well described. Only *Slc7a5* and *Slc7a8* (L-type amino acid transporters Lat1 and Lat2, respectively) were expressed at levels comparable with *Mct8* in the neurons. Both Lats have been characterized previously as T_3 transporters with low K_M values (Friesema et al., 2001) and have been identified as astrocytic T_3 transporters (Francon et al., 1989; Blondeau et al., 1993; Chantoux et al., 1995).

Developmental expression of thyroid hormone transporters in the mouse brain

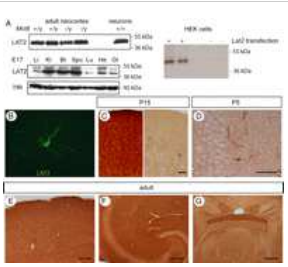
To define the developmental expression patterns of thyroid hormone transporters in the mouse brain, we performed qPCR on cortical samples in mice covering four developmental stages: E14, birth (P0), P5, and P20 (supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material). Several transporter genes, including *Lat2*, were coexpressed with *Mct8* at all time points tested. To gain more insight into their cell type specificity, we consulted the Allen Brain Atlas (Lein et al., 2007). In agreement with a recent report (Roberts et al., 2008b), we found that both *Lat1* and *Slco1c1* were clearly expressed in the choroid plexus and in brain microvessels. Strongest signals with a neuron-like distribution were observed for *Lat2* (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

Lat2 expression in the mouse brain

To study the function of Lat2-mediated T_3 transport during mouse development, we developed a specific antiserum against mouse Lat2. With this antiserum, we detected a single band of 45 kDa in mouse brain, primary neurons, and kidney (Fig. 5 *A*) (data not shown). Specificity of the antiserum was further assessed by recombinant expression of Lat2 in HEK293 cells and Western blotting. The 45 kDa band was the only band detected by the antiserum and was specific for *Lat2*-transfected cells. Lat2 protein is prominently expressed in the developing brain and spinal cord at E17, and kidney is the only organ with a similarly strong expression (Fig. 5 *A*). Immunocytochemical staining for Lat2 on cultured primary neurons revealed widespread expression in both the somatic and neuritic compartments (Fig. 5 *B*). Next, we determined the cellular expression pattern of Lat2 in the developing mouse brain by immunohistochemistry. Preabsorption of the antiserum with Lat2 peptide completely abolished immunostaining in the mouse brain (Fig. 5 *C*). At P5, Lat2 immunoreactivity is detected in neurons located in the cerebral cortex and hippocampus (Fig. 5 *D*). Similar as in the case of Mct8, Lat2 immunoreactivity is widely distributed throughout the cerebral cortex and hippocampus of the adult mouse (Fig. 5 *E*, *F*). A likely reason for the blurred immunohistochemical signal may be its dendritic localization and its reported expression in cortical astrocytes (Blondeau et al., 1993). Strikingly, the stratum lucidum was mostly devoid of Lat2 as it is devoid of Mct8 immunoreactivity. Since Lat2 is expressed in mouse cortex and hippocampus in a pattern overlapping the expression of Mct8, we speculate that Lat2 is a candidate alternative T_3 transporter during neuronal development that may compensate for the lack of Mct8 in *Mct8* $^{-/y}$ mice.

Figure 5.

Developmental expression of Lat2 in the mouse brain. ***A*** , Western blot against Lat2. Top left panel, Mouse



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cerebral cortex and cultured primary neurons. Lat2 protein abundance is not different between *Mct8* $^{+/y}$ and *Mct8* $^{-/y}$ mice. Bottom left panel, Strong Lat2 expression in brain and spinal cord at E17. Li, Liver; Ki, kidney; Br, brain; Spc, spinal cord; Lu, lung; He, heart; GI, gastrointestinal tract. Molecular weight markers are indicated on the left. TfR served as loading control. Right panel, Specificity of the Lat2 antiserum. Only one band of the appropriate size is detected in HEK cells transfected with *Lat2* expression plasmid. ***B–G*** , Immunocytochemical staining for Lat2. ***B*** , Cortical neurons cultured for 7 d. ***C*** , Specificity of the antiserum. In the right panel, the antibody was preincubated with the blocking peptide before immunostaining. P15 cerebral cortex is shown. ***D*** , Hippocampus, CA3 region, P5. ***E*** , Adult cerebral cortex. ***F*** , Adult hippocampus. ***G*** , Anterior commissure in the adult mouse. Note the differential Lat2 staining in the posterior and the anterior one-half of the commissure. Scale bars: ***D*** , 50 μm; ***C*** , ***E–G*** , 100 μm.

