Thyroid hormone and development: the importance of transporters and deiodinases

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Thyroid hormone and development: the importance of transporters and deiodinases

Schildklierhormoon en ontwikkeling: het belang van transporters en dejodases

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CONTENTS

Chapter 1

General introduction

Part of this chapter is based on

Thyroid hormone transport by monocarboxylate transporters
W. Edward Visser, Edith C.H. Friesema, Jurgen Jansen and Theo J. Visser
Best Practice & Research Clinical Endocrinology & Metabolism 2007;2:223-36

Thyroid hormone transport in and out of cells
W. Edward Visser, Edith C.H. Friesema, Jurgen Jansen and Theo J. Visser
Trends in Endocrinology & Metabolism 2008;19:50-6

INTRODUCTION

Thyroid hormone, which is the common name for the prohormone T4 (3,5,3′,5′-tetraiodothyronine; thyroxine) and the bioactive hormone T3 (3,5,3′-triiodothyronine), is indispensable for normal development and metabolism of all tissues. Its effects on metabolism are clearly illustrated by the clinical manifestations in primary thyroid diseases. In hypothyroidism, symptoms such as cold intolerance, constipation, weight gain and bradycardia result from a generalized slowing of metabolic processes. In contrast, heat intolerance, weight loss and increased heart rate which are observed in patients with hyperthyroidism are explained by an increased metabolism. The prerequisite of thyroid hormone for normal development is clearly exemplified by patients with cretinism. Insufficient supply of iodine, which is a principal component of thyroid hormone, during critical periods of development may result in severe and permanent growth impairments, deafness, motor and mental retardation.

Clinical effects of an altered thyroid state arise from changes in thyroid hormone physiology at the cellular level. Thyroid hormone homeostasis requires adequate function of transporter proteins, deiodinating enzymes and nuclear receptors at the level of thyroid hormone target tissues.

THYROID HORMONE SYNTHESIS

Thyroid hormone is produced by the thyroid gland, which is located ventrocaudal of the thyroid cartilage. Histologically, the thyroid is mainly composed of spheric follicles. Each follicle is constituted of a colloid lumen surrounded by epithelial cells or thyrocytes. A smaller population of different cells, C-cells, are dispersed between the follicles and are involved in the Ca^{2+}-homeostasis. Thyroid hormone synthesis is a multi-step process which takes place in the follicles.

Because iodide is a principal component of thyroid hormone, sophisticated mechanisms ensure efficient handling of this trace element. Iodide transport across the basolateral membrane of the thyrocyte is facilitated by the Na/I symporter (NIS; SLC5A5). The apical iodide flux into the colloid lumen is mediated, at least partially, by pendrin (PDS; SLC26A4), although other transporter proteins may also play a role (1). Once in the colloid, iodide is oxidized by thyroperoxidase (TPO). This reaction requires hydrogen peroxide, which is generated by the dual oxidase type 2 (DUOX2). The maturation factor DUOXA2 is needed for proper function of DUOX2. The oxidized iodide is rapidly incorporated by TPO into specific tyrosyl residues of thyroglobulin (TG). This organification reaction results in the formation of mono- and diiodotyrosines (MIT and DIT). TPO also catalyzes the coupling of iodotyrosines to form T3 and T4 as well as some other

iodothyronines. The hormone containing TG molecules are internalized by the thyrocyte and subsequently hydrolyzed by lysosomes, which ultimately results in the release of the iodothyronines and the non-coupled iodotyrosines. The latter are deiodinated by the iodotyrosine dehalogenase 1 (DEHAL1), whereas rT3 (3,3',5'-triiodothyronine) and lesser iodothyronines may be deiodinated by the type 1 deiodinase (2). These enzymes may have a scavenger function by recycling iodide, which is particularly relevant in iodine-insufficient situations. Finally, the iodothyronines are released into the circulation. In humans, the thyroid gland secretes predominantly T4 and to a lesser extent T3 under iodine-sufficient conditions. In other species such as rats and mice, the T4/T3 secretion ratio is somewhat less (3). Mutations in several proteins involved in thyroid hormone synthesis have been reported to result in dyshormonogenesis and subsequent congenital hypothyroidism, which clearly demonstrates their importance in human physiology (4-6). Several steps in the thyroid hormone biosynthesis pathway such as the mechanism by which the thyrocyte itself is protected against the high intracellular thyroid hormone concentrations or the mechanisms by which thyroid hormone is secreted into the bloodstream are elusive.

HYPOTHALAMUS-PITUITARY-THYROID AXIS

Under physiological conditions, the hypothalamus-pituitary-thyroid axis maintains serum concentrations of thyroid hormone within a narrow range. The thyroid gland is stimulated by thyroid-stimulating hormone (TSH), acting through its receptor (TSH-receptor) to produce thyroid hormone. TSH originates from the pituitary gland, where its secretion and bioactivity is stimulated by hypothalamus-derived thyrotropin releasing hormone (TRH). Thyroid hormone negatively regulates TRH and TSH production, which constitutes the so-called negative feedback loop. For a long time, the predominance of the pituitary in the negative feedback was believed. However, in recent years the preeminent role of the hypothalamus in this system has been emphasized (7). In addition to the classical feedback system, it has been proposed that extra control mechanisms such as the TSH-dependent regulation of TRH (short-loop) and even of TSH (ultra-short-loop) may exist (8).

THYROID HORMONE METABOLISM

Thyroid hormone is subject to different chemical modifications, which modulate its serum and tissue availability and bioactivity. In addition to deiodination, which is regarded as the most important pathway, alternate routes of thyroid hormone metabolism exist, which will be discussed first.

Alternate pathways of thyroid hormone metabolism

Alternate pathways of thyroid hormone metabolism include conjugation of the phenolic hydroxyl group, net decarboxylation and deamination of the alanine-side chain and ether-link cleavage (Fig. 1). The latter is thought to represent a minor pathway in physiology.

The precise mechanisms by which the acetic acid derivatives tetraiodothyroacetic acid (Tetrac) and triiodothyroacetic acid (Triac) are generated are unknown, but possibly involves decarboxylation and subsequent oxidative deamination of iodothyronines. Although their physiological role is elusive, the receptor-specific affinities of these compounds led to the evaluation of their potential therapeutic use in certain diseases (9).

Recently, the endogenous existence of a novel class of putatively iodothyronine-derived substances, the so-called thyronamines, has been discovered (10). Injection of 3-iodothyronamine (T1AM) in mice induced dramatic effects including an acute reduction in heart rate and body temperature. Circulating T1AM concentrations in human serum appear to exist in the same order of magnitude as T3 and T4 (11). Future studies will reveal their importance for human physiology and the possible applications in disease.

Conjugation of iodothyronines with glucuronic acid or sulfate, which is facilitated by ER-located UDP-glucuronyltransferases and cytosolic sulfotransferases respectively, increases their water-solubility. In the hepatoenteric cycle, glucuronidated iodothyronines such as T3G and T4G are excreted in the bile and partially hydrolyzed. Subsequently, the liberated T3 and T4 may be reabsorbed and re-enter the circulation. Sulfation of iodothyronines accelerates degradation by deiodination (12). In addition, biologically inactive sulfated thyroid hormone may serve as a pool contributing to the availability

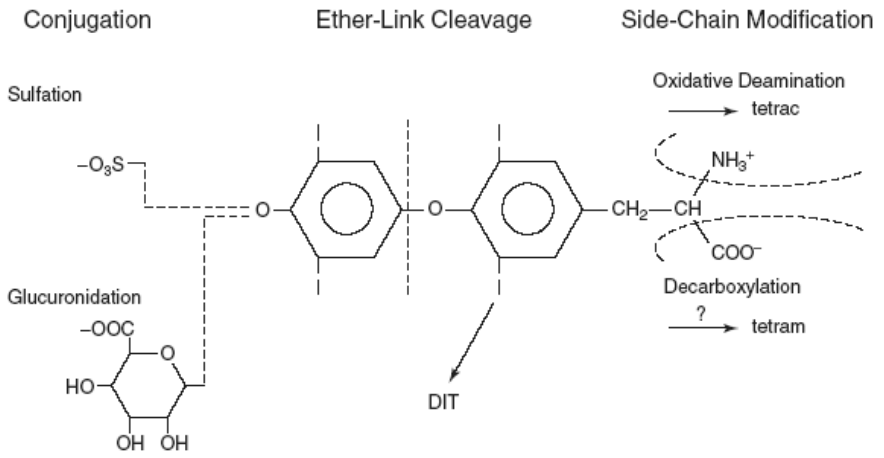

Fig. 1. Alternate pathways of thyroid hormone metabolism. Adapted from Wu *et al*. Thyroid 2005.

of thyroid hormone, because, for example, bioactive T3 may be recovered from T3S by sulfatases in selective tissues (9). This function may be particularly relevant during development, because serum iodothyronine sulfate levels are much higher in the fetus than in adults (13, 14).

Deiodination

A principal regulatory role to achieve appropriate intracellular thyroid hormone concentrations is reserved for the 3 deiodinating enzymes. The deiodinases type 1 (D1), type 2 (D2) and type 3 (D3) have distinct roles based on differences in mode of action, substrate preference and tissue expression (Table 1). The deiodinases D1 and D2 remove an iodide atom from the outer ring of the iodothyronine molecule. These enzymes have an activating function as they are capable of converting T4 to T3. D3 inactivates T4 and T3 by inner ring deiodination to the transcriptionally inactive rT3 and 3,3'-T2 (3,3'-diiodothyronine), respectively (15, 16).

Although being the first deiodinase identified, the physiological role of D1 is still enigmatic (17). Based on the abundant expression of D1 in liver and kidney, it seems ideally sited to serve whole body thyroid hormone metabolism. D1 may contribute to serum T3 levels by converting T4 to T3. This is based on several observations including the outer ring deiodination activity of D1, the observation that the D1-blocking drug PTU reduces the serum T3 levels with approximately 30% in athyreotic subjects substituted with L-thyroxine and on effects of genetic variation in the *DIO1* gene on serum thyroid hormone levels (18-21). This concept is challenged by studies in mice with decreased or absent D1 activity which showed normal serum T3 concentrations (22, 23). Furthermore, the catalytic efficiency of D1 is some orders of magnitude larger for rT3 and sulfated iodothyronines compared to T4. Therefore, it has been proposed that D1 has mainly a scavenging function by removing iodide atoms from receptor-inactive iodothyronines. The thus obtained iodide is available for reuse by the thyroid gland (see under Thyroid

Table 1. Characteristics of the iodothyronine deiodinases.

	D1	D2	D3
Preferred substrates	rT3, sulfated iodothyronines	T4, rT3	T3, T4
K_m	10^{-7}, 10^{-6}	10^{-9}	10^{-9}
Tissue expression	Liver, kidney, thyroid	Brain, pituitary, brown adipose tissue, muscle	Brain, fetal tissues
Physiological role	Clearance of rT3 and sulfated iodothyronines, production of serum T3	Local T3 production	Clearance of T3 and T4

hormone synthesis). In fasting and critical illness a diminished D1 activity has been observed in several tissues (24). To which extent the reduced D1 activity plays a causative role in the changed serum thyroid hormone parameters (mostly low T3 concentrations) in these conditions is presently unclear. It may be inferred that the decreased D1 activity results from these conditions, as D1 is highly responsive to thyroid hormone. In fact, D1 is currently the most sensitive marker for peripheral thyroid state (25). No human mutations have been reported from which a physiological role of D1 might be deduced.

D2 serves essentially as a thyroid hormone activating enzyme. The extent to which D2 contributes to serum T3 levels is unclear, but D2 has an unequivocal role in regulating local thyroid hormone homeostasis (16). In D2-expressing tissues (central nervous system, pituitary, brown adipose tissue), intracellular T3 concentrations are mainly achieved by activity of this enzyme, which is nicely illustrated in studies of D2 activity in brown adipose tissue (26). D2 is inversely regulated by its substrates T4 and rT3. This process is importantly regulated at the posttranslational level by an intricate ubiquitination-deubiquitination system positioned for a rapid adjustment of cellular thyroid hormone levels (16). To date, no mutations in the human *DIO2* gene have been reported. Recently, human mutations in the SECIS-binding protein SBP2, which is a critical protein in selenoprotein synthesis, were described (27, 28). Patients demonstrated elevated T4, rT3 and TSH along with decreased T3 levels. Fibroblasts from SBP2 patients exhibited markedly reduced D2 activity. The serum thyroid hormone changes are reminiscent of the thyroid state observed in *Dio2* knockout animals, although the other deiodinases may also be affected.

D3 is the major thyroid hormone inactivating enzyme. It terminates the action of its preferential substrate T3 and prevents activation of T4. An important role in developmental biology has been attributed to D3 given its expression in fetal tissues. Indeed, in addition to largely abnormal serum thyroid hormone levels, *Dio3* knockout mice demonstrate significant neonatal mortalility and growth retardation (29). In adult tissues, D3 activity is detectable in brain, pituitary, placenta and skin. Furthermore, evidence is accumulating that D3 is re-activated in certain disease states such as hemangiomas, critical illness, tumors and hypoxic-ischemic injury (16, 30). Whether the D3-induced local hypothyroidism aggravates or alleviates the different disease states is currently under debate (31). The *DIO3* gene is preferentially expressed from the paternal allele, because *DIO3* is subject to imprinting. In cases of uniparental disomy (UPD), in which two copies of the same chromosome are derived from one parent, imprinted genes may have increased or reduced expression. In animals with maternal UPD12 or paternal UPD12 (*Dio3* is located on mouse chromosome 12), T3 levels were increased or decreased, respectively, which suggests that genetically determined aberrant *Dio3* expression may contribute to the phenotype observed in these models (32). However, in humans with maternal UPD14 or paternal UPD14 (*DIO3* is located on human chromosome 14), no

thyroid dysfunction has been reported (33). As is the case for the other deiodinases, no mutations in *DIO3* have been described so far.

Taken together, the deiodinases serve as important regulators of serum thyroid hormone concentrations and serum-independent local thyroid hormone homeostasis.

THYROID HORMONE ACTION

Thyroid hormone acts via genomic and nongenomic pathways. Genomic actions of thyroid hormone are initiated by binding of T3 to its nuclear receptors. Several receptor isoforms are encoded by the *THRA* (thyroid hormone receptor α) and *THRB* (thyroid hormone receptor β) genes (34). Like other members of the nuclear hormone receptor family, the T3 receptors (TRs) function as ligand-dependent transcription factors. A number of co-activators and co-repressors interact with the TR to form functional transcriptional units. Binding of this transcriptional complex to T3 responsive elements (TREs) in the promoter region of T3-responsive genes results in activation or suppression of gene transcription (34). Although various TR isoforms are differentially expressed, suggesting isoform-specific effects, a large overlap in target genes has been detected indicating some redundancy (35). Possibly, isoform-specific transcriptional properties on common target genes allow fine-tuning of expression levels (36).

The nongenomic (or extranuclear) actions of thyroid hormone have only recently been recognized (37). Effects of the receptor-inactive rT3 on the actin cytoskeleton in brain development as well as 3,5-T2 effects on mitochondrial energy metabolism indicate important effects of other iodothyronines beyond the classical concepts of thyroid hormone action (37, 38). Cytosolic TRs are recognized to mediate some nongenomic effects, although other proteins such as the αVβ3 integrin, located in the plasma membrane, are also involved (39, 40). The rapid effects of thyronamines may act through the trace amine-associated receptor TAAR1 (10).

The clinical effects of thyroid hormone observed in humans are the result of the influence of the hormone on these processes at the molecular level. However, which genes are regulated by thyroid hormone in humans is largely unexplained as most knowledge is derived from animal models, even of the classical genomic pathway. Again, such results should be treated with care as species-specific metabolism properties exist. Obviously, studies in human tissues are important to validate animal models. Given the important clinical effects of thyroid hormone on metabolism, knowledge of the net effects on metabolic important tissues such as muscle, fat and liver are needed.

THYROID HORMONE TRANSPORT

Because the active sites of the deiodinases and the TRs are located intracellularly, thyroid hormone metabolism and action require transport of the hormone from extracellular compartments (e.g. the bloodstream) across the plasma membrane. Based on their lipophilic nature, it was assumed previously that translocation of iodothyronines across the lipid bilayer of cell membranes occurred by diffusion. However, experimental evidence over the last three decades and clinical studies in recent years show clearly that thyroid hormone traverses the cell membrane mainly through transporters (41, 42).

Several early observations have indicated that thyroid hormone uptake has different characteristics across cell types, with regard to ligand specificity, energy (ATP) dependence, Na^+-dependence and interactions with a variety of compounds (41). This suggested that thyroid hormone uptake might be facilitated by different types of transporters. In recent years, this hypothesis was confirmed by the molecular identification of thyroid hormone transporting proteins. These include the Na^+/taurocholate cotransporting polypeptide (43, 44), fatty acid translocase (45), multidrug resistance-associated proteins (46), amino acid transporters (reviewed in (47)) and members of the organic anion-transporting polypeptide (OATP) family (reviewed in (48)) and monocarboxylate transporter (MCT) family (reviewed in (49) and see under MCT family).

The majority of the thyroid hormone transporters currently known accepts a wide variety of compounds. To date, only OATP1C1 (50-52), MCT8 (53) and MCT10 (54) are reported to have relatively high specificity towards iodothyronines. The human *SLCO1C1* gene encoding the OATP1C1 protein is highly expressed in brain capillaries of rats and mice (51, 52, 55, 56). Although the cell types are unknown, OATP1C1 is widely distributed in human brain, including significant expression in the choroid plexus (50, 57). OATP1C1-expressing cells show preferential transport of T4 and rT3 (50-52). Recently, the sulfated iodothyronine T4S has been added to the list of substrates (58). These studies suggest an important role for OATP1C1 in T4 transport across the blood-brain-barrier. MCT10, originally designated as a T-type amino acid transporter because of its involvement in aromatic amino acid transport, also mediates T3 and T4 uptake (54). MCT10 is widely distributed, including the entire intestine, kidney, liver and placenta (59-61). However, no patients with mutations in either OATP1C1 or MCT10 or corresponding animal models have been reported so far. Thus, the precise *in vivo* function of these transporters remains to be elucidated.

Because a considerable amount of work presented in this thesis is related to MCT8, the whole MCT family will be discussed briefly and subsequently the characteristics of MCT8 in (patho)physiology will be emphasized.

The MCT family

Originally, it was believed that transport of monocarboxylates such as lactate and pyruvate takes place via diffusion (62). When a specific system of monocarboxylate transport was recognised, extensive exploration of the different characteristics of this transport suggested a family of MCTs. The first member of this family was shown to facilitate proton-linked transport of lactate and pyruvate, and therefore named monocarboxylate transporter 1 (MCT1).

The MCT family comprises 14, possibly 15 members (62, 63). So far, only MCT1-4 and MCT6 have been shown to transport monocarboxylates (62, 64). MCT1, MCT2, MCT4, MCT9 and MCT14 appear not to facilitate uptake of (sulfated) iodothyronines (W.E. Visser, unpublished observations). The role of the orphan transporters MCT5, MCT7, MCT9 and MCT11-14(15) remains to be elucidated.

The human *MCT* (renamed: solute carrier family 16; SLC16) genes encoding the different MCTs are located on autosomal chromosomes except *MCT8* (*SLC16A2*), which has an X-chromosomal location. The human MCTs are proteins ranging from 426 (MCT13) to 613 (MCT8) amino acids. All MCTs have 12 putative transmembrane domains (TMDs), typical for transporter proteins, with the N- and C-terminus located intracellularly. The sequence varies substantially in the long C-terminal end, in the large intracellular loop between TMDs 6 and 7 and in the N-terminus. Two highly conserved sequences (part of TMD 1 and TMD 5) are considered as characteristic of the MCT family. Different residues in the C-terminal part of MCT1 are important for its ligand specificity (65-67).

Tissue distribution varies substantially among the MCTs. MCT1 is ubiquitously expressed, whereas MCT3 is only located in the retinal pigment epithelium and choroid plexus epithelia (62). Halestrap and Meredith discuss the expression of the various MCTs in more detail (62). Regulation of MCTs occurs by transcriptional as well as post-transcriptional processes (62). It is believed that substrate demand plays an important role in regulation. For example, in skeletal muscle MCT1 is up-regulated during chronic stimulation, whereas down-regulation is seen in denervated muscle (62). Expression of MCTs in various tissues changes also during development (62). In addition, hormones may alter MCT function. It has been established that MCT1-4 require ancillary proteins for proper cellular function. Ancillary proteins are widely distributed proteins, which play important roles in cellular activities. CD147 (Basigin) is an extensively expressed transmembrane protein of the immunoglobulin superfamily (68). MCT1, MCT3 and MCT4 interact with CD147, whereas MCT2 is linked to Gp-70 (Embigin) (67, 69-71). However, in erythrocytes, MCT1 is also shown to interact with Gp-70 (72). This suggests that interaction between MCT and ancillary protein is cell-specific, depending on which ancillary protein is present. The association with ancillary proteins is required for routing of the MCTs to the plasma membrane as well as for their catalytic activity (70).

Monocarboxylates play central roles in cellular metabolism. Some tissues depend on glycolysis for their energy requirement, of which lactic acid is the end product, and all tissues become dependent on the glycolysis pathway during hypoxia (73). Therefore, rapid transport of lactic acid across the plasma membrane of mammalian cells is essential (62). Many tissues express more than one MCT, reflecting facilitation of influx as well as efflux by different MCTs in various cell types (62). A well-known example is the distribution of MCTs in the brain. MCT1 and MCT4 are predominantly expressed in astrocytes, whereas MCT2 is present in neurons. MCT1 and MCT4 export lactate from the astrocytes, thus preventing accumulation of lactate in these cells, which would eventually inhibit glycolysis. MCT2 facilitates influx of this extracellular lactate into neurons, providing an additional energy substrate (74).

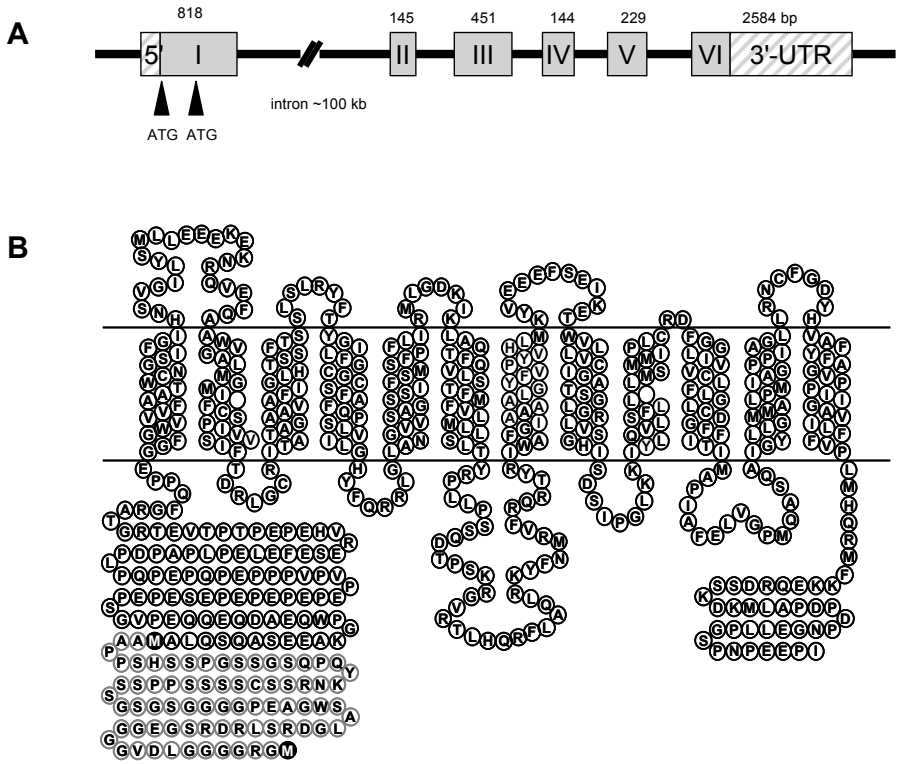

Fig. 2. (A) Genomic structure of h*MCT8*. Arrowheads indicate possible translation start sites. (B) Putative structure of hMCT8 with 12 transmembrane domains and the N- and C-terminal domains located intracellularly. The putative long variant of hMCT8 is indicated in grey.

MCT8

The human (h) *MCT8* gene is located on the X-chromosome (at Xq13.2) and contains 6 exons (Fig. 2A). Its homology to the MCT family justifies its classification. h*MCT8* encodes two hMCT8 proteins of 613 and 539 amino acids, depending on which of the two putative translation start sites (TLSs) is used (Fig. 2B). It is currently unknown whether there are two human MCT8 proteins expressed *in vivo*, and if so, whether they are subject to differential expression and regulation. The latter may be inferred from studies showing different isoform ratios depending on the cell type used (75). Non-primate *MCT8* genes lack the first TLS, but are homologous with h*MCT8* downstream from the second TLS (76). Like the other MCTs, MCT8 has 12 putative TMDs and an N-terminus which is enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) residues (abbreviated as PEST domain), which is why MCT8 was previously named XPCT (X-linked PEST-containing transporter). Previously, it was assumed that proteins containing PEST sequences often undergo rapid degradation (77). However, this concept was challenged recently when global protein stability in mammalian cells was investigated (78). No enrichment of PEST sequences in labile proteins was found. Thus, the function of the PEST domain in MCT8 is still elusive.

More than a decade after characterization of the h*MCT8* gene, rat (r) Mct8 was identified as a specific and active thyroid hormone transporter (53). Oocytes injected with r*Mct8* mRNA showed a rapid uptake of the iodothyronines T4, T3, rT3 and T2 but not of sulfonated iodothyronines, aromatic amino acids or monocarboxylates. Recently, the functional characterization of hMCT8 was described (79). Cells transfected with hMCT8 cDNA displayed a marked T3 and T4 uptake. It appears, that like rMct8 also hMCT8 transports rT3 as effective as T4 and T3 (W.E. Visser, unpublished observations). Cells cotransfected with hMCT8 and one of the deiodinases exhibited a significant increase in iodothyronine metabolism compared with cells transfected with deiodinase only. These findings clearly show that hMCT8 increases the availability of iodothyronines for intracellular metabolism by the different deiodinases.

The initially high thyroid hormone uptake rate by hMCT8 quickly reaches a plateau phase, indicating a rapid balance between thyroid hormone uptake and export. Indeed, direct measurement of T4 and T3 efflux showed rapid cellular release of the hormones in hMCT8-expressing cells (80). To investigate this in more detail, cells were cotransfected with MCT8 and μ-crystallin (CRYM), an intracellular binding protein with a high affinity for iodothyronines, to increase intracellular thyroid hormone-binding capacity. T4 and T3 efflux from cells co-expressing hMCT8 and hCRYM was significantly diminished. These findings substantiate the concept that hMCT8 is not only important for uptake but also for export of thyroid hormone.

MCT8 shows a broad tissue distribution in all species studied. *In situ* hybridization studies revealed that *Mct8* mRNA is significantly expressed in mouse liver, kidney, thy-

roid and brain (81, 82). In mouse brain, *Mct8* mRNA is predominantly expressed in the choroid plexus of the ventricles and in the neo- and allocortical regions as well as in capillary endothelial cells (57, 83). In rats, the Mct8 protein has been detected in heart and brain (53, 84).

In humans, MCT8 is expressed in many tissues, in particular in liver, heart and brain (85). Expression of MCT8 mRNA and protein in human placenta shows an increase during gestation (86). *MCT8* mRNA is upregulated in placentae associated with intrauterine growth retardation during the early third trimester of pregnancy, possibly suggesting a compensatory mechanism to increase thyroid hormone transport. MCT8 is also clearly localized in neurons of the paraventricular, supraoptic and infundibular nuclei of the hypothalamus and in glial cells of the ependymal lining of the third ventricle and median eminence (87). In the human pituitary, the folliculostellate cells, rather than the TSH-producing cells, show MCT8 expression (88). MCT8 expression is also found in human brain microvessels, hippocampus, choroid plexus and in some cortical regions (57, 89). A systemic detailed analysis of *MCT8* expression in human brain has not been reported yet.

The biological importance of MCT8 for brain development became apparent when mutations therein were associated with X-linked psychomotor retardation and deranged thyroid hormone levels (90, 91). It has been recognized that clinical features of patients with MCT8 mutations resembled those of Allan-Herndon-Dudley syndrome (AHDS) patients. Indeed, MCT8 mutations were found in all AHDS families tested, thereby providing a molecular basis for a syndrome already described in 1944 (92). Most affected males show a homogeneous neurological phenotype (reviewed in detail in (93)). There is a generalized low muscle tone with an inability to hold the head up, which usually progresses to spasticity. In addition, AHDS patients display episodic involuntary movements, which occur spontaneously or are triggered by stimuli. Most of the patients are unable to sit upright, crawl, stand or walk. Development of speech is absent in most affected individuals. However, in a few families motor and speech development are somewhat less impaired, resulting in limited walking and verbal communication. The cognitive impairment is dramatically shown in the severe mental retardation with usually IQs below 40. MCT8 mutations were also diagnosed in patients with a phenotype similar to AHDS, who were initially classified as having Pelizaeus-Merzbacher-Like disease (PLMD) (94). Recently, we identified a family in which the impact of a MCT8 mutation on neurological development was much less (95). In chapter 5 this family is described in detail along with the molecular characterization of the mutated MCT8. To date, only one female has been described in which a chromosomal break resulting in disruption of the *MCT8* gene was associated with the AHDS phenotype (96). The existence of a phenotype resembling AHDS in this female is presumably due to infavorable nonrandom X-inactivation. All other females with heterozygous *MCT8* mutations do not express neurological abnormalities.

Mutations in *MCT8* are spread over the entire coding region of the gene. Although it may be too early to localize hotspot regions, some mutations have been identified twice in distinct families. The identification of more than 40 affected families in a few years indicates that mutations in *MCT8* are not an uncommon cause of X-linked mental retardation. It is clear that many of the mutations, such as large deletions and truncating mutations, are devastating for the function of MCT8. The effects on MCT8 function are less obvious with amino acid substitutions, deletions or insertions. Therefore, different mutations have been introduced in MCT8 cDNA by site-directed mutagenesis and functionally tested (75, 81, 97, 98). In contrast to wild-type MCT8, transport and metabolism of T3 is completely absent with most MCT8 mutants. However, some mutants show significant residual activity, which is cell-type dependent (75, 95, 98). Interestingly, most patients in the families with MCT8 mutants demonstrating residual activity had developed some walking and speech capacities. These observations suggest that diverse mutations affect MCT8 function differentially and may be the cause of phenotypic variations.

Patients with MCT8 mutations have abnormal thyroid function tests. TSH levels are roughly doubled compared to non-carriers (81). Mean serum T4, free T4 and rT3 levels are approximately 40% decreased compared to healthy controls (81). In most patients, the most characteristic finding is the markedly elevated T3 levels. In heterozygous (female) carriers, TSH levels are comparable to familial non-carriers (81, 99). However, other thyroid hormone values seem to be intermediate between patients and non-carriers.

Recently, two different *Mct8* knock-out (KO) mouse models were generated to gain insight into the mechanisms underlying the neurological and endocrinological abnormalities in MCT8 patients (82, 100). Serum thyroid parameters in the Mct8 KO mice replicate the abnormalities seen in human patients. T4 and rT3 levels are decreased, TSH levels are modestly increased and T3 levels are markedly increased in *Mct8* KO mice compared to wild-type mice. The liver content of T3 is increased as well as the T3-responsive D1 mRNA levels and D1 activity, supporting the observation that *Mct8* KO mice do not differ in T3 uptake into the liver compared to controls.

Different aspects of the hypothalamus-pituitary-thyroid axis were investigated in *Mct8* KO mice. TRH transcript levels are strongly increased in the hypothalamic PVN neurons in *Mct8* KO mice. The elevated *TRH* expression is suppressed by T4, but not by T3 administration, indicating that these neurons are able respond to locally produced T3. In contrast to the "hypothyroid" state in the hypothalamus, the pituitary appears to be "euthyroid", as transcript levels of thyroid hormone-responsive genes were not altered in *Mct8* KO mice (82). However, the pituitary in the *Mct8* KO animals is relatively insensitive to T3. Only administration of high T3 concentrations is able to suppress TSH in *Mct8* KO animals rendered hypothyroid.

Brains of the *Mct8* KO mice show a significantly lower content of T4 in parallel with the decrease in serum T4. This is underscored by the increased D2 mRNA levels and activities, which are negatively regulated by T4 (15). In *Mct8* KO mice brain T3 levels are also markedly decreased despite the strong increase in serum T3. The cerebral uptake of T3, but not T4, is dramatically impaired in these animals. The low brain T3 content in *Mct8* KO animals is associated with low brain D3 activity, which is positively regulated by thyroid hormone.

Except for minor abnormalities such as somewhat decreased anxiety-related behavior revealed by extensive behavioral testing, the most remarkable finding in *Mct8* KO animals is the absence of neurological disturbances, despite the low levels of T4 and T3 in the brain (82, 89, 100). This may be explained in different ways. First, brain development in mice may respond differently to thyroid hormone deficiency than in humans. Second, species-specific cerebral patterning of MCT8 may account for the differences (101). Third, it has been proposed that the lack of *Mct8* may be compensated sufficiently by other thyroid hormone transporters in mouse brain, such as LAT2, but not in human brain, to secure a relatively normal development (89). Fourth, human MCT8 may transport other ligands, which are essential for normal human brain development. Fifth, the putative long MCT8 protein in humans may differ in expression of subcellular localization in addition to the short MCT8 protein, the only form present in mice.

Thus, although *Mct8* KO mice are a suitable tool to study endocrine abnormalities, it remains a challenge to unravel the precise molecular mechanisms involved in the pathogenesis of the psychomotor retardation in human patients with MCT8 mutations.

Integrating the findings in humans with *MCT8* mutations and in *Mct8* KO mice models may lead to the following considerations. The modestly increased serum TSH levels in patients seem to fit with the low free T4 levels, but in view of the strongly elevated serum T3 the TSH levels appear inappropriately high. Studies in the *Mct8* KO mice indicate a relative thyroid hormone insensitivity in the hypothalamus and pituitary. The inappropriate TSH levels in human *MCT8* patients correspond with a partial hypothalamic and pituitary resistance. The presence of MCT8 in human hypothalamus and pituitary fit with this hypothesis (87, 88).

In keeping with the assumed function of MCT8 in neuronal T3 uptake, it is fully understandable that MCT8 mutations result in a diminished intracellular T3 concentration. Considering the crucial role of thyroid hormone in normal brain development (see under Thyroid hormone and brain development), it is conceivable that neurological defects will be the consequence of this neuronal T3 deprivation. It is currently unknown whether all T3-dependent neurons express MCT8. Therefore, it cannot be excluded that certain types of neurons express other prominent T3 transporters, instead of or in addition to MCT8. Depending on the presence of additional transporters, MCT8 may function primarily in the import or in the export of T3. In the former case, inactivation of

MCT8 will result in decreased intracellular T3 levels, and in the latter case in an increased intracellular T3.

Changes in deiodinase activities contribute to the abnormal thyroid hormone levels in subjects with MCT8 mutations. The accumulation of T3 may be the result of blocking T3 entry in D3-expressing cells, which in turn leads to a decrease in T3 clearance. This would be followed by an increase in renal and hepatic D1 activities and consequent T3 production, which further stimulates T4 to T3 conversion. However, preliminary studies in *Mct8/Pax8* double KO mice do not support this explanation. The increased D1 activity may also contribute to decreased circulating T4 and rT3 concentrations. Recently, it was shown that the T3/T4 ratio increases with age in *Mct8* KO mice, thereby underscoring the prominent role for D1 in the origin of the thyroid hormone abnormalities (102).

It is feasible that the liver in MCT8 patients is in a hyperthyroid state as is the case in *Mct8* KO mice, because sex hormone-binding globulin (SHBG) concentrations are markedly increased in serum (81). Since SHBG production in the liver is positively regulated by T3, SHBG levels are indicative for the thyroid hormone status in the liver. The hyperthyroid state in the liver is explained by the elevated serum T3 levels and a presumed lack of importance of MCT8 for hepatic T3 uptake. If MCT8 in liver is more important for T3 efflux, its inactivation may even further increase intracelluar T3. The low muscle and fat mass in MCT8 patients may be the result of tissue 'wasting', because these tissues are in a hypermetabolic state as they are exposed to high T3 levels. Apparently, muscle and fat do not require MCT8 for thyroid hormone uptake.

In conclusion, while it is apparent that MCT8 has a crucial role in proper brain development, the contribution of MCT8 to thyroid hormone transport in other tissues is less clear. The exact mechanisms which play a role in the generation of tissue-specific hypo- or hyperthyroidism remain to be elucidated.

Currently, the therapeutic options for MCT8 patients are limited. The harmful effects of thyroid hormone deprivation on early brain development are almost certainly irreversible. Therefore, postnatal thyroid hormone treatment is expected to have limited positive consequences. Only mutations that partially inactivate MCT8 may benefit from thyroid hormone therapy. Theoretically, T3 analogs that are taken up by alternative transporters in cells in which MCT8 is disrupted may also have a place in the treatment of MCT8 patients (103). Supportive therapy, like appropriate diet to prevent aspiration and anticonvulsant therapy, may alleviate some of the secondary somatic problems. Recently, PTU plus L-thyroxine treatment, the standard treatment regimen in primary hyperthyroidism, resulted in normalisation of serum thyroid hormone values in a MCT8 patient (104). Furthermore, beneficial effects on body weight and heart rate were noted. Unfortunately, no neurological improvement was seen. In addition, positive effects of the thyroid hormone analogue 3,5-diiodothyropropionic acid (DITPA) were reported on the thyroid hormone serum concentrations, deiodinase activities and T3-responsive

genes in brain of Mct8 KO animals (103). Normalisation of serum thyroid hormone parameters and weight gain were achieved in an identical twin with a MCT8 mutation treated with the combination of PTU plus L-thyroxine and DITPA treatment (105). Although standardized treatment options are limited in MCT8 patients, the detection of MCT8 mutations is important for providing a diagnosis to family members, carrier identification and prenatal diagnosis.