Expression of MCT8 and LAT2 in the human brain

Mct8-deficient mice replicate the endocrine, but not the neurological phenotype of human patients suffering from mutations in *MCT8*. We therefore speculated that one possible explanation could be differential species-specific expression patterns of thyroid hormone transporters in humans and mice. During development, mice may express more thyroid hormone transporters than humans at their blood–brain barrier (BBB) or in neurons compared with humans and thus may be less vulnerable to the lack of one of these transporters, MCT8. To investigate which thyroid hormone transporters are expressed in the human brain, we first performed qPCR on adult human cortical cDNA and found significant expression of L-type transporters (*SLC7A*), *SLCO4A1*, and *SLCO1C1* in addition to *MCT8* (Fig. 6 *A*). Western blotting supported protein expression of LAT2 and MCT8 in brain (Fig. 6 *B*). We then determined the expression pattern of MCT8 in human fetal brain during development by immunohistochemistry. MCT8 expression is first detected in blood vessels at gestational week 25 (GW25) (Fig. 6 *E*) and maintained at GW32 and GW40 (Fig. 6 *C*). Later, MCT8 immunoreactivity is also found in developing neurons with punctate staining in white matter, possibly representing cortical axons. From GW32, the mature MCT8 expression pattern is established in the hippocampus and cortex, but immunoreactivity is still weak (Fig. 6 *C*). By GW40, prominent MCT8 staining is achieved resembling essentially the pattern observed in mice: In the dentate gyrus, granule cell dendrites are strongly labeled in the molecular layer, whereas their axons in the stratum lucidum are apparently lacking MCT8. Most hilar and pyramidal cells are strongly stained for MCT8 (Fig. 6 *C*). In the cerebral cortex, pyramidal cells show MCT8 immunoreactivity from GW30 with increasing intensity until birth (data not shown). Similar as in the mouse brain, MCT8 is located on the apical side of the choroid plexus (Fig. 6 *E*). We then tested our newly developed antibody against LAT2 and confirmed its applicability for immunohistochemistry in paraffin-embedded tissue by robust labeling of kidney tubules (supplemental Fig. 3, available at www.jneurosci.org as supplemental material) and adult human cerebral cortex (Fig. 6 *F*). LAT2 immunoreactivity is clearly detectable in adult neurons. During human fetal development, microglia is clearly LAT2 positive, but not neurons (Fig. 6 *D*). We thus suggest that a major difference regarding T_3 transporters between mice and humans is the absence of LAT2 from developing neurons in the human. Thus, human neurons may depend exclusively on MCT8 expression for T_3 uptake during a critical time of development.

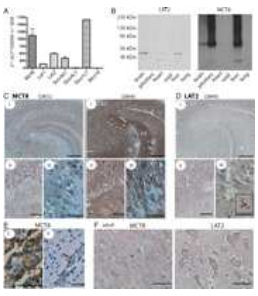

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Figure 6.

Developmental expression of thyroid hormone transporters in the human brain. ***A*** , qPCR detection of thyroid hormone transporters in adult human brain cDNA. Values are calculated according to the ΔCt method in relation to β-actin as a housekeeping gene. ***B*** , Multiple tissue Western blot on adult human membrane fractions for LAT2 and MCT8. The antibodies detect a protein of the same size as in mouse brain. Left, Molecular weight markers. ***C*** , Immunohistochemical detection of MCT8. Labeling of MCT8 increases with time in the developing human brain. ***i*** , Hippocampus. Scale bar, 500 μm. ***ii*** , CA3. Scale bar, 100 μm. ***iii*** , CA3. Scale bar, 50 μm. GW32 and GW40 are shown. ***D*** , Immunohistochemistry for LAT2 in GW40 hippocampus detects microglial, but not neuronal staining (***iii*** , inset). Scale bars are as in ***C*** . ***E*** , MCT8 in GW25 choroid plexus (***i***) (scale bar, 50 μm) and cortical gray matter vessel (***ii***) (scale bar, 50 μm). ***F*** , Hippocampal CA3 neurons in the adult human brain stained for MCT8 and LAT2. Scale bars, 50 μm. Capillaries are indicated by asterisks.