THYROID HORMONE AND BRAIN DEVELOPMENT

The importance of thyroid hormone for normal brain development is known for over 100 years. As the fetal thyroid gland develops during the second trimester of the pregnancy, the fetal brain is highly dependent on sufficient maternal thyroid hormone supply. From the second trimester onwards, the contribution of the fetal thyroid gland to fetal thyroid hormone levels gradually increases.

The most frequent causes of insufficient thyroid hormone availability during development are iodine insufficiency, maternal hypothyroxinemia and congenital hypothyroidism. Patients with neurological cretinism, caused by iodine deficiency and subsequent maternal hypothyroxinemia, are characterized by severe mental retardation. Although iodine deficiency is easily corrected by iodine supplementation, it remains the most frequent cause of preventable mental retardation (106, 107). Even mildly decreased maternal thyroid hormone levels affect neuropsychological development (108). Furthermore, untreated congenital hypothyroidism, irrespective of the cause, affects neurological development. Neonatal screening programs coupled to early thyroid hormone replacement therapy are evaluated as an efficient prevention of neurological impairment due to congenital hypothyroidism (109).

The molecular events underlying the effects of thyroid hormone on brain development are mainly derived from rat and mouse models. Therefore, results should be interpreted with caution if extrapolated to humans. Neuronal cell migration and myelination are classically identified as thyroid hormone-dependent processes (110, 111). In addition to well-known T3-dependent genes such as *Reelin* (*Reln*), *Hairless* (*Hr*), *Neurogranin* (*Nrgn* or *RC3*), a large number of novel genes which are changed in a different thyroid state have been identified using microarray approaches (112-117).

The current hypothesis of thyroid hormone homeostasis in brain assumes that thyroid hormone during brain development is locally controlled by functional units of astrocytes and neurons. Mainly based on immunohistochemical studies, this process is thought to involve at least the following steps (Fig. 3) (118). First, T4 is transported across the blood-brain barrier through OATP1C1. Second, T4 is taken up into astrocytes by an as-yet-unknown transporter. Third, T4 in astrocytes is converted to T3 by D2. Fourth, the

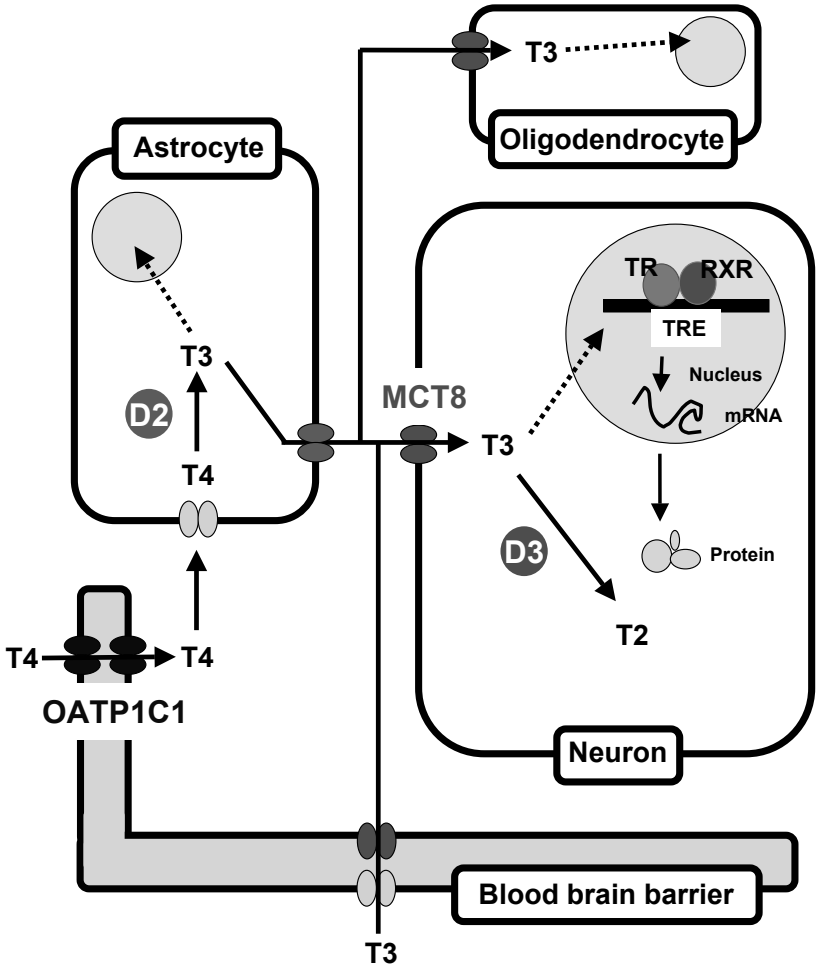

Fig. 3. Model of TH regulation in brain. Transporters (paired ovals) are required for uptake and release of T3 and T4. The deiodinases (circles) activate or inactivate TH. The type 2 deiodinase (D2) converts T4 to T3, thereby activating TH, whereas the type 3 deiodinase (D3) degrades T3 to T2. Ultimately, T3 binds to the nuclear TH receptor (TR) and modulates gene expression of T3-target genes.

biologically active T3 is released from the astrocytes by another unidentified transporter. Fifth, neuronal uptake of T3 is facilitated by MCT8. Sixth, T3 exerts its genomic action by binding to its nuclear receptor. Finally, T3 is degraded by D3 to T2, and both T3 and T2 may also leave the cells through MCT8. This model may be extended by the suggestion that MCT8 plays an important role in thyroid hormone transport across the blood-brain barrier as well as into oligodendrocytes (82, 94, 100, 119). Furthermore, transporters are required to ensure proper access of receptor-inactive iodothyronines, which are sup-

posedly involved in non-genomic actions in brain development (120). In addition, MCT8 has been implicated in the differentiation of embryonic stem cell into neural cells (121). Too low or too high local thyroid hormone concentrations may lead to abnormal thyroid hormone signalling and eventually result in abnormal brain development.

The abovementioned concept is largely supported by the finding of MCT8 mutations in humans resulting in mental retardation. Furthermore, patients with severe thyroid hormone resistance due to mutations in *THRB* including mental retardation have been reported (122-125). The deleterious effects on brain of such a severe mutation have been characterized in detail in a *Thrb* knock-in model (126).

To which extent the other supposed key players in local thyroid hormone metabolism are physiological relevant for human brain development is currently unknown. Mice harbouring mutations in *Thra* exhibit neurological dysfunction, mostly characterized by extreme anxiety (127). However, no humans with *THRA* mutations have been identified. The concerted action of the deiodinases D2 and D3 likely plays a key role in ensuring adequate thyroid hormone levels in brain. However, *Dio2* KO mice do not have gross neurological dysfunction (and for the *Dio3* KO mice no neurological information is available yet) (29, 128). No mutations in either *DIO2* or *DIO3* have been reported in humans. The thus far reported mutations in *SBP2* result in relatively mild clinical features (see also under Thyroid hormone metabolism), although it is speculated that complete loss-of-function mutations may result in a more severe phenotype (27, 28). Recently, it has been reported that polymorphisms in *OATP1C1* are associated with depression scores, but not with neurocognitive function, in hypothyroid patients on T4 replacement therapy (129). No *Oatp1c1* mouse model or *OATP1C1* mutations have been reported.

Obviously, cognitive functions markedly differ between humans and other species, which may be attributed to the species-specific patterning and temporal expression of the brain transcriptome (101, 130). Thus, it is highly important to realize that genetically engineered animals may be at best regarded as models. Therefore, it is of preeminant importance to systematically investigate spatiotemporal expression of genes important in thyroid hormone metabolism in human brain. Furthermore, genes which do not give rise to neurological features in mice, may certainly underlie human neurological disorders, most clearly exemplified by mutations in *MCT8*.

THYROID HORMONE AND AGING

Complex genetic and environmental factors contribute to variation in effects of thyroid hormone. Among these factors, age is a major component which largely influences variables in general. As aging and physiology are interrelated, it is no surprise that also thyroid hormone physiology changes during life. The high similarity between general

aging symptoms and symptoms due to hypothyroidism, suggests that the net effects of thyroid hormone decline during aging. Indeed, several indices suggest a functional decrease of thyroid function in humans during aging (131). Although (subclinical) hypothyroidism is negatively associated with morbidity and mortality, accumulating evidence suggests that in older individuals a lowering of thyroid function may be beneficial (132-135).

It has been shown that changes in endocrine systems are associated with longevity in certain animal models. Much attention has been drawn to the worm Caenorhabditis elegans and the fruitfly Drosophila which show several-fold extended life span if the growth hormone/insulin-like growth factor 1 (GH/IGF1) pathway is suppressed (136). In addition, in mice with mutations in endocrine signalling pathways, such as the *Igf-receptor* KO or Klotho-overexpressing mice, life span is significantly increased (137, 138). Other hormonal systems may be involved as well. Over one decade ago, Ames dwarf mice, which lack GH, prolactin and TSH due to a mutation in *Prop-1*, which is required for normal pituitary development, were found to have a roughly 50-60% increased life span (139).

The current view on aging proposes that stochastic damage to important biomolecules contributes to the aging process (140). As nuclear DNA is in principle irrenewable, damage to this class of biomolecules may have irreversible consequences. In support of this view, it has been shown that DNA damage accumulates with advancing age (141). In addition, several intricate DNA repair systems attempt to safeguard the integrity of the genome (142). The biological relevance of these mechanisms for aging are dramatically demonstrated in patients who harbor mutations in genes involved in DNA repair. In these so-called progeria syndromes, patients are characterized by premature aging in several organs and die at a young chronological age.

The stochastic process of damage accumulation and genetic factors that influence aging are seemingly in contrast. Recently, fascinating studies in mice deficient in DNA repair attempted to reconcile these paradoxical views. Very short-lived animals with mutations in DNA repair demonstrated a marked suppression of the GH/IGF1 axis as well as changes in defense and antioxidant systems highly similar to naturally aged animals (143, 144). Interestingly, the same response was observed in normal mice exposed to DNA damaging agents. These findings may well explain the paradox of random damage accumulation and genetic factors modulating the aging process. Random damage drives the age-related functional decline, which triggers a 'survival' response producing adaptive changes in multiple pathways including the endocrine systems (145). Thus, endocrine signalling may determine the rate of aging. Dampening the GH/IGF1 axis and oxidative metabolism reduces energy metabolism, thereby reallocating the sources from growth and proliferation to maintainance and somatic preservation.

Several studies investigating effects of thyroid hormone on life span support the hypothesis that the lowered endocrine systems are beneficial. An early study showed a clear reduction in life span, if dessicated thyroid tissue was administered to rats (146). As a corollary, moderate hypothyroidism induced by neonatal T4 treatment in rats clearly prolonged life span (147). Interesting models to investigate the relationship between aging, DNA damage and thyroid hormone changes are DNA repair mutants. Addressing this issue is a clinically relevant question as it may provide basal information whether decreased thyroid hormone action is beneficial in terms of survival during aging. This may influence the decision whether older subjects with subclinical hypothyroidism should be treated or not, which becomes even more relevant in Western populations with a steadily growing number of elderly people.

OUTLINE OF THE THESIS

The first chapters reveal several characteristics of the thyroid hormone transporter MCT8, including its homodimeric structure (**chapter 2**) and ubiquitination (**chapter 3**). In **chapter 4** we show that the specific thyroid hormone transporters MCT8 and MCT10 do not bind the affinity label BrAcT3. The subsequent chapters describe mechanisms of disease in patients with mutations in MCT8. In **chapter 5**, we demonstrate that MCT8 is functionally relevant in skin fibroblasts by showing decreased transport of thyroid hormone in fibroblasts derived from patients with (novel) MCT8 mutations. Furthermore, we show that mutations in MCT8 which differentially affect complete transport or predominantly efflux may underlie variation in the clinical phenotype. In **chapter 6** we describe the analysis of the fibroblast transcriptome from MCT8 patients. Linking the fibroblast transcriptomes to several comprehensive spatiotemporal gene expression profiles of human brain, provides insight into the molecular pathways underlying the neurological abnormalities of MCT8 patients. In **chapter 7**, we investigate the possible involvement of the SLC10 transporter family in thyroid hormone transport and characterize the SLC10A1 transporter in more detail for thyroid hormone transport. **Chapter 8** describes the characteristics of thyroid hormone transport in human umbilical vein endothelial cells as a model for the endothelium, which may serve as the first barrier of the transport of the hormone from plasma into tissues.

Based on the importance of thyroid hormone for normal brain development and the discovery that mutations in MCT8 result in mental retardation, we hypothesized that other key players involved in local thyroid hormone homeostasis in brain may underlie mental retardation. Therefore, a large cohort of subjects with mental retardation of unknown origin (the TOP-R study; the thyroid hormone origin of psychomotor retardation study) was collected, of which the characteristics are described in **chapter 9**. In **chapter**

10 several *MCT8* mutations in males with mental retardation in this cohort are identified. In vitro studies reveal the pathogenicity of these mutations. **Chapter 11** deals with preliminary data on the sequence analysis of the genes encoding D2 (*DIO2*) and D3 (*DIO3*).

Although the typical clinical symptoms related to a different thyroid state are well-known, knowledge of the underlying molecular events in humans is largely lacking. In **chapter 12**, we demonstrate that gene expression profiles in skeletal muscle largely differ dependent on the thyroid state and identify a large number of putatively novel T3-responsive genes. Finally, in **chapter 13** the relationship between aging, DNA damage and thyroid hormone changes is studied in different DNA repair mutants and naturally old mice.

A general discussion of the findings described in this thesis is presented in **chapter 14**.

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Chapter 2

Evidence for a homodimeric structure of human monocarboxylate transporter 8

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ABSTRACT

The human monocarboxyate transporter 8 (hMCT8) protein mediates transport of thyroid hormone across the plasma membrane. Association of hMCT8 mutations with severe psychomotor retardation and disturbed thyroid hormone levels has established its physiological relevance but little is still known about the basic properties of hMCT8. In this study we present evidence that hMCT8 does not form heterodimers with the ancillary proteins basigin, embigin or neuroplastin, unlike other MCTs. In contrast, it is suggested that MCT8 exists as monomer and homodimer in transiently and stably transfected cells. Apparently, hMCT8 forms stable dimers, as the complex is resistant to denaturing conditions and dithiothreitol. Cotransfection of wild-type hMCT8 with a mutant lacking amino acids 267-360 resulted in formation of homo- and heterodimers of the variants, indicating that transmembrane domains 4-6 are not involved in the dimerization process. Furthermore, we explored the structural and functional role of the 10 Cys residues in hMCT8. All possible Cys>Ala mutants did not behave differently from wild-type hMCT8 in protein expression, cross-linking experiments with $HgCl_2$ and transport function. Our findings indicate that individual Cys residues are not important for the function of hMCT8 or suggest that hMCT8 has other yet undiscovered functions in which cysteines play an essential role.

INTRODUCTION

Thyroid hormone (TH), the common name for the prohomone T4 and the active hormone T3, is essential for normal metabolism and development of many tissues. Because TH metabolism and action takes place intracellularly, transport of TH is required across the plasma membrane. This process takes place via TH transporters (1, 2). The majority of the currently known TH transporters accepts a wide variety of compounds and demonstrate a relatively low apparent affinity towards TH. So far, only OATP1C1 (3-5), MCT8 (6) and MCT10 (7) are reported to have high specificity for TH. Recently, the biological importance of MCT8 became apparent when loss-of-function mutations in MCT8 were associated with severe psychomotor retardation and disturbed TH levels, also known as Allan-Herndon-Dudley Syndrome (AHDS) (8).

Evidence has accumulated that MCT1-4 which transport monocarboxylates such as pyruvate and lactate, require ancillary proteins for normal cellular function. Apparently depending on the cell type, MCT1 interacts with basigin (BSG, CD147) or the homologous protein embigin (EMB, GP70), MCT3 and MCT4 only with BSG, and MCT2 only with EMB (9-12). The association of these MCTs with their partners is required for translocation to the plasma membrane and transport activity (11).

Western blotting of lysates of cells transfected with hMCT8 revealed besides a band of 61 kDa, in accordance with the calculated molecular mass of hMCT8, a band suggesting a molecular mass approximating 260 kDa (13). It is currently unknown whether this complex represents the association of hMCT8 with an ancillary protein or not. The relevance of this question is that mutations in possible protein partners of hMCT8 may also result in neurological abnormalities similar to AHDS. In addition, basic properties of the hMCT8 protein are mostly unclear. In this paper we investigate the possible dimerization and the effects of Cys mutants on the characteristics of the hMCT8 protein.

MATERIALS AND METHODS

Animals

The 5A11/Bsg-knockout mouse strain was generated using a neomycin gene-insertion strategy, as described previously (14). Care and handling of these animals was in accordance with the guidelines established by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC).

Materials

[3′-^{125}I]T3 was obtained from GE Healthcare (Little Chalfont, UK); nonradioactive iodothyronines from Henning (Berlin, Germany); 4,4′-diisothiocyanatostilbene-2,2′-disulfonate

(DIDS) and $HgCl_2$ from Sigma (St. Louis,); bis(sulfosuccinimidyl)suberate (BS^3) from Pierce (Etten-Leur, The Netherlands); and FuGENE6 transfection reagent from Roche Diagnostics (Almere, The Netherlands).

Cell culture

COS1 and JEG3 cells were cultured in six-well culture dishes with DMEM/F12 medium supplemented with 9% heat-inactivated FBS and 100 nM sodium selenite. Depending on the experiment, cells were transfected with 500 ng pcDNA3.hMCT8 (wt or mutated; from our MCT8 cDNA library (15)) alone or in combination with 500 ng pCMV-SPORT6. hBSG, pcDNA3.hEMB, rat neuroplastin 55 (rNP55) or rNP65 in pOPRSVICat. The full-coding sequence of human MCT3 cDNA was amplified from human RPE and cloned into the pTargeT (pTT) vector (Promega, Madison, WI). COS1 cells were transfected with 1 μg hMCT3 or hMCT8 using Fugene-6.

Flp-293-hMCT8 cells, HEK293 derived cells (Invitrogen) stably transfected with hMCT8, were grown in DMEM/F12 medium supplemented with 9% heat-inactivated FBS, 100 nM sodium selenite and 100 μg/ml hygromycin.

RT-PCR

Total RNA was isolated from 1 x 10^6 cells using the High Pure RNA isolation kit (Roche Diagnostics) according to the manufacturer's guidelines. cDNA was synthesized using 0.5 μg RNA and TaqMan RT reagent (Roche Diagnostics) in a total volume of 50 μl. The oligonucleotide primers of human GAPDH, BSG, EMB, NP55 and NP65 were designed across introns (Table 1).

TH uptake experiments

TH uptake studies were performed as described previously (13). Briefly, after 48 h transfection, COS1 and JEG3 cells were washed with incubation medium (Dulbecco's PBS containing 0.1% D-glucose and 0.1% BSA). Uptake of T3 was tested by incubation of the cells for 5 min at 37 C with 1 nM (2 x 10^5 cpm) [^{125}I]T3 in 1.5 ml incubation medium. After incubation, cells were briefly washed with the medium, lyzed with 0.1 M NaOH and counted in a gamma counter.

Western blotting

SDS-PAGE was performed as reported recently, except for different concentrations (0-250 mM) of dithiothreitol (DTT) and 7.5% acrylamide gels (13, 16). The purified polyclonal antibody 1306, which has been raised against the C-terminal part of hMCT8, was used for Western blotting.

Table 1. Oligonucleotide primers of ancillary proteins.

	Forward (5'-3')	Reverse (5'-3')	Product (bp)
GAPDH	TTGTCATACCAGGAAATGAGC	GTTGACTCCGACCTTCACCTT	942
EMB	ATCTGGGGATTTGAATGCAG	TAGTGCACGGCACCAGTAAG	432
BSG	ATCCAGTGGTGGTTTGAAGG	GTCAGAACACATCAACGAGGG	545
NP55	GAGATCCAGTGGTGGTACGC	CATCACTGGCCATAAACGGAG	557
NP65	AGGAGGACGGGGAAGGAC	CATCACTGGCCATAAACGGAG	868

Immunofluorescence

Transfected COS7 cells and frozen sections of mouse brain were labelled with MCT3 and MCT8 antibodies as previously described (17). The MCT8 antibody was produced in rabbits immunized with the carboxyl terminal cytoplasmic tail of MCT8 by Dr. Ian Simpson (College of Medicine, Penn State University, Hershey, PA). Images were obtained on a laser scanning confocal microscope (Zeiss LSM510) with a 63x oil objective.

Cross-linking experiments

Cross-linking of proteins on intact cells was carried out using the membrane-impermeable reagents DIDS (20 µM), BS^3 (1-5 mM)) or $HgCl_2$ (1 µM - 1 mM). Subsequently, cells were harvested in 100 mM phosphate (pH 7.2), 2 mM EDTA (PE) buffer, and washed twice with cold PBS.

Cloning and side-directed mutagenesis

The cloning of wild-type (wt) hMCT8 has been described recently (13). The hMCT8 Cys>Ala mutants C184A, C231A, C244A, C281A, C283A, C436A, C481A, C491A, C497A and C546A were generated by side-directed mutagenesis using the QuickChange kit (Stratagene, Amsterdam, The Netherlands). The presence of the desired mutations was confirmed by DNA sequencing.

Affinity-labeling with BrAc[^{125}I]T3

Affinity labeling was carried out as described elsewhere (13). At 80% confluency, COS1 cells in six-well plates were cotransfected with pcDNA3.hMCT8 and pcDNA3.rD1. After 48 h, the cells were washed with DMEM/F12 and preincubated with DMEM/F12 at 37 C. Subsequently, BrAc[^{125}I]T3 (2 x 10^5 cpm/well) was added and cells were incubated for 4 h at 37 C. Cells were washed with PBS and harvested in 200 µl PE buffer. After protein correction (Bradford assay), SDS-PAGE loading buffer (4 x) was added with a final concentration of 10 mM DTT. Samples were analyzed by SDS-PAGE (10% gels, Pierce, The Netherlands). Radioactivity was measured by phosphor imaging (Typhoon 9200).

RESULTS

Western blotting of lysates of COS1 and JEG3 cells transfected with hMCT8 both demonstrated in addition to the 61 kDa monomer a high molecular mass band, possibly representing the association of hMCT8 with an ancillary protein (13). Therefore, we measured mRNA expression of the ancillary proteins BSG, EMB, NP55 and NP65 in these cell lines. COS1 cells showed high expression of EMB and NP65 mRNA, low expression of NP55 mRNA, and no expression of BSG mRNA. JEG3 cells showed marked expression of BSG, NP55 and NP65 mRNA but EMB mRNA was undectectable (Fig. 1A).

Western blotting of lysates of cells cotransfected with hMCT8 and each of the investigated ancillary proteins did not show an increased intensity of the hMCT8-related bands (data not shown). Because ancillary proteins do not necessarily induce protein synthesis, but rather stimulate protein translocation and functional activity, we tested T3 uptake in cotransfection studies. Cotransfection of hMCT8 and hEMB, hBSG, rNp55 or rNp65 in COS1 (Fig. 1B) and JEG3 (Fig. 1C) cells did not result in increased T3 uptake compared to hMCT8 alone.

COS1 cells transfected with hMCT8 cDNA were labeled with MCT8 antibody. The immunofluorescence staining pattern observed is consistent with cell membrane labelling indicating that the protein was trafficked to the cell surface (Fig.1D). In contrast, in cells transfected with MCT3 cDNA, the MCT3 protein, which is known to require BSG for trafficking to the cell surface, is retained in the ER (Fig. 1D).

To rule out that (other) endogenous ancillary proteins are sufficient for proper function of hMCT8, we attempted to knock-down their endogenous expression. However, different knock-down strategies using RNAi were unsuccessful (data not shown). Therefore, we performed IHC in tissues of Bsg-/- animals.

In the choroid plexus, in which no Emb and Np have been reported (unpublished data and (18), a clear Mct8 signal was observed at the apical plasma membrane in tissue from both wild-type and Bsg-/- mice (Fig. 1E). Also in several other tissues, a clear plasma membrane localization of Mct8 was observed, in contrast with Mct1 and Mct3, which showed a cytoplasmic staining in the Bsg-knockout mice (17). Taken together, these data suggest that MCT8 does not require these ancillary proteins for normal function.

Previously, Western blotting was performed after separation of the hMCT8 transfected cell lysate on a 12% SDS-PAGE gel (13). To explore the possibility of homodimerization for hMCT8, COS1 cells were transfected and analyzed on 7.5% SDS-PAGE gels and subsequent Western blotting. This revealed three major bands at 60, 120 and 250 kDa, suggesting the existence of hMCT8 as monomer, dimer and tetramer (Fig. 2, lane 2). Faint higher molecular mass bands are visible, possibly representing multimeric hMCT8 complexes. In addition, there are multiple faint bands between 75 and 100 kDa, the

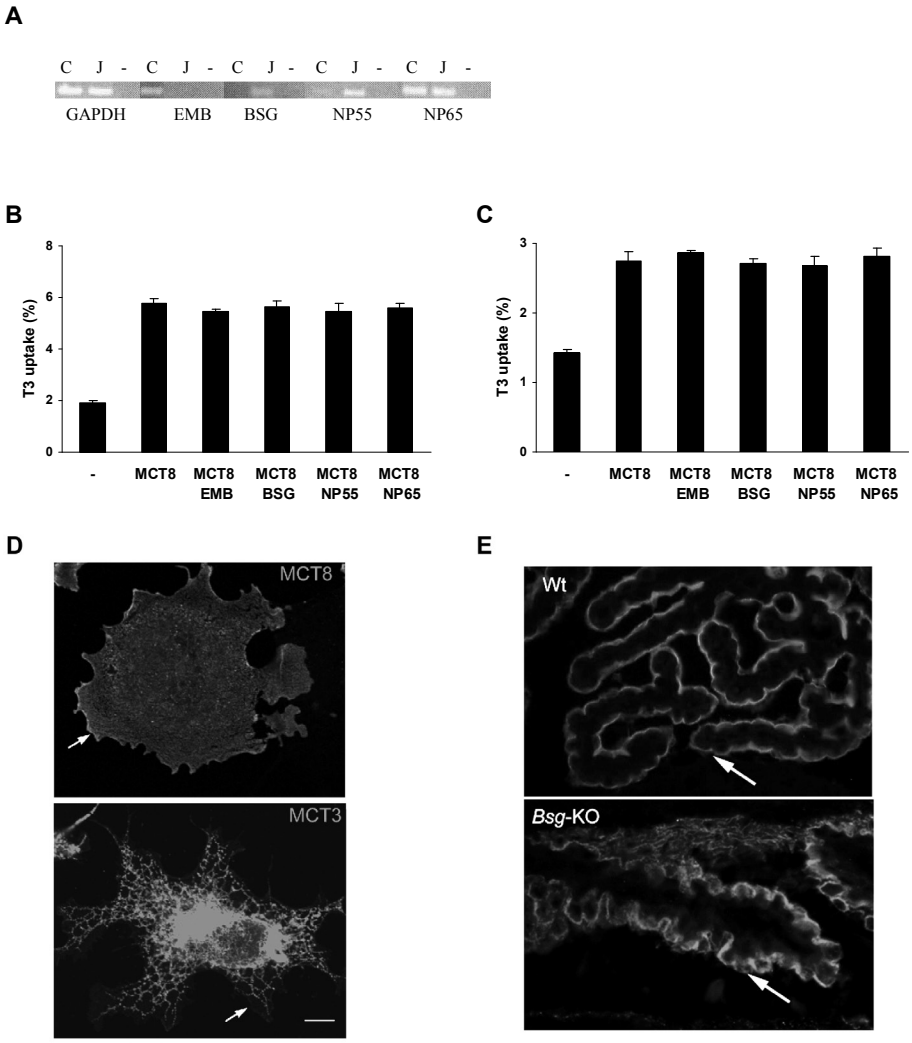

Fig. 1. (A) RT-PCR (35 cycles) of embigin (EMB), basigin (Bsg), neuroplastin 55 (NP55) and Neuroplastin 65 (NP65) mRNA levels in COS1 (C) and JEG3 (J) cells. GAPDH (27 cycles) was used as control. Uptake after 5 min incubation with 1 nM [3′-^{125}I]T3 in COS1 cells (B) and JEG3 cells (C) cotransfected (ratio 1:1) with hMCT8 and hBSG, hEMB, rNp55 and rNp65. (D) Immunofluorescence localization of MCT3 and MCT8 in COS1 cells transfected with hMCT8 (upper panel) or hMCT3 (lower panel). Arrows indicate the plasma membrane. Bar equals 10 μm. (E) Immunofluorescence localization of Mct8 in the choroid plexus in wt (upper panel) and Bsg knockout (lower panel) mice. Arrows indicate the apical plasma membrane of the choroid plexus epithelium.

nature of which remains unknown. The same results were obtained after transfection of JEG3 cells with hMCT8 (data not shown).

Because the bands at 120 and 250 kDa are resistant to denaturing conditions and even 10 mM DTT, indicating strong protein-protein interactions, we performed SDS-PAGE

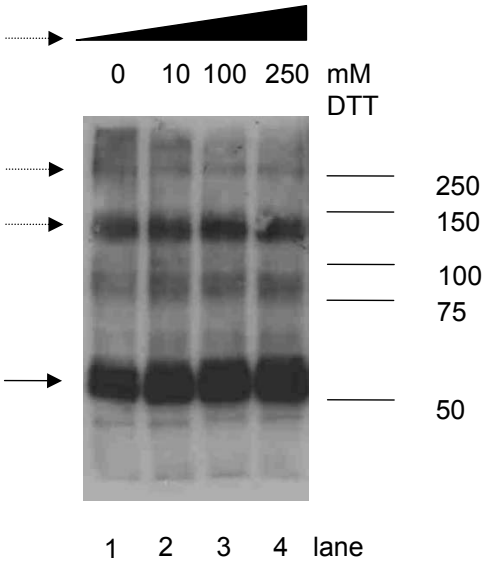

Fig. 2. Expression of hMCT8 in COS1 cells. Lysates of cells transfected with hMCT8 were separated by SDS-PAGE (7.5%) and analyzed by Western blotting. Expression of hMCT8 protein is shown with increasing DTT concentrations (0-250 mM). The blot was probed with hMCT8-specific antibody 1306. The solid arrow indicates hMCT8 monomer; the dashed arrows point to hMCT8 dimers.

after treatment with increasing DTT concentrations. Fig. 2 shows that with increasing concentrations of DTT the 250 kDa band and the higher molecular mass bands disappear, suggesting that disulfide bonds are involved in the oligomerization of hMCT8. In addition, exposure of intact COS1 cells transfected with hMCT8 to $HgCl_2$, which is a known Cys cross-linker suggests an increased intensity of the higher molecular mass bands (Fig. 3A). This cross-linking is a reversible process, because the cross-linked oligomeric hMCT8 species decreased with DTT treatment (Fig. 3B). Flp293-hMCT8 cells also demonstrated a 250 kDa band after $HgCl_2$ treatment, consistent with the presence of tetrameric hMCT8 (Fig. 3C). The cross-linkers DIDS, which is known to crosslink MCT1 and BSG, and BS^3 did not have any effect on the hMCT8 protein pattern (data not shown).

The increased oligomerization of hMCT8 with $HgCl_2$ treatment and its reversion with DTT treatment suggested the involvement of Cys residues in this process. hMCT8 contains 10 Cys residues, which may participate in disulfide bond formation. Therefore, we investigated the effects of all possible Cys to Ala mutations. Western blots demonstrated no changes in protein patterns of any of the Cys mutants compared to wt hMCT8 (Fig. 4A). In addition, we did not detect differences between mutants and wt hMCT8 after $HgCl_2$ treatment (Fig. 4B). To determine whether the Cys>Ala substitutions result in an

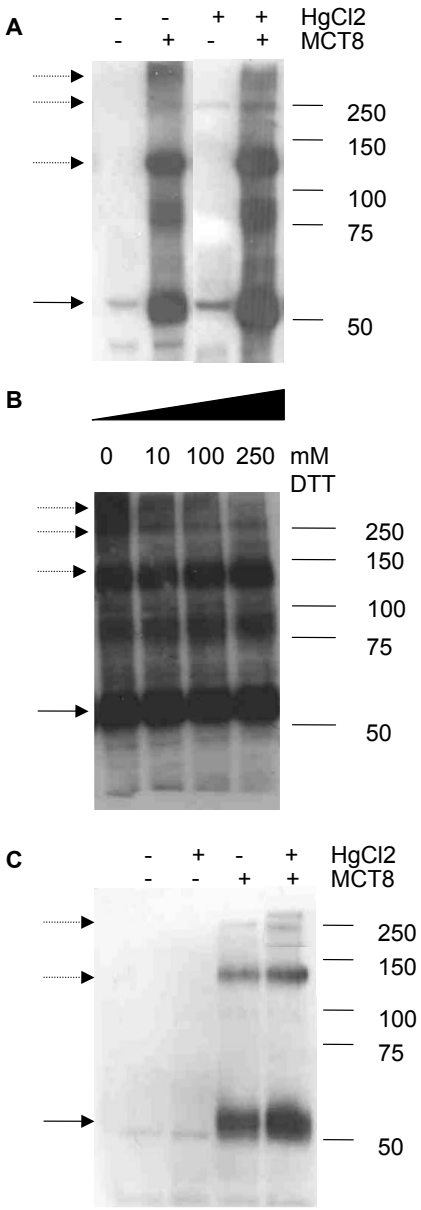

Fig. 3. Crosslinking of hMCT8 with $HgCl_2$ (10 μM). (A) The effect of $HgCl_2$ on the expression of hMCT8 in transiently transfected COS1 cells (B) The effect of increasing concentrations of DTT (0-250 mM) on crosslinked hMCT8 in COS1 cells. (C) Expression of lysates from Flp293-hMCT8, HEK293 cells stably transfected with hMCT8, with and without $HgCl_2$ treatment. The solid arrow indicates hMCT8 monomer; the dashed arrows point to hMCT8 dimers.

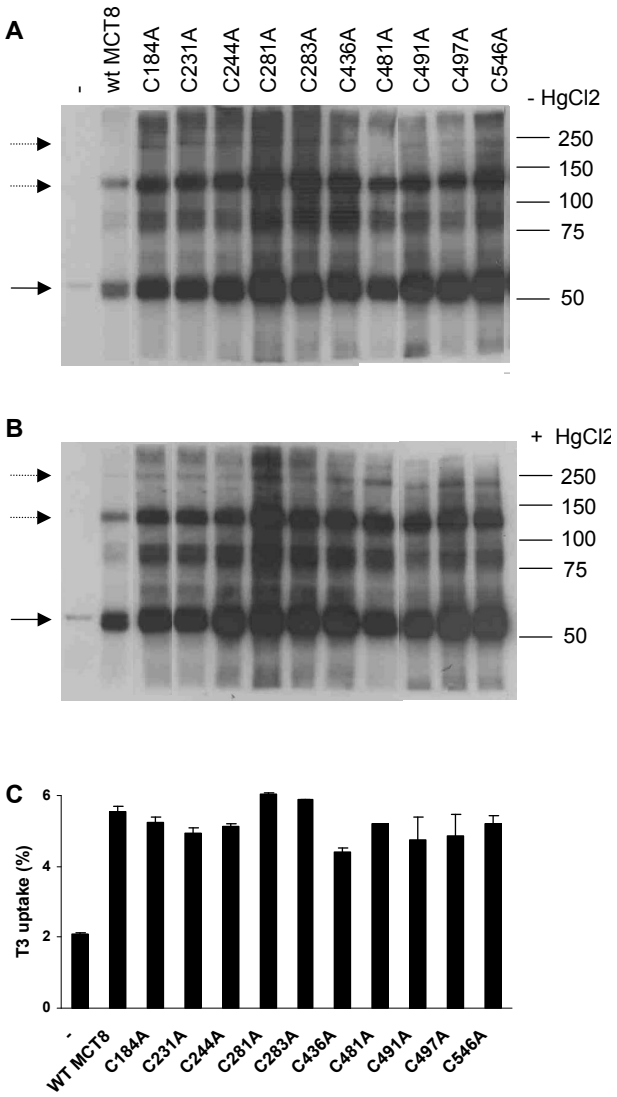

Fig. 4. Expression of cysteine-to-alanine hMCT8 mutants in COS1 cells. Western blotting of mutant hMCT8 without (A) and with (B) 10 μM $HgCl_2$ treatment. The solid arrow indicates hMCT8 monomer; the dashed arrows point to hMCT8 dimers. (C) Uptake of [3′-^{125}I]T3 (5 min) in COS1 cells transiently transfected with cysteine-to-alanine mutants as compared to wt hMCT8.

altered MCT8 function, transport of T3 was measured. No differences in T3 uptake were observed between the various Cys mutants and wt hMCT8 (Fig. 4C).

The oligomerization of hMCT8 was further investigated using cDNA coding for a hMCT8 splice variant identified in one of our patients that results in the deletion of ami-

no acids 267-360 (15). The splice site mutant results in a monomeric hMCT8 protein of about 51 kDa, corresponding to the 94 amino acid loss. (Fig. 5A, lane 2). The bands of 110 and 240 kDa represent oligomeric mutant hMCT8. When wt hMCT8 and the splice site mutant were cotransfected, an intermediately migrating band of ~115 kDa appeared, demonstrating heterodimerization between wt hMCT8 and the splice site mutant (Fig. 5A, lane 3). This confirms that the hMCT8 protein forms homodimers *in vitro*.