Discussion

Despite the important role of thyroid hormones for brain development and function, we are only beginning to identify the molecules that mediate the uptake of thyroid hormones *in vivo* at the BBB or into neurons and astrocytes (Roberts et al., 2008a,b). Major interest in the molecular identity of brain thyroid hormone transporters was raised after the identification of mutations in *MCT8* as the cause of mental retardation in human patients (Dumitrescu et al., 2004; Friesema et al., 2004; Schwartz et al., 2005). Previous reports using *Mct8*-deficient mice supported the observation that mutations in MCT8 cause the unusual thyroid hormone constellations in human patients (Dumitrescu et al., 2006; Trajkovic et al., 2007). However, unlike the human subjects, mice lacking functional Mct8 did not develop obvious signs of neurodevelopmental retardation. Trajkovic et al. (2007) demonstrated that cerebral uptake of ^{125}I-T_3 was greatly impaired in *Mct8*-deficient mice, whereas uptake of ^{125}I-T_4 was almost normal. This finding implicated a role for Mct8 in T_3 uptake at the BBB, consistent with our data presented here and a recent report by Roberts et al. (2008b). Accordingly, Oatp-14, a T_4-specific transporter, mediates T_4 uptake at the BBB (Sugiyama et al., 2003; Roberts et al., 2008b). Moreover, type 2 deiodinase (Dio2), an astrocytic enzyme capable of converting T_4 to active T_3, is upregulated in *Mct8*-deficient mouse brain and thus indicates a relative lack of T_3 behind the BBB (Dumitrescu et al., 2006; Trajkovic et al., 2007).

In the rodent brain, *Mct8* mRNA is expressed in neurons (Heuer et al., 2005). Thus, it was generally concluded that Mct8 represents the neuronal T_3 transporter. We set out to test this hypothesis and performed T_3 uptake experiments on primary cortical neuron cultures prepared from wild-type and *Mct8*-deficient mice. Mct8 is quantitatively the most important T_3 transporter in cultured cortical neurons. Using BCH and Prob as pharmacological inhibitors, we found that a significant fraction of neuronal T_3 uptake is independent of Mct8. We may have even underestimated their quantitative contribution, if inhibition was incomplete as in the case of the Mct8 inhibitor BSP. The existence of a Mct8-independent T_3 uptake mechanism may explain the general lack of an obvious hypothyroid phenotype in *Mct8*-deficient mice and is compatible with the normal dendritic development of Purkinje cell dendrites in *Mct8*-deficient cerebellar cultures (Trajkovic et al., 2007). We performed a comprehensive immunohistochemical study but were unable to identify structural defects compatible with developmental hypothyroidism in *Mct8*-deficient mouse brains. In addition, we subjected *Mct8*-deficient mice to an extensive behavioral and neurological screen. Again, no neurological defects were observed, but for the first time significant behavioral abnormalities were noted. These were, however, not consistent with changes observed in hypothyroid animals (as expected for the T_3 transport deficiency into neurons) or mice deficient in nuclear *TRα1* receptors, *TRα*$^{+/R384C}$ mice (Venero et al., 2005), *TRα1* $^{-/-}$ mice (Guadaño-Ferraz et al., 2003) and *TRα*$^{0/0}$ mice (Wilcoxon et al., 2007), since these animals display increased anxiety-related behavior. *Mct8*-deficient mice displayed decreased anxiety-related behavior similar to rats transiently treated with T_4 after birth (Yilmazer-Hanke et al., 2004). Rather, some of our results pointed to increased T_3 signaling resulting in enhanced pain perception (Edmondson et al., 1990; Bruno et al., 2005, 2006). However, in contrast to hyperthyroid rodents (Redei et al., 2001; Sala-Roca et al., 2002), there was no indication for hyperactivity in *Mct8* $^{-/y}$ mice as shown by unaltered locomotion. We thus speculate that neurons in specific brain regions with direct access to the increased circulating T_3 levels may be exposed to enhanced T_3 signaling, if their T_3 uptake is independent of Mct8. In contrast, other neurons may completely rely on Mct8 for T_3 import and thus become hypothyroid in the absence of Mct8 (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Accordingly, we detected mRNAs encoding several potential thyroid hormone transporters in cortical neurons. Some of these were formerly known as components of the BBB (e.g., Lat1/*Slc7a5* and Oatp-14/*Slco1c1*) (Boado et al., 1999; Sugiyama et al., 2003; Chu et al., 2008; Roberts et al., 2008b). Lat1 and Lat2 are BCH-sensitive T_3 transporters and may account for one-half of Mct8-independent T_3 import into neurons. The remaining T_3 uptake capacity is sensitive to Prob consistent with a potential role of *Slco1c1*.