Intriguingly, whereas the monomeric expression of the splice site mutant was decreased, the oligomeric bands seem more intense compared to wt hMCT8. Therefore, we performed Western blotting of a series of hMCT8 mutants to investigate the expres-

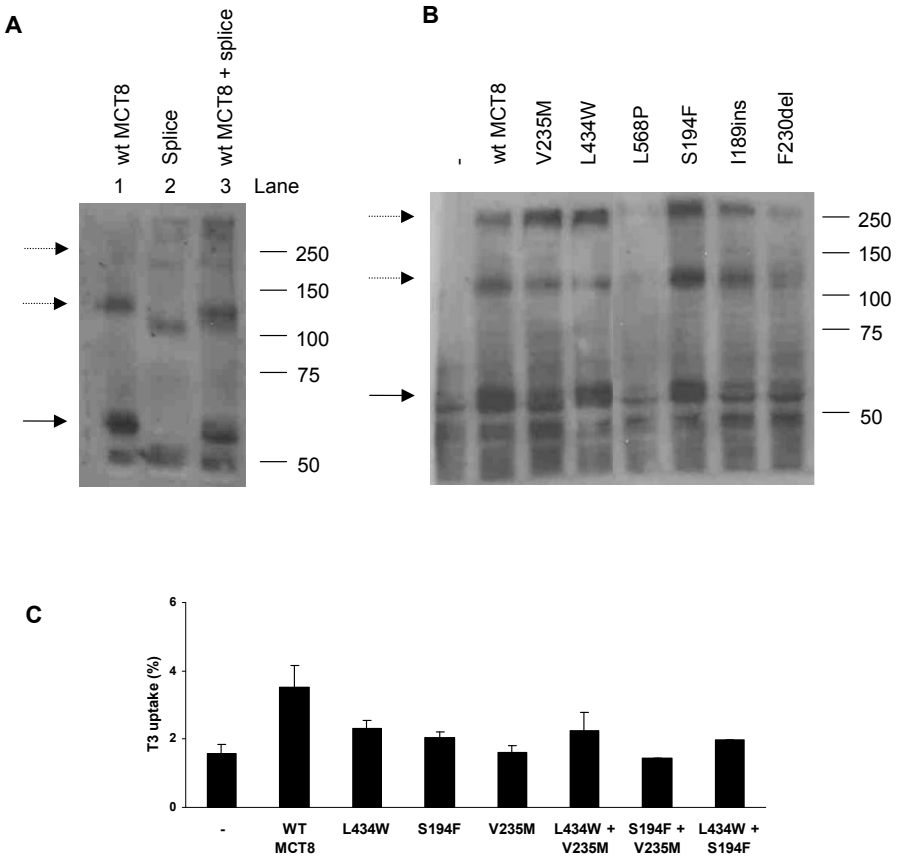

Fig. 5. Expression of mutant hMCT8 in JEG3 cells, corresponding to mutations in MCT8 patients. (A) Expression of hMCT8 (lane 1), a splice site mutant, which lacks amino acids 267-360 (lane 2) and cotransfection of wt and splice site mutant hMCT8 (lane 3). (B) Expression of different hMCT8 mutants. The solid arrow indicates wt hMCT8 monomer; the dashed arrows point to wt hMCT8 dimers and oligomers. (C) Uptake of 1 nM [3'-^{125}I]T3 (5 min) in JEG3 cells (co)transfected with a (combination of) hMCT8 mutants.

sion of the oligomeric bands. Most MCT8 mutants (V235M, S194F, I189ins), which show monomeric protein expression, have a relatively increased intensity of oligomeric bands compared to wt hMCT8 (Fig. 5B).

To investigate whether oligomerization could be functional for transporter activity, we wondered whether cotransfection of two mutant hMCT8 plasmids could (partially) rescue transport activity. We used the L434W and S194F mutants, which show some residual activity, and the V235M mutant, which has no residual function (19). Cotransfection of a combination of the mutants did not result in a clear recovery of function (Fig. 5C).

Recent results suggested that hMCT8 undergoes affinity-labeling by BrAc[^{125}I]T3 (13). Because Cys is a likely target for affinity-labels such as BrAcT3, the Cys>Ala mutants enabled us to explore whether Cys residues are responsible for affinity-labelling of hMCT8. BrAc[^{125}I]T3 incubation of intact COS1 cells cotransfected with hMCT8 (wt or Cys mutants) and rD1 resulted in a clear labeling of a 29 kDa and a ~60 kDa protein, corresponding to the molecular mass of rD1 and hMCT8, respectively (Fig. 6). However, no differences in intensity of these bands was noticed between the Cys mutants and wt hMCT8.

DISCUSSION

In the present study, we explored dimerization of the TH transporter hMCT8. The well-studied MCT family members MCT1-4 all require ancillary proteins for their expression and normal function (9-12, 20). BSG is the most common ancillary protein as it is capable of making functional complexes with MCT1, MCT3 and MCT4. However, depending on the cell, MCT1 may also associate with EMB, which is the requisite ancillary protein for MCT2. In contrast with EMB, BSG is widely expressed and believed to be the most important ancillary protein in adult life.

Our data obtained using COS1 as well as JEG3 cells demonstrate that neither co-expression of BSG or EMB, which are differentially expressed in the cell types studied, nor of the homologous NP55 or NP65 isoforms result in increased hMCT8 expression or activity. The observation that hMCT8, in contrast with MCT3, is localized at the plasma membrane in transfected COS1 cells, suggests that the lack of effect of exogenous ancillary proteins is not due to sufficient levels of endogenous ancillary proteins in our MCT8 overexpression system.

This is supported by similar co-expression studies which demonstrate that MCT1 is only properly trafficked if it is associated with BSG (9). Notably, these experiments were done in COS cells, indicating that endogenous ancillary proteins are insufficient for effective trafficking of MCT1 in an overexpression model.

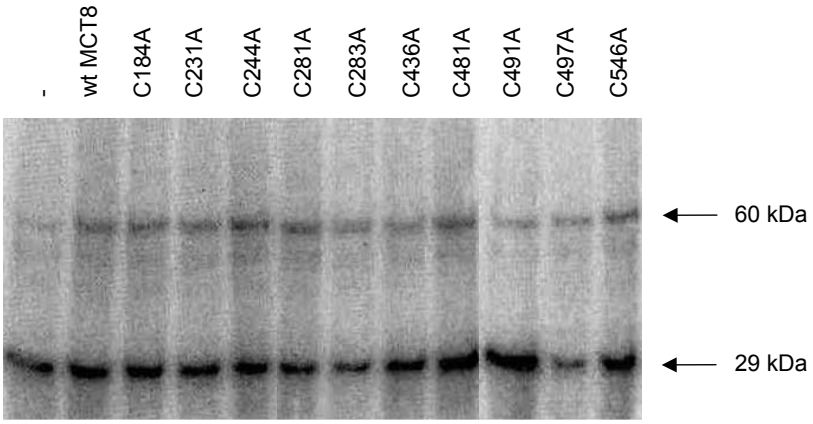

Fig. 6. Affinity labeling of cells transfected with wt and cysteine-to-alanine hMCT8 mutants. COS1 cells were cotransfected with wt hMCT8 or cysteine-to-alanine mutants and rD1. COS1 cells cotransfected with pcDNA3 and rD1 were used as control. Intact cells were incubated for 4 h at 37 C with BrAc[^{125}I]T3.

Furthermore, our results are supported by the observation that in Bsg knockout mice Mct8 is normally expressed at the plasma membrane in the choroid plexus where Bsg is the main ancillary protein. In contrast, Mct1, Mct3 and Mct4 are not trafficked to the plasma membrane in many tissues in these animals.

Taken together, our results suggest that the studied ancillary proteins are not required for proper function of hMCT8. This is not surprising, because the amino acid homology between hMCT1, hMCT2, hMCT3 and hMCT4 is 38-58%, whereas hMCT1-4 show only 21-25% amino acid identity with hMCT8. However, this does not preclude a role for other as-yet-unknown regulatory proteins in the proper cellular control of hMCT8.

In previous experiments using 12% SDS-PAGE gels, only a 61 and a 260 kDa band were observed when Western blots were made of hMCT8-expressing cell lysates (13). In the present study we investigated the hMCT8 protein pattern using 7.5% SDS-PAGE gels, as lower percentage acrylamide gels separate large proteins better. Our present findings clearly show hMCT8-related protein bands of 60, 120 and 250 kDa, and even higher molecular mass bands. As hMCT8 has no predicted phosphorylation or glycosylation sites, the present data strongly suggest that hMCT8 forms homodimers and tetramers.

Cotransfection of wt hMCT8 (predicted molecular mass 59.5 kDa) with a splice site mutant (predicted molecular mass 49 kDa) revealed heterodimerization, thereby confirming the existence of hMCT8 as a homodimer. This is in agreement with a study in which an alternative approach was used to demonstrate homodimerization of wt

hMCT8 and heterodimerization of wt hMCT8 with the A224V hMCT8 mutant (21). The high molecular mass bands on Western blots suggest the presence of tetramers and even multimers of a higher order, possibly octamers. The existence of these higher-order multimers may be aggresomes, which is a general cellular response occurring when the capacitiy of the degrading pathway is exceeded by the production of aggregation-prone misfolded proteins (22). However, the observation that not only overexpressing cells, but also stably transfected cells show hMCT8 oligomerization, indicate that these oligomers represent relevant quaternary structures of hMCT8.

The hMCT8 dimer and tetramer are DTT-reducible and appear further stabilized by the Cys cross-linker $HgCl_2$, suggesting that disulfides as well as free cysteines are involved in this process. Thus, we hypothesized that Cys residues are important for hMCT8 dimerization. Indeed, Cys residues are involved in the dimerization of other 12 transmembrane domain transporter proteins which are also DTT-reducible and $HgCl_2$-reactive (23-25). However, we did not observe differences in dimerization between any of the possible Cys>Ala mutants and wt hMCT8, which argues against the involvement of Cys residues in the dimerization process. This is strengthened by the fact that high concentrations of DTT affected only higher-order multimers. Our data suggest that although Cys residues may be necessary for the formation of higher-order multimers, other amino acids may play a more prominent role in the formation of hMCT8 dimer and tetramer. However, it should be stressed that we have mutated only individual Cys residues, and therefore it is not excluded that the function of one Cys residue may be taken over after mutation by another Cys residue. It should also be mentioned that Cys residues may not only be involved in intermolecular protein binding through disulfide formation but also through complexation with metals such as Zn^{2+}. Indeed it has been demonstrated that the dopamine transporter exists as a Zn^{2+}-containing protein, which stabilizes the dopamine-dopamine transporter interaction (26). Because the amino group-targeted cross-linkers BS^3 and DIDS did not stabilize hMCT8 dimers, Lys residues do not seem to be present at suitable distances in the MCT8 dimerization domain(s).

Apparently, the loss of residues 267-360 in the splice site mutant does not hamper dimerization. Prediction programs suggest that the regions of the splice site mutant corresponding to TMDs 1,2 and 7-12 in wt hMCT8 have not been changed. In contrast, the 94 amino acid loss result in the deletion of TMDs 4-6. Additionally, the amino acids which form TMD 3 in wt hMCT8, do not traverse the plasma membrane in the splice mutant. If this is correct, it means that the region comprising TMDs 3-6 is not necessary for dimerization.

BrAc[^{125}I]T3 is a well-known affinity-label for a number of proteins interacting with TH, for example D1 and protein disulfide isomerase (27). Recently, we demonstrated that hMCT8 facilitates uptake of BrAc[^{125}I]T3 (7). In addition, a clear labeling of a 55 kDa protein was observed, which we assumed represented BrAcT3-labeling of hMCT8 itself.

We reasoned that BrAc[^{125}I]T3 was targeted to a reactive amino acid such as cysteine in the T3 binding site of hMCT8.

However, in none of the cell lysates transfected with the Cys>Ala mutants BrAc[^{125}I]T3-labeling of the 60 kDa protein changed, which is now well explained if the 60 kDa labeled protein is not hMCT8. Indeed, the protein disulfide isomerase is labeled by BrAc[^{125}I]T3 and not hMCT8 (**Chapter 3**). Apparently, the transport of BrAc[^{125}I]T3 by the Cys mutants was not hampered, because rD1 was equally labeled in cells cotransfected with the various mutants compared to wt hMCT8.

As hMCT8 is a plasma membrane protein and no decreased function or protein expression of the Cys mutants were seen, there is no doubt that all these mutants are properly trafficked to the plasma membrane.

Taken together, the Cys mutants did not behave differentially compared to wt hMCT8 in any of experiments we carried out. This seems remarkable as Cys residues usually have important biological functions, because the reactive thiol group is among others involved in forming disulfide bridges, catalytic sites and post-translational modifications. Our findings may indicate that Cys residues are not important at all for the function of hMCT8 or suggest that hMCT8 has other yet undiscovered functions in which Cys residues play an essential role. Again, it should be stressed that our results were obtained with individual Cys>Ala mutants. To exclude redundancy in function, the study of a single mutant in which all Cys residues are mutated is needed.

Although it is clear that hMCT8 forms homodimers, the role of hMCT8 dimerization remains tentative. Therefore, further studies are required to establish the function of hMCT8 dimerization.

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Chapter 3

Ubiquitination and turnover of the human monocarboxylate transporter 8

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** both authors contributed equally to these studies*

ABSTRACT

Transporter proteins are essential for cellular uptake of thyroid hormone. Monocarboxylate transporter 8 (MCT8) is a specific thyroid hormone transporter, which plays a pivotal role in human brain development, as mutations in MCT8 result in psychomotor retardation. In the present study we investigated turnover and regulation of the MCT8 protein. As MCT8 contains N-terminal PEST domains, rapid protein turnover was expected. We used a stable cell line to determine MCT8 protein expression. siRNA-induced knock-down revealed a ~50% decrease in MCT8 protein, but only after 72 h. In addition, inhibition of protein synthesis for 24 h with cycloheximide did not affect MCT8-mediated T3 uptake, suggesting that MCT8 is not rapidly degraded. Incubation with the proteasome inhibitor MG132 resulted in a ladder of MCT8-containing bands on western blot, suggestive of ubiquitination. Pull-down of 6xHis-tagged ubiquitin from lysates of cells co-transfected with MCT8 revealed MCT8-containing complexes between 60 and 120 kDa, in support of MCT8 ubiquitination. As PEST domains have been implicated in ubiquitination, the N-terminal PEST domains in MCT8 were deleted. No MCT8-related protein was detected in cells transfected with this variant, indicating an essential role of these domains for proper MCT8 expression.

Our studies indicate that MCT8 is not rapidly degraded, despite the presence of PEST domains. Furthermore, we provide evidence that MCT8 expression is affected by the ubiquitin-proteasome pathway.

INTRODUCTION

In recent years, it has been established that plasma membrane located transporter proteins are required for cellular uptake of thyroid hormone (TH) (1). Among the transporters identified, monocarboxylate transporter 8 (MCT8) is a specific transporter of iodothyronines (2). Its physiological relevance is beyond doubt as patients lacking functional MCT8 have psychomotor retardation and abnormal serum TH levels (3, 4). Several *in vitro* studies have addressed the effects of MCT8 mutations on TH transport (5-8). However, the regulation of MCT8 expression and function is still elusive.

Perhaps the most intriguing structural aspects of human (h) MCT8 are the N-terminal PEST domains. These domains with proline (P) and glutamic acid (E) repeats were already noted at the identification of the *MCT8* gene by Lafrenière *et al*. in 1994 (9). They called the gene X*PCT*, for X-linked PEST containing transporter (it is now officially termed *SLC16A2*). PEST domains are hydrophilic stretches of 12 or more amino acids containing at least one proline (P), glutamic acid (E) or aspartic acid (D) and one serine (S) or threonine (T), flanked by lysine (K), arginine (R) or histidine (H) residues (10). Many PEST-domain containing proteins, such as p53, HSP70 and ornithine decarboxylase (ODC) have very short half-lives of 0.5 to 2 h. Involvement of PEST domains in this rapid turnover was demonstrated, for example, by Ghoda *et al*., who truncated the mouse ODC protein just before the C-terminal PEST domain (11). The shortened protein showed to be at least 10-fold more stable than the native protein. Adding the PEST domain of mouse ODC to the stable *Trypanosom*a ODC converted it to a rapidly degraded protein (12). The ProtParam protein analysis program (www.expasy.org/tools/protparam.html) regards full-length hMCT8 as an unstable protein, but predicts a half-life of over 30 h (in reticulocytes), which does not suggest rapid protein turnover. hMCT8 without PEST domains is predicted to be a stable protein, but with a similar half-life as the native protein. These intriguing predictions prompted us to study whether hMCT8 is in fact rapidly degraded, and if a hMCT8 variant devoid of PEST domains is more stable.

One of the most important pathways for intracellular protein degradation is the ubiquitin-proteasome pathway. It allows for tight regulation of protein breakdown. Proteins targeted by this system are ubiquitinated, a process in which 8.5 kDa ubiquitin molecules are covalently attached to a lysine residue in the target protein. This highly specific process involves ubiquitin-activating enzyme (E1), ubiquitin-carrier enzymes (E2) and ubiquitin ligases (E3) (13). A polyubiquitin chain is formed by binding an additional ubiquitin to the lysine in position 48 of the previous ubiquitin molecule. This polyubiquitin chain serves as a recognition marker for the proteasome, in which the tagged protein is degraded. Many different E3 enzymes have been identified, recognizing specific motifs on target proteins. These include primary and secondary protein structures, but also post-translational modifications like phosphorylation or binding

to ancillary proteins. Various proteins containing PEST domains are degraded via the ubiquitin-proteasome pathway. Examples are the intracellular domain of Notch 1 (14), NPDC-1 (neural proliferation and differentiation control protein 1) and P53 (15, 16).

In the present studies we tested the hypothesis that the N-terminal PEST domains target MCT8 for rapid protein turnover, possibly via the ubiquitin-proteasome pathway. We constructed a stable yellow fluorescent protein (YFP) labeled MCT8-expressing cell line to study MCT8 protein expression after transfection with MCT8 siRNA using fluorescence assisted cell sorting (FACS). Furthermore, we incubated MCT8 transfected cells with MG132, a potent inhibitor of the proteasome (17), aiming to detect an increase in (poly)ubiquitinated MCT8 molecules. Co-transfection of cells with MCT8 and His-tagged ubiquitin enabled specific isolation of such molecules. Finally, we constructed a MCT8 variant without N-terminal PEST domains in order to study effects on protein stability and function. We demonstrate that MCT8 is indeed ubiquitinated. However, we were not able to detect a rapid decrease of MCT8 expression after inhibition of its synthesis, suggesting that MCT8 is not subject to rapid protein degradation.

MATERIALS AND METHODS

Construction of a stable cell line expressing hMCT8-YFP

The coding sequence of human (h) MCT8 (18) was subcloned into a pEYFP-N1 expression vector (Clontech, BD Biosciences, Breda, The Netherlands) using *Hind*III and *Age*I. To enable this, the hMCT8 stop codon was transformed into an *Age*I restriction site by site-directed mutagenesis. The resulting vector pEYFP-N1-hMCT8 codes for a hMCT8-YFP fusion protein, with the YFP linked to the C-terminal end of hMCT8 via a 4-amino acid spacer (Pro-Val-Ala-Thr). Functional characteristics of the fusion protein were compared to unlabeled hMCT8 in transient transfection studies in JEG3 cells. To obtain stably hMCT8-YFP transfected Flp-in 293 cells (Invitrogen, Breda, The Netherlands), the chimeric coding sequence was subcloned into pcDNA5/FRT (Invitrogen) using *Hind*III and *Not*I. HEK293 derived Flp-in 293 cells (Invitrogen) were co-transfected with pcDNA5/FRT-hMCT8-YFP and pOG44 (Invitrogen), a Flp-recombinase containing vector, in various ratios using FuGene-6 transfection reagent (Roche Applied Science, Almere, The Netherlands). Transfected cells were selected using hygromycin (100 µg/ml medium). Four single colonies were cultured separately and compared functionally. With the four clones having equal characteristics, one was named Flp293-hMCT8Y and selected for further studies.

Construction of the hMCT8 variants (hMCT8-ΔPEST and K86A mutant)

The h*MCT8* gene codes for a protein containing three N-terminal PEST domains, two of which are included in the short hMCT8 variant that starts at the second putative ATG start site. This variant, which is homologous to the rodent MCT8, is encoded by the pcDNA3-hMCT8 vector used in our studies. We constructed a hMCT8 variant without PEST domains by introducing two *Eco*RI restriction sites, one starting at nucleotide position 10 from the second ATG, and one at position 271. Deleting 87 codons by *Eco*RI digestion and religation, the vector (pcDNA3-hMCT8-ΔPEST) codes for a protein with only 9 amino acids before the first predicted transmembrane domain. The hMCT8 K86A mutant was generated by side-directed mutagenesis using the Quick-Change kit (Stratagene, Amsterdam, The Netherlands). The presence of the desired mutations in an otherwise unaltered cDNA was confirmed by DNA sequencing.

Cell culture, transfections, T3 uptake studies and fluorescence microscopy

Flp-in 293 cells stably transfected with hMCT8 (Flp239-hMCT8 cells) and stably transfected controls (Flp239-C cells) were kindly provided by Drs. Alex Ianculescu and Tom Scanlan (UCSF, San Francisco, CA). All stable Flp293 cells were cultured in DMEM/F-12 medium supplemented with 9% heat-inactivated FBS, 100 nM sodium selenite, penicillin/streptomycin 1% and hygromycin (100 µg/ml). HEK293, COS1 and JEG3 cells were cultured in DMEM/F12 with FBS and sodium selenite, but without antibiotics. All transient cDNA transfections were performed using 3 µl FuGene-6 transfection reagent and 1 µg plasmid DNA (11). Uptake of T3 was tested as reported previously (12). Briefly, cells were incubated with 1 nM (2 x 10^5 cpm) [^{125}I]T3 in 1.5 ml incubation medium at 37 C. After incubation, cells were washed with the medium, lyzed with 0.1 M NaOH and counted in a gamma counter. Fluorescence microscopy of hMCT8-YFP expressing JEG3 cells was performed as described previously (11).

siRNA transfections and FACS analysis

Flp293-hMCT8Y cells were transfected with 5-10 nM SLC16A2 chimera RNAi (Abnova, Taipei, Taiwan) using HiPerfect siRNA transfection reagent (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Small interfering RNA (siRNA) directed against house keeping gene MAPK1 (Qiagen) was used as negative control. Flp293-C cells transfected with Allstars Alexa Fluor 488-labeled nonspecific siRNA (Qiagen) served to determine transfection efficiency. After 24, 48 and 72 h, cells were trypsinized and fixated in 4% paraformaldehyde. Mean fluorescence of 10^4 cells was determined by FACS analysis on a Beckman-Coulter Flow Cytometer.

Inhibition of protein synthesis, the proteasome and ubiquitination of MCT8

Flp293-hMCT8 and Flp293-C cells were cultured in 6-well plates. At confluence, cells were incubated for 1, 3, 6 or 24 h with culture medium containing 25 or 50 µg/ml cycloheximide (CHX) (Sigma-Aldrich, Zwijndrecht, The Netherlands), an inhibitor of protein synthesis, or 1 µM MG132 (Calbiochem, San Diego, CA), a potent inhibitor of the proteasome. After incubation, we tested T3 uptake and analyzed cell sonicates by western blotting using purified hMCT8 polyclonal antibody 1306 as reported previously (6).

HEK293 cells were transiently co-transfected with pcDNA3-hMCT8 and a pcDNA3.1 vector coding for 6xHis-tagged ubiquitin (pcDNA3.1-6HUB) in a 2:1 ratio. Controls were transfected with pcDNA3-hMCT8 or pcDNA3.1-6HUB only, complemented with empty pcDNA3 vector to equalize total cDNA amount. After 24 h, cells were incubated for 4 h with medium containing 20 µM MG132. Cells were then harvested and diluted to 10^7 cells per 500 µl in 6 M urea in PBS. After sonication, supernatants were incubated for 2 h at 4 C with 10 µl 5% Ni-NTA beads (Qiagen). Beads were washed and eluted according to the manufacturer's protocol. Eluates were diluted to 0.4 mg protein per ml and analyzed by western blotting using hMCT8 antibody 1306.

RESULTS

Function of YFP-labeled MCT8

The function of the hMCT8-YFP fusion protein encoded by the pEYFP-N1-hMCT8 vector, was tested by measuring T3 uptake in transiently transfected JEG3 cells (Fig. 1A). Compared to empty pcDNA3-transfected controls, 3.3, 3.9 and 3.0-fold increases of intracellular T3 were measured after 2, 5 and 10 min, respectively. Fold increases were slightly higher than in cells transfected with pcDNA3-hMCT8 (3.0, 3.3 and 2.4-fold after 2, 5 and 10 min). T3 uptake in JEG3 cells transiently transfected with pcDNA5/FRT-hMCT8-YFP, the vector constructed for stable transfection into Flp-in 293 cells, showed similar results (data not shown). Fluorescence microscopy of JEG3 cells transiently transfected with pEYFP-N1-hMCT8 showed clear distribution of the hMCT8-YFP fusion protein at the plasma membrane (Fig. 1B). T3 uptake in Flp293-hMCT8Y cells after 5 min was 2.6-fold higher than in Flp293-C controls, showing roughly the same induction of transport as in non-YFP tagged Flp293-hMCT8 cells (2.8-fold) (Fig. 1C).

Stability of MCT8

Flp293-hMCT8Y cells were used to study MCT8 expression after transfection with MCT8 siRNA as the YFP tag enables rapid determination of MCT8 protein levels by FACS. The efficiency of the HiPerfect siRNA transfection protocol was determined at 45, 58 and 42% after 24, 48 and 72 h, respectively, in Flp293-hMCT8 cells using an Alexa Fluor 488-labeled

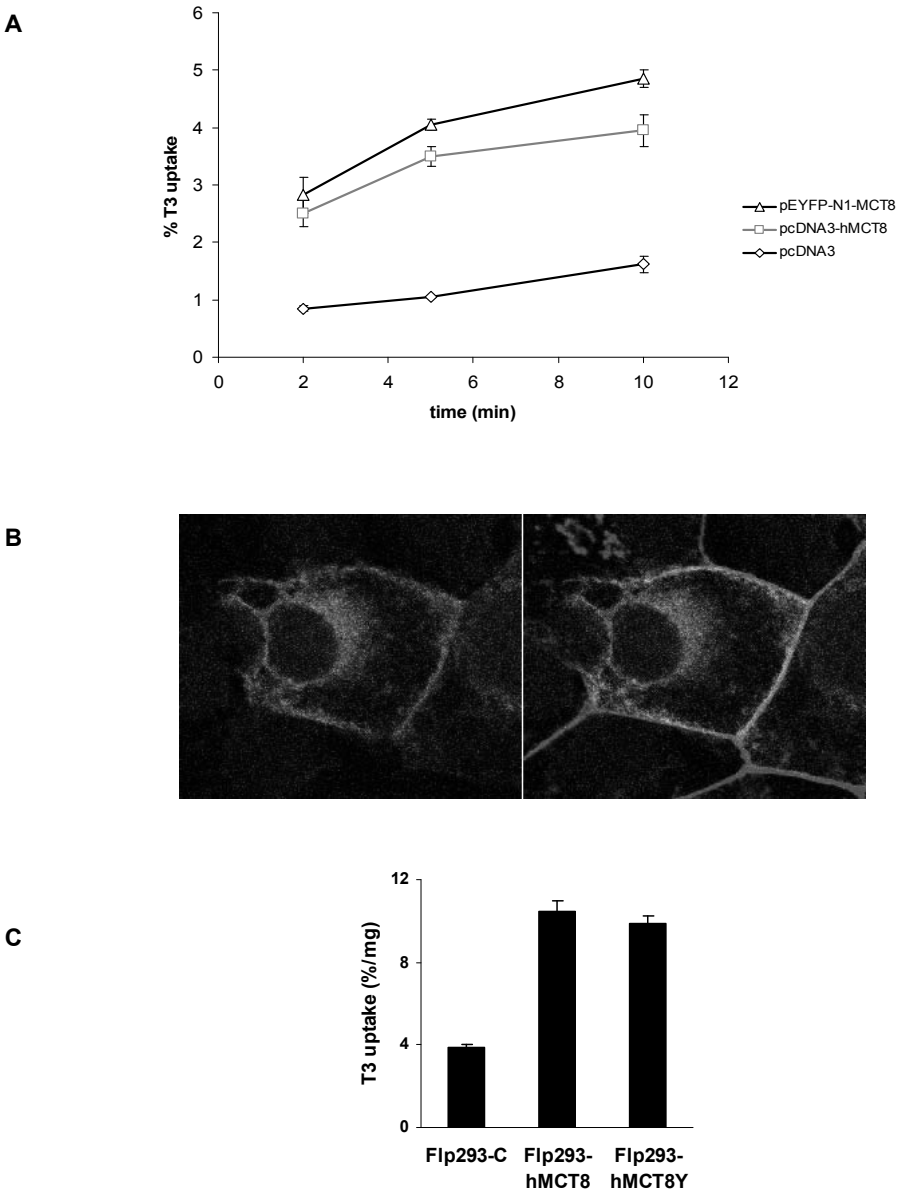

Fig. 1. (A) Uptake of T3 after 2, 5 and 10 min in JEG3 cells transfected with pEYFP-N1-hMCT8, pcDNA3-hMCT8 and empty pcDNA3 controls. (B) Cellular distribution of the hMCT8-YFP fusion protein (green) in JEG3 cells transfected with pEYFP-N1-hMCT8. Right panel: plasma membrane localization is indicated by co-localization with tight-junction protein ZO-1 (red). (C) Uptake of T3 after 5 min, shown as percentage of total T3 added taken up per mg protein in Flp293-C, Flp293-hMCT8 and Flp293-hMCT8Y cells. No significant differences in T3 uptake between YFP-labeled and non-labeled MCT8 are detected.

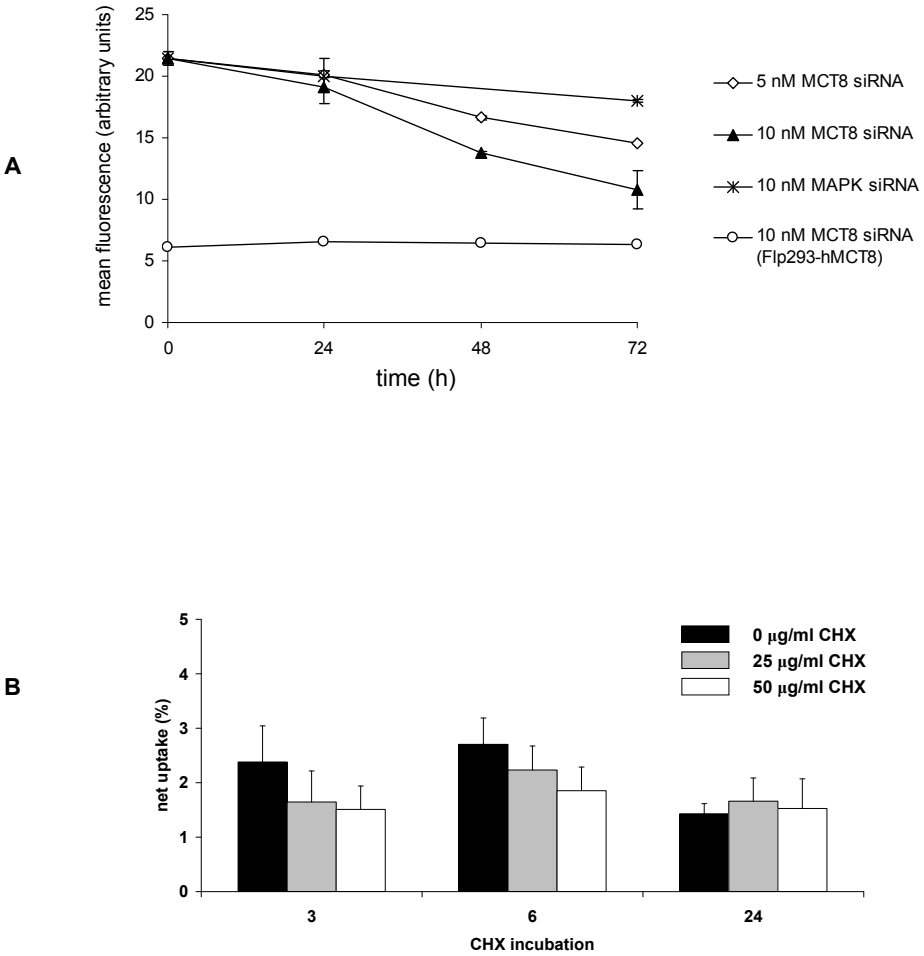

Fig. 2. (A) Mean fluorescence intensity of Flp293-hMCT8Y cells 0-72 h after transfection with MCT8 siRNA. A significant, dose-dependent decrease in intensity is observed after 24 h of transfection. No significant changes in fluorescence are observed after transfection with MAPK siRNA in Flp293-hMCT8Y cells and with MCT8 siRNA in non-YFP labeled Flp293-hMCT8 cells. (B) Uptake of T3 after 5 min in Flp293-C and Flp293-hMCT8 cells after 3, 6 and 24 h incubation with 0, 25 and 50 μg/ml CHX. Uptake in Flp293-C is subtracted from uptake Flp293-hMCT8 cells to correct for background signals.

nonspecific siRNA. Transfection of Flp293-hMCT8Y cells with 5 or 10 nM MCT8 siRNA did not lead to a significant decrease of fluorescence intensity within 24 h (Fig. 2A). However, a dose-dependent decrease was observed after 48 and 72 h. After 72 h, transfection with 10 nM MCT8 siRNA resulted in a ~50% decrease of mean YFP fluorescence, indicating a complete inhibition of hMCT8Y expression in transfected cells. Transfection of Flp293-hMCT8Y cells with siRNA targeting the housekeeping gene MAP-kinase resulted in an

insignificant decrease of YFP signal after 72 h. Transfection of untagged Flp293-hMCT8 cells with MCT8 siRNA showed that the background fluorescence signal of these cells is not affected by MCT8 knock-down. These controls confirm that the observed decreases in fluorescence signal in Flp293-hMCT8Y cells reflect reduced expression of YFP tagged MCT8.

However, we must consider that although the YFP tag does not interfere with the production, trafficking and function of MCT8, it might affect its degradation. Therefore, we incubated Flp293-C and Flp293-hMCT8 cells with different concentrations of CHX to inhibit protein synthesis. Figure 2B shows that even after 24 h CHX treatment, no decrease in net T3 uptake is observed in cells expressing hMCT8.

Inhibition of the proteasome and ubiquitination of MCT8

Incubation of Flp293-hMCT8 cells for 1-24 h with 1 μM MG132 resulted in an increase in MCT8-containing protein complexes between 60 and 120 kDa in size, as detected by western blotting (Fig. 3A). No clear increase in intensity of these bands was observed in lysates of Flp293-C cells, although HEK293 cells are known to express MCT8 endogenously. Functional analyses of Flp293-hMCT8 and Flp293-C cells showed that MG132 treatment did not significantly change T3 uptake (Fig. 3B). Because ubiquitination may explain the phenomenon of increased intensity of MCT8-containing bands after MG132 treatment, we used a pull-down approach to investigate this possibility. Sonicates of hMCT8 + 6HUB co-transfected HEK293 cells were analyzed by western blotting using MCT8 specific antibody 1306 before and after incubation with Ni coated beads to obtain 6HUB-linked proteins (Fig. 3C).

Pre-incubation samples show bands of ~60 and ~120 kDa, the expected size for MCT8 monomers and dimers (19), in hMCT8 and hMCT8 + 6HUB co-transfected cells. Bands of ~240 kDa are also observed, suggesting the presence of MCT8 tetramers. Large quantities of MCT8 containing complexes with sizes between ~60 and ~120 kDa and higher are present in the MCT8 + 6HUB co-transfected cells. In smaller amounts these are also observed in cells transfected with hMCT8 only. A ~60 kDa band is also observed in 6HUB only transfected cells, in keeping with endogenous expression of MCT8 monomers in HEK 293 cells.

Analysis of His-tagged ubiquitinated protein complexes pulled down from lysates of hMCT8 + 6HUB co-transfected cells using Ni-coated beads, shows the presence of MCT8. A faint ~60 kDa band is also seen in hMCT8 only transfected cells, suggesting incomplete removal of non-His-tagged proteins in this sample, or nonspecific staining. Faint bands of ~60 kDa and higher are also detected in 6HUB only transfected cells.