Why is the neurological phenotype of *MCT8*-deficient patients so much more severe than the phenotype of *Mct8*-deficient mice? One may argue that the mouse brain may not represent a good model for the human brain. Human neurons may be much more sensitive to deviations from adequate T_3 supply than mouse neurons. Especially higher cortical functions like speech are simply not present in mice and cannot be studied in the rodent model. In our eyes, it is at present not possible to reject this argument in general, but there is no indication of motor deficits in *Mct8*-deficient mice, although this is a prominent feature of the patients.

Another question is whether a mouse with total deletion of the *Mct8* gene is a model for the human patients given the large number of missense, nonsense, and splice site mutations that may allow for expression of MCT8 fragments with potential dominant-negative actions. However, patients with total or partial deletions of *MCT8* or patients harboring mutations that *in vitro* do not allow for protein expression do not have a weaker phenotype. Conversely, the only patients who are apparently less severely affected harbor missense mutations (S105F, L434W, and L568P). Finally, the *Mct8* $^{-/y}$ mouse is a good model for the human disease regarding the endocrine phenotypes.

Roberts et al. (2008b) reported that rodent, but not human, brain microvessels express Oatp-14. The authors propose that Oatp-14 may compensate for the loss of Mct8 in the mouse, but not in the human brain. However, Oatp-14 is significantly more active toward T_4 than T_3 (Sugiyama et al., 2003). If this proposed mechanism was entirely true, *MCT8*-deficient patients should resemble congenital hypothyroid patients, since thyroid hormone transport beyond the BBB would be generally impaired—and this is not observed. Also, this explanation does not take into account the expression of Lat1 at the BBB (Roberts et al., 2008a).

A fourth hypothetical explanation states that *Mct8*-deficient mice do not display neurological defects, because homeostatic mechanisms in neurons and other cells (other alternative transporters, deiodinase upregulation and downregulation) collectively succeed in establishing sufficiently high neuronal T_3 levels to eventually prevent functional hypothyroidism. We have explored this possibility in more detail and therefore tested the hypothesis that differential species-specific spatiotemporal expression patterns of neuronal thyroid hormone transporters account for the more severe neurological phenotype of *MCT8*-deficient patients. Based on our functional and expression data in cultured neurons, we speculate that *Lat2* may compensate in the mouse, but not in the human brain for the lack of Mct8. Therefore, we compared LAT2 expression in the developing human and mouse brain. Although *Lat2* is significantly expressed in primary cortical neurons isolated at the time point when corticogenesis is in full progress in the mouse, there is rather low LAT2 expression in developing human neurons. At this time, it appears as if MCT8 is the critical T_3 transporter during human brain development. Only in the adult, LAT2 expression increases. Our results further suggest that MCT8 is located in the dendritic compartment and we can only speculate at present whether it may be involved in synaptic function (Ruiz-Marcos et al., 1994). Such a role would be of utmost importance during the formation of functional connections. A role for thyroid hormone in these processes is well documented by the neurodevelopmental retardation occurring in severely hypothyroid children. Since thyroid hormone transporter expression is apparently developmentally and cell type-specifically regulated, it may be difficult to interpret the phenotypes associated with the lack of one T_3 transporter. In the mouse, it appears as if at least a fraction of the brain gains access to the elevated plasma T_3 levels in *Mct8*-deficient mice, and thus a mixed phenotype is created with respect to thyroid state. The same situation may occur in *MCT8*-deficient patients. Although their neurons may be deprived of T_3 signaling during critical periods of development, they may, at least in part, be exposed to the exceedingly high T_3 levels after birth, when T_3 transport relies more on LAT2, as we showed for CA3 pyramidal neurons. In keeping with the proposed role of thyroid hormone signaling during development, it has recently been demonstrated that normalizing peripheral thyroid hormone levels does not improve the neurological deficits of *MCT8*-deficient patients but only ameliorates the peripheral hyperthyroid phenotype (Wémeau et al., 2008).

Footnotes

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