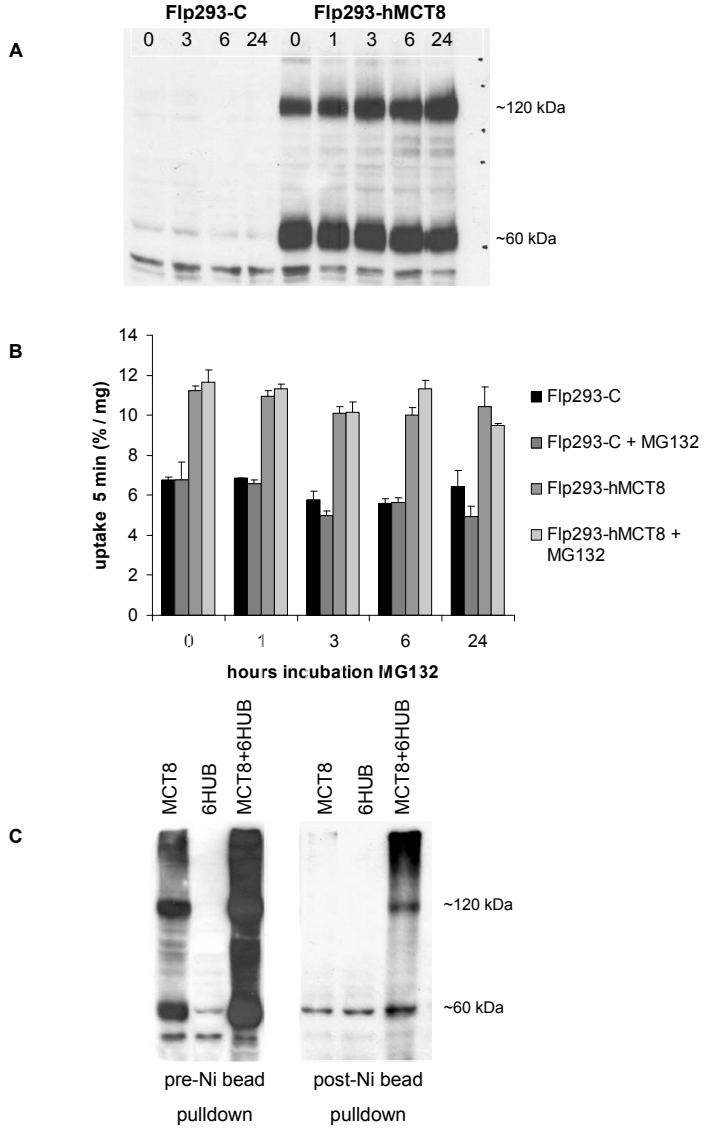

Fig. 3. (A) Western blot of homogenates of Flp293-C and Flp293-hMCT8 cells after 0-24 h incubation with 1 µM MG132 probed with MCT8 specific antibody 1306. Note the increase of MCT8-containing fragments between 60 and 120 kDa, suggesting (poly)ubiquitinated MCT8. (B) Uptake of T3 after 5 min in Flp293-C and Flp293-hMCT8 cells treated for 1-24 h with 1 µM MG132. No significant differences in uptake are observed after inhibition of the proteasome. (C) Western blot of HEK293 homogenates after transfection with hMCT8, 6xHis-tagged ubiquitin (6HUB) and hMCT8 + 6HUB. Left: samples prior to pull-down of ubiquitinated proteins with Ni beads. Ubiquitinated MCT8 is observed in hMCT8 and hMCT8 + 6HUB transfected cells. Right: samples after Ni-bead pull-down, confirming the presence of MCT8 in these ubiquitinated proteins.

Function of hMCT8-ΔPEST and K86A

Next, we investigated whether the PEST domains may be involved in the ubiquitination process. Therefore, we constructed a MCT8 variant devoid of the N-terminal PEST domains (pcDNA3-hMCT8-ΔPEST) (Fig. 4A and B). However, transfection of JEG3 or COS1 cells with pcDNA3-hMCT8-ΔPEST in the presence or absence of 1 μM MG132 did not increase T3 uptake compared to empty pcDNA3 transfected controls. Western blotting of sonicates of these cells did not identify MCT8 specific bands (data not shown). From this it was concluded that this hMCT8-ΔPEST variant is not expressed at the protein level.

Finally, we mutated the most N-terminal located lysine residue to alanine (K86A) to study its possible involvement in MCT8 ubiquitination. However, no change in function (T3 uptake) or protein pattern (with or without MG132) on western blot was observed (data not shown).

DISCUSSION

The N-terminal PEST domains in hMCT8 suggests the protein is subject to rapid turnover, possibly via the ubiquitin-proteasome pathway. To enable quantitative analyses of MCT8 expression in cells, we constructed a Flp293 cell line stably expressing hMCT8 with a C-terminal YFP tag. Benefits of such a cell line include rapid determination of protein expression by FACS and the possibility to study spatio-temporal aspects of expression in live cells. We demonstrate that YFP-labeled MCT8, both in transiently and stably transfected cells, functions similar to non-labeled MCT8 regarding the uptake of T3. Not surprisingly, we show that the hMCT8-YFP fusion protein is expressed at the plasma membrane of transfected cells, the functional localization of the transporter. From this it appears that the 26.9 kDa YFP tag does not interfere with the synthesis, trafficking or function of MCT8.

Inhibition of the proteasome by MG132 results in an intracellular increase of (poly) ubiquitinated proteins. We detected a clear increase of (poly)ubiquitinated MCT8 in Flp293-hMCT8 cells after 1-24 h incubation with 1 μM MG132. Co-transfection of hMCT8 and 6xHis-tagged ubiquitin in HEK293 cells enabled the identification of hMCT8 in isolated ubiquitinated protein complexes, confirming that hMCT8, at least in HEK293 cells, is ubiquitinated. Ubiquitinated MCT8 is found in Flp293-hMCT8 and in hMCT8 only transfected HEK293, indicating that a complete ubiquitination machinery, including a specific E3 ubiquitin ligase, is present in these cells. Mutation of the most N-terminal lysine residue at position 86 did not affect MCT8 function or turnover. Apparently, this lysine is not involved in the ubiquitination of the protein. Which lysine is involved in this process needs to be elucidated in future studies by one-by-one mutation of all lysine residues in hMCT8.

The observed increase of (ubiquitinated) MCT8 after stimulation with MG132 does not lead to an increased T3 uptake. It is known that ubiquitination plays an important role in internalization of membrane proteins (20), thus inactivating them prior to the actual degradation in the proteasome. It is therefore likely that ubiquitinated MCT8 is not active or not expressed at its functional localization in the plasma membrane. It is however possible that proteins are de-ubiquitinated and relocated to their functional position in the cell, as has been shown, for example, for the type 2 deiodinase (21). As yet, we have not studied whether this occurs in our system.

We demonstrate a specific decrease of hMCT8-YFP expression in Flp293-hMCT8Y cells after transfection with a MCT8 specific siRNA. However, this effect was not rapid, within hours, as may be expected from a rapidly degraded protein. This indicates that MCT8 is not a rapid-turnover protein, in keeping with the predicted protein half-life of over 30 h by the ProtParam program. The observation that inhibition of protein synthesis with increasing concentrations of CHX up to 24 h did not decrease MCT8-mediated T3 uptake supports this conclusion. Furthermore, in contrast to the previous belief that PEST sequence-containing proteins are subject to rapid degradation (10), it has recently been demonstrated by whole proteome profiling that rapidly degraded proteins are not enriched in PEST domains (22).

Our attempts to study the expression and functional characteristics of a hMCT8 variant without PEST domains were unsuccessful so far, because no MCT8 protein was produced by cells transfected with pcDNA3-hMCT8-ΔPEST. It is therefore likely that the N-terminal sequence of MCT8 is vital for normal protein expression or stabilization.

In conclusion, we demonstrate that, in spite of the N-terminal PEST domains, hMCT8 most likely is not subject to rapid protein degradation. In contrast, elements within or close to these domains appear essential for normal protein synthesis and expression. We demonstrate that MCT8 is a target for the ubiquitin-proteasome pathway, indicating specific and controlled breakdown. Although more extensive work needs to be done, our studies so far provide a first look at the regulation of MCT8 expression.

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Chapter 4

The thyroid hormone transporters MCT8 and MCT10 transport the affinity-label N-bromoacetyl-[^{125}I] T3 but are not modified by it

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Submitted

Chapter 5

Novel pathogenic mechanism suggested by *ex vivo* analysis of MCT8 (SLC16A2) mutations

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* Both authors contributed equally to these studies

Human Mutation 2009;30:29-38

ABSTRACT

Monocarboxylate transporter 8 (MCT8; SLC16A2) facilitates cellular uptake and efflux of 3,3',5-triiiodothyronine (T3). Mutations in MCT8 are associated with severe psychomotor retardation and high serum T3 and low 3,3',5'-triiodothyronine (rT3) levels. Here we report 3 novel MCT8 mutations. Two subjects with the F501del mutation have mild psychomotor retardation with slightly elevated T3 and normal rT3 levels. T3 uptake was mildly affected in F501del fibroblasts and strongly decreased in fibroblasts from other MCT8 patients, while T3 efflux was always strongly reduced. Moreover, type 3 deiodinase activity was highly elevated in F501del fibroblasts, whereas it was reduced in fibroblasts from other MCT8 patients, probably reflecting parallel variation in cellular T3 content. Additionally, T3-responsive genes were markedly upregulated by T3 treatment in F501del fibroblasts but not in fibroblasts with other MCT8 mutations. In conclusion, mutations in MCT8 result in a decreased T3 uptake in skin fibroblasts. The much milder clinical phenotype of patients with the F501del mutation may be correlated with the relatively small decrease in T3 uptake combined with an even greater decrease in T3 efflux. If fibroblasts are representative of central neurons, abnormal brain development associated with MCT8 mutations may be the consequence of either decreased or increased intracellular T3 concentrations.

INTRODUCTION

The thyroid gland synthesizes two iodothyronines, thyroxine (3,3',5,5'-tetraiodothyronine, T4) and 3,3',5-triiiodothyronine (T3), which together are called thyroid hormone (TH). Most T3, the major biologically active TH, is generated from the prohormone T4 by the deiodinating enzymes D1 and D2 (1). The deiodinase D3 inactivates T4 to 3,3',5'-triiodothyronine (rT3) and T3 to 3,3'-diiodothyronine (T2) (1). The genomic actions of T3 are mediated by nuclear T3 receptors (TRs) (2). As the active centers of the deiodinases and the TRs are located intracellularly, TH metabolism and action require transport of the hormone across the plasma membrane.

Accumulating evidence indicates that uptake of TH into the cell is facilitated by transporter proteins (3). Recently, several classes of transporter proteins have been characterized at the molecular level (4). In contrast to most known TH transporters that accept a wide variety of ligands, organic anion transporting polypeptide 1C1 (OATP1C1), monocarboxylate transporter 8 (MCT8; HUGO-approved code SLC16A2; MIM#300095) and MCT10 express a high selectivity towards TH (4).

TH is critically involved in the development of the CNS during fetal and neonatal life. Minor changes in only one of the factors involved in modulating and mediating TH effects on the brain, may have deleterious neurological effects. Only recently, the first mutations in a TH transporter have been identified (5, 6). Males with loss-of-function mutations in MCT8 show severe neurological deficits, with axial hypotonia, spastic quadriplegia and impaired or absent speech, and muscle hypoplasia. This X-linked syndrome is known as the Allan-Herndon-Dudley syndrome (AHDS, OMIM#300523). Thyroid function tests show low to low-normal serum T4 levels, normal or moderately increased TSH levels, low rT3 levels and strongly elevated T3 levels. Because MCT8 facilitates cellular T3 uptake and is highly expressed in neurons, it is likely that inactivation of MCT8 results in an impaired supply of T3 to neurons (7). Considering the importance of TH for normal brain development, it is understandable that the resulting neuronal T3 deprivation results in neurological damage.

Initially, MCT8 was shown to facilitate T3 and T4 uptake (8). However, we recently demonstrated that MCT8 also functions as an efficient T3 and T4 exporter (9). The biological relevance of this function and the possible contribution to the pathogenesis of the MCT8 syndrome is currently unknown.

Here, we present 3 new Dutch families with mutations in MCT8. We used skin fibroblasts of MCT8 patients as an *ex vivo* model to elucidate the pathogenic mechanisms resulting in neurological deficits. We provide evidence that MCT8 mutations may differentially affect cellular influx and efflux of T3. This may suggest that, depending on the mutation, decreased as well as increased intracellular T3 concentrations may result in neurological abnormalities in MCT8 patients.

MATERIALS AND METHODS

Materials

[3'-^{125}I]T3 and [3',5'-^{125}I]T4 were purchased from GE Healthcare (Little Chalfont, UK). Nonradioactive iodothyronines were obtained from Henning (Berlin, Germany). 12-O-tetra-decanoylphorbol-13-acetate (TPA) was obtained from Sigma (St. Louis, MO). Real-time PCR primers and probes were purchased from Biosource (Nivelles, Belgium). Oligonucleotides were synthesized by Invitrogen (Paisly, UK). FuGENE6 transfection reagens was obtained from Roche Diagnostics (Almere, The Netherlands).

Serum analysis

Serum FT4, T3 and TSH were measured by Vitros ECI technology (Ortho-Clinical Diagnostics, Beerse, Belgium) and rT3 was measured by an in-house radioimmunoassay. Neonatal screening data were obtained from the Dutch Health Administration after informed consent of the parents.

Genetic analysis

The *MCT8* gene (RefSeq, NM_006517.3) was analyzed using standard primers as described previously (10). We designed additional primers for patient P6 (Table S1).

Cloning and site-directed mutagenesis

The cloning of wild-type (wt) human MCT8 cDNA was described recently (8). The mutations of patients P2, P3 and P4 were introduced in the MCT8 cDNA using the QuickChange Site-Directed Mutagenesis protocol (Stratagene, Amsterdam, The Netherlands). DNA sequencing confirmed the presence of the introduced mutations.

Cell cultures and transfection

We obtained human skin fibroblasts from patients P1-4 by punch biopsy after informed consent by the parents. Fibroblasts from 3 non-affected subjects were used as controls (kindly provided by Dr. B. Thio, Erasmus MC). We grew fibroblasts in 75 cm^2 flasks in DMEM/F12 medium (Invitrogen) supplemented with 9% FBS (heat-inactivated; Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 100 nM sodium selenite (Sigma). At confluency, fibroblasts were harvested and seeded at equal densities in six-well dishes for TH transport assays and in 28 cm^2 dishes for metabolism experiments and RNA isolation.

JEG3 cells were cultured in six-well culture dishes with DMEM/F12 medium plus 9% FBS and 100 nM sodium selenite. For TH transport studies, cells were transfected with pcDNA3.hMCT8 (wt or mutant) using empty pcDNA3 as control.

For iodothyronine metabolism experiments, cells were co-transfected with pcDNA3. hMCT8 (wt or mutant) and pCIneo.hD3, as previously described (10).

Immunoblotting (IB) and immunocytochemistry (ICC)

The MCT8-specific (C-terminal) polyclonal antibody 1306 was used for IB and ICC. For ICC, the plasma membrane was stained with the zona occludens 1 (ZO1) antibody (Invitrogen). IB and ICC were performed as reported recently (8, 10).

TH transport experiments

At confluency, fibroblasts were washed with incubation medium (Dulbecco's PBS containing 0.1% D-glucose and 0.1% BSA). TH uptake was tested by incubation of the cells for 30-60 min at 37 C with 1 nM ($2x10^5$ cpm) [^{125}I]T3 or [^{125}I]T4 in 1.5 ml incubation medium. After incubation, cells were washed with the medium, lyzed with 0.1 M NaOH and counted in a gamma counter.

For measurement of TH efflux, cells were loaded for 1 h with incubation medium containing 1 nM ($2x10^5$ cpm) [^{125}I]T3 or [^{125}I]T4. After removal of the medium, cells were washed and incubated for 10-30 min with incubation medium without ligand. Finally, medium was removed, and cells were washed with incubation medium, lyzed with 0.1 M NaOH and counted in a gamma counter. Values were corrected for protein concentrations (Bradford assay).

This procedure was adapted to JEG3 cells with minor modifications. After 48 h transfection, JEG3 cells were incubated for 10-30 min with incubation medium containing [^{125}I]T3 or [^{125}I]T4.

TH metabolism experiments

To maximize D2 activity, fibroblasts were incubated for 24 h with DMEM/F12 plus 6% charcoal-treated FBS and 100 nM sodium selenite at confluency. To induce D3 activity, fibroblasts were stimulated for 6 h with 0.1 µM TPA in DMEM/F12 plus 9% FBS. Subsequently, cells were washed with DMEM/F12 plus 0.1% BSA, and incubated for 4-72 h at 37 C with 1 nM ($1x10^6$ cpm) [^{125}I]T4 (D2 activity) or for 3 h at 37 C with 1 nM ($1x10^6$ cpm) [^{125}I]T3 (D3 activity) in DMEM/F12 plus 0.1% BSA. After incubation, medium was sampled, processed and analyzed by HPLC as previously described (8).

The intact-cell metabolism of T3 in JEG3 cells was investigated as described previously (10).

T3 effects on fibroblast

For the T3 stimulation experiment, culture medium was replaced with DMEM/F12 plus 6% charcoal-treated FBS and 100 nM sodium selenite. After 48 h, medium was refreshed with the same medium containing 10 nM T3, and the incubation was continued for 6 h.

Total RNA was isolated from $1x10^6$ fibroblasts using the High Pure RNA isolation kit (Roche). cDNA was synthesized using 0.5 µg RNA and TaqMan RT reagent (Roche). For semiquantitative PCR of MCT8, the sense primer 5'-TGCAGCAGCAGAAACAAGTACC-3' and the antisense primer 5'-GCACACAATGGCAAGAAAGG-3' were used.

SYBR Green I (Eurogentec) was used as detector dye for quantitative PCR of the T3-responsive genes ZAKI 4α, GLUT1 and MCT4, and the primer sequences are presented in Table S2. mRNA levels are expressed relative to that of the house-keeping gene cyclophilin A.

Statistical analysis

All results are the mean of at least duplicate determinations from representative experiments. Values are expressed as means ± SE. Statistical significance was determined using the Student's *t* test for unpaired observations.

RESULTS

Clinical features

Patients P1 and P2 have been reported previously (5, 7, 10). Briefly, both patients have severe psychomotor retardation, characterized by truncal hypotonia, quadriplegia, mental retardation and absence of speech.

Patients P3-6 are newly identified. Patient P3 is a 4 yr old boy born to non-consanguineous parents. He presented at the age of 9 mo when gross motor milestones were not reached with axial hypotonia, headlag, spastic quadriplegia, microcephaly and a myopathic face. MRI showed delayed myelination and thinning of the corpus callosum.

Patient P4 is a boy who at the age of 16 mo presented with a global developmental delay. He had mild axial hypotonia and mild spastic tetraparesis. He functioned as a 12 mo old infant with respect to communication skills. At the age of 18 mo, his head balance was much better than observed in other MCT8 patients; he was able to crawl and he used his hands to grasp toys. A slightly delayed myelination was detected by brain MRI.

Patient P5 is a 38 yr old brother of the mother of patient P4 (Fig. 1A). His gross motor milestones were delayed. At a recent examination there was a mild spastic tetraparesis with good head balance. He was able to walk with some support and to communicate by speaking, although slurred and influently. Furthermore, he was capable of reading by combining separately spelled letters and of writing simple sentences (without verbs) by computer.

At the age of 4 mo, patient P6 presented with a severe delay in mental and motor development, characterized by a severe axial hypotonia and headlag. He had micro-

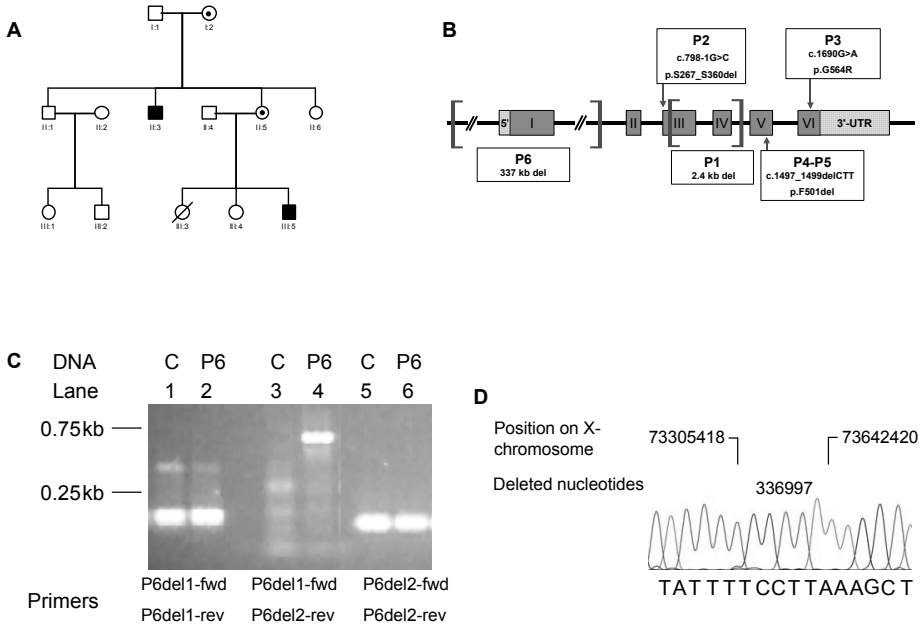

Fig. 1. (A) Pedigree of the family with the F501del mutation. Filled squares indicated affected males. Circles with a dot represent unaffected carriers. A symbol crossed by a slash means that this subject has deceased. Patient P4 and P5 are denoted as II:3 and III:5, respectively. (B) *MCT8* gene structure with the location of the different mutations in patients P1-6. (C) PCR analysis of a deletion in the *MCT8* gene in patient 6 (P6) compared to a control. No DNA is amplified in P6 in the 336 kb large region between the 2 amplicons in the left and right lanes. Combination of P6del1-fwd and P6del2-rev generates an amplicon in DNA of P6, but not in control DNA. (D) Partial sequencing profile of the amplicon in lane 4 refines the borders of the deletion in P6 to a loss of 336,997 nucleotides.

cephaly, low muscle mass, and developed spastic quadriplegia. Delayed myelination and thinning of the corpus callosum were demonstrated by MRI.

Table 1 shows the serum thyroid parameters determined in the patients. In all patients, serum FT4 levels were at or below the lower limit of normal, and serum T3 was increased although only slightly so in patient P5. Serum rT3 was decreased in patients P1-3 and P6 and normal in patients P4 and P5. Neonatal screening results could be retrieved for patients P3, P4 and P6, showing decreased T4 levels in patients P3 and P6, and a normal T4 concentration in patient P4.

Mutation analysis

Based on the combination of developmental delay and elevated serum T3, DNA from patients P1-6 was tested for mutations in *MCT8*. The results are graphically depicted

in Fig. 1B. A deletion of almost 2.4 kb with borders located in exon 3 and intron 4 (c.970_1392+1952del) was found in patient P1, and a mutation in the acceptor splice site of intron 2 (c.798-1G>C) was identified in patient P2, as described previously (5, 7).

In patient P3 a c.1690G>A mutation was found, which results in a Gly to Arg substitution at position 564 (p.G564R). A 3-bp deletion (c.1497_1499delCTT) was identified in patients P4 and P5, causing a deletion of Phe at position 501 (p.F501del). We were not able to amplify exon 1 in patient P6. We further investigated the extension of the deletion by PCR using sets of primers aligning to chromosome X from 3.3 Mb upstream to 105 kb downstream of exon 1. Eventually, we identified two most proximal primer sets (P6del1fwd/rev, P6del2fwd/rev), which each yielded PCR products of the expected size (Fig. 1C). Combination of P6del1fwd and P6del2rev resulted in an amplicon of ~0.75 kb in patient P6 but not in a control. Sequencing of this amplicon refined the borders of the deletion, demonstrating a deletion of 336,997 nucleotides, which includes 252 kb of the upstream region of *MCT8*, exon 1, and 84 kb of intron 1 (Z83843.3:g1322_MCT8:c650-15128del; Fig. 1D).

Delineation of the mutations at the mRNA level

To determine the effects of the mutations on mRNA structure, we performed a PCR on cDNA prepared from fibroblasts using a forward primer located just before the second putative translation start site and a reverse primer located just after the stop codon of the *MCT8* gene. Figure 2A demonstrates a band of ~1.8 kb for controls and patients P3 and P4, corresponding to the predicted length of the coding region of *MCT8*. In patient P1, a vague band appears of ~0.9 kb. Sequence analysis indicated that exons 3, 4 and 5 have been deleted from the mRNA (r.798_1621del; Fig. 2B).

A 1.5 kb PCR product was obtained in patient P2. Sequence analysis of this product indicated the use of an alternative splice site downstream in exon 3, resulting in the deletion of 282 nt from the mRNA (r.798_1079del) and a predicted loss of 94 aa from the protein (p.S267_S360del).

In addition to the full-length 1.8 kb band, a 1.5 kb band was also demonstrated in controls and patients P3 and P4. This splice variant has the same sequence as the MCT8 mRNA in patient P2. Densitometric analysis demonstrated a 41% increased intensitiy of the splice variant in patient P4 compared to controls, suggesting enhanced splicing (Fig. 2C).

IB and ICC

Using IB and ICC, we were not able to detect MCT8 in fibroblasts from controls and patients. Subsequently, JEG3 cells, which do not express endogenous MCT8, were transfected with wt-MCT8 or the splice site (P2), G564R (P3) or F501del (P4/P5) mutants. On IB, minimal MCT8 expression was seen with the G564R mutant, whereas the F501del

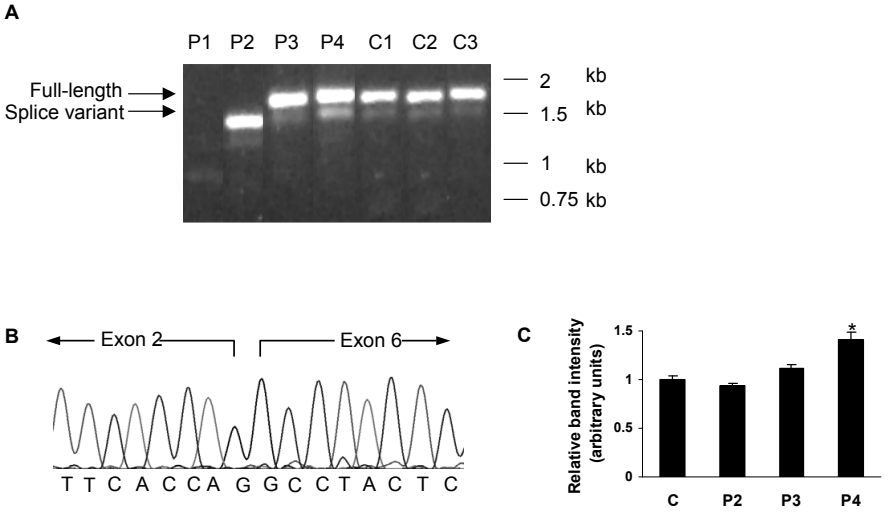

Fig. 2. MCT8 mutations at the mRNA level. (A) RT-PCR of MCT8 mRNA in skin fibroblasts of patients 1-4 (P1-4). The 1.8 kb band indicates full-length MCT8 mRNA and the 1.5 band suggests an alternative splicing variant. (B) Part of sequencing profile of cDNA derived from mRNA in patient P1 demonstrating a loss of exons 3, 4 and 5. (C) Densitometric analysis of the MCT8 splice variant (lower band) detected in controls and patients P2-4. Significance represent values obtained in patient fibroblasts compared to control fibroblasts. * $P < 0.005$.

mutant showed only slightly diminished protein expression compared to wt-MCT8 (Fig. 3A). A band of ~50 kDa was detected for the splice site variant, in agreement with the 94 aa loss. ICC was carried out to assess the cellular distribution of the F501del mutant. Figure 3B shows a clear membrane distribution of the F501del mutant, similar to wt-MCT8.

TH uptake and efflux

Fibroblasts from patients P1-4 and controls were incubated for 30 min with [^{125}I]T3. This resulted in a reduction of T3 uptake in fibroblasts from patients P1-3 by ~70% and in patient P4 by ~50% compared to controls (Fig. 4A). Uptake of [^{125}I]T4 in fibroblasts from all patients was ~40% of controls (Fig. 4B).

Subsequently, we measured uptake of ^{125}I-labeled T3 and T4 after incubation for 30 or 60 min. There was no significant difference in T4 uptake between patients and controls. However, although initial T3 uptake rates were higher in controls than in patients, the T3 uptake rate between 30 and 60 min was significantly higher in patient P4 (0.23%/mg protein/min) than in controls and patients P1-3 (0.07-0.08%/mg protein/min), indicating that the equilibrium phase has not been reached in patient P4 (Fig. 4C,D).

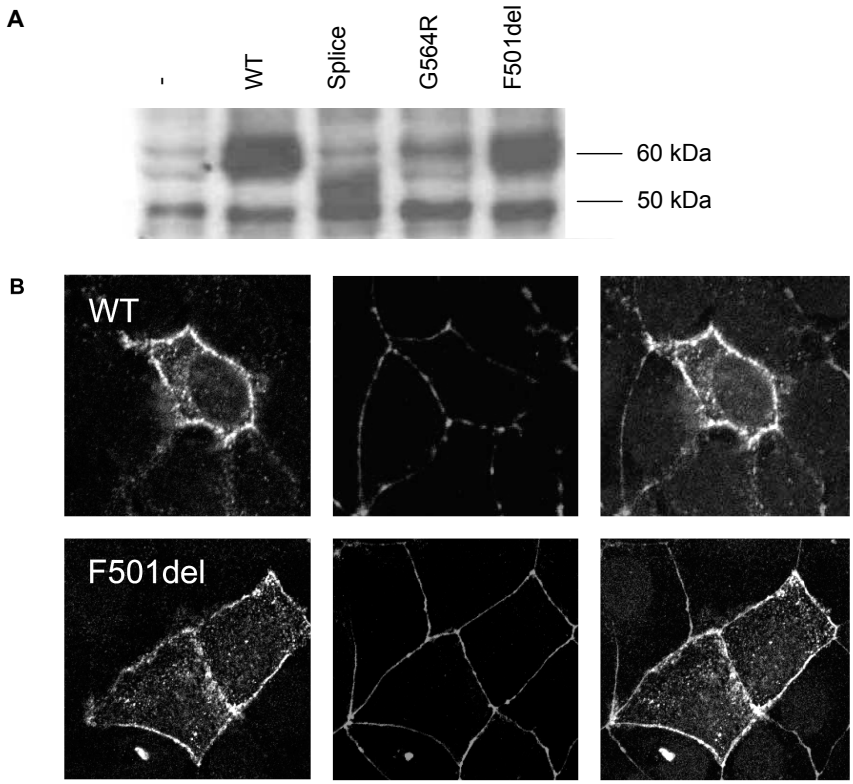

Fig. 3. MCT8 mutations at the protein level. (A) Immunoblot analysis of JEG3 cell lysates transfected with wild-type or mutant MCT8. (B) Immunofluorescent detection of JEG3 cells transfected with wt and the F501del mutation in MCT8. The plasma membrane was stained by a ZO1 antibody.

Furthermore, we tested the characteristics of the mutants in transfected JEG3 cells, providing the same cellular background for all mutants. After transfection, cells were incubated for 10 or 30 min with ^{125}I-labeled T3 or T4. No induction of T3 and T4 uptake was seen in the splice site and G564R mutants (Fig.s 4E,F). Both T3 and T4 uptake by the F501del mutant increased from ~55% to ~75% of wt-MCT8 after 10 to 30 min of incubation.

Because the above results suggest an impaired T3 efflux from fibroblasts of patient P4, we directly tested T3 efflux from fibroblasts. Figure 5A shows a higher T3 efflux rate (1.6%/mg protein/min) in controls than in patients P1-3 and P4 (both 0.6%/mg protein/min). Similarly, T4 efflux rate was higher in controls compared with patients' fibroblasts (Fig. 5B).

Figure 5C,D shows that in transfected JEG3 cells efflux of both T4 and T3 by the F501del mutant is markedly slower than with wt-MCT8.

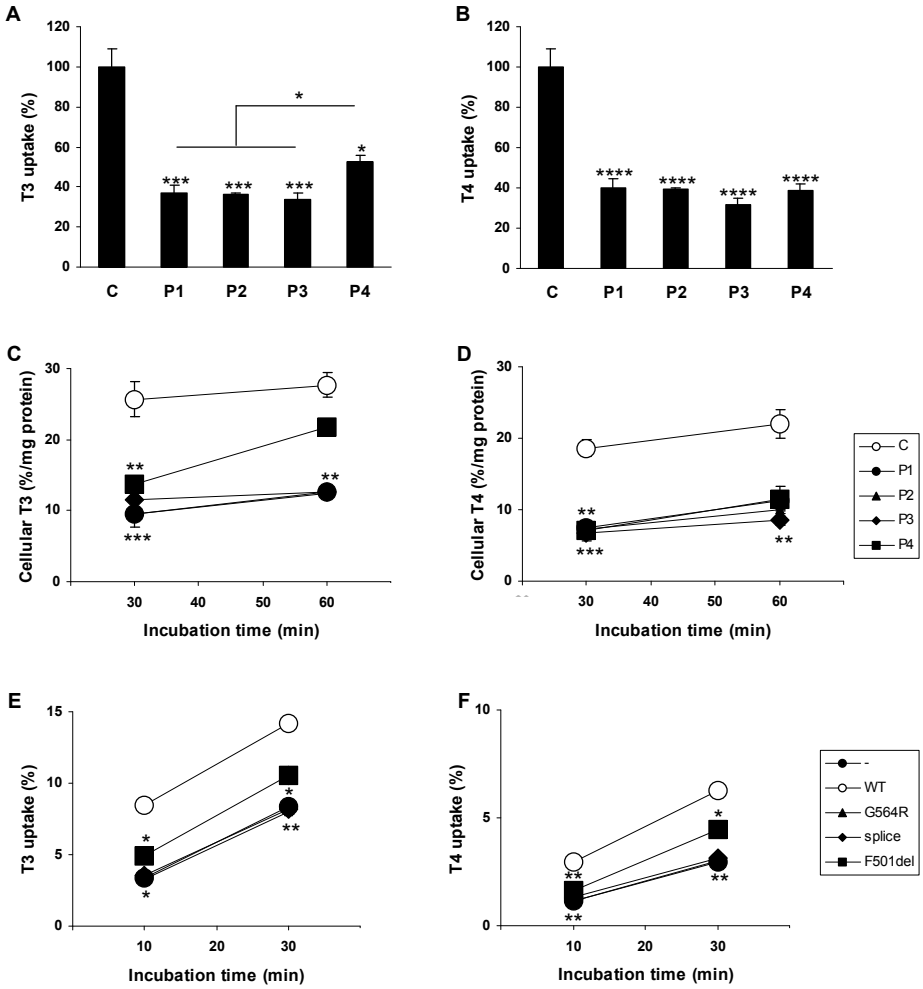

Fig. 4. Uptake of ^{125}I-labeled T3 (A) and ^{125}I-labeled T4 (B) after 30 min incubation in fibroblasts of MCT8 patients. T3 and T4 uptake in control fibroblasts is defined as 100%. Uptake of [^{125}I]T3 (C) and [^{125}I]T4 (D) after 30 and 60 min incubation in fibroblasts of MCT8 patients. Results are corrected for protein concentrations. Significances represent values obtained in patient fibroblasts compared to control fibroblasts. Uptake of [^{125}I]T3 (E) and [^{125}I]T4 (F) after 10 and 30 min incubation in wt or mutant MCT8-transfected JEG3 cells are shown as percentage of added ligand. Significances represent values of empty vector or mutant MCT8 compared to wt MCT8. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$.

In addition, we studied the metabolism of T3 in intact JEG3 cells cotransfected with wt or mutated MCT8 and human D3. Cells expressing wt-MCT8 demonstrated 22% metabolism of T3, whereas the splice site and G564R mutants produced results similar to the empty vector (Fig. 6). We observed 13% T3 metabolism in cells expressing the F501del mutant.

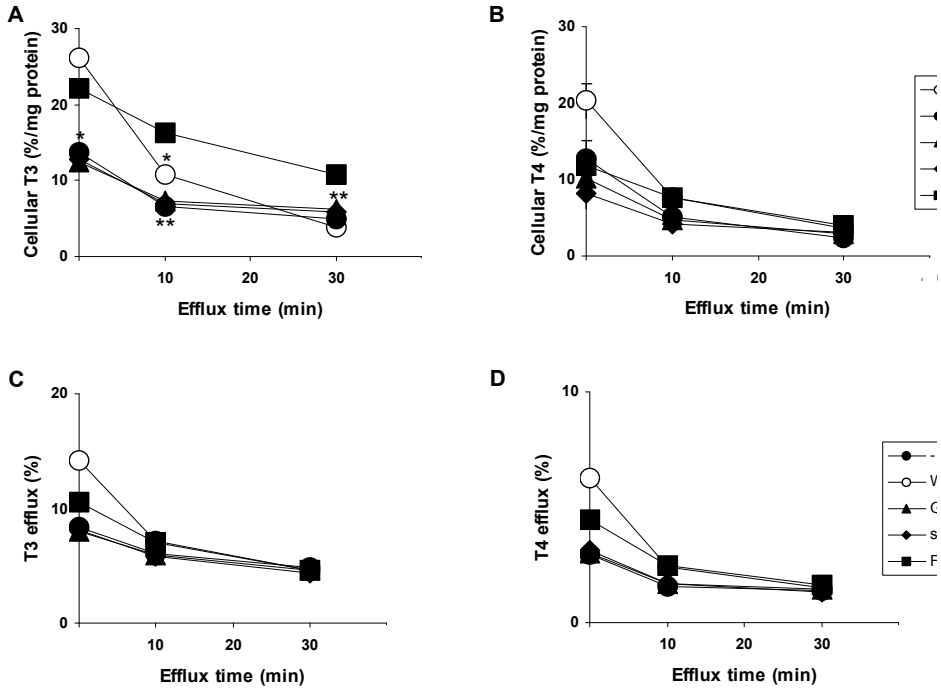

Fig. 5. Efflux of cellular T3 and T4 from fibroblasts of MCT8 patients relative to control fibroblasts measured after 10 and 30 min after a 60 min loading with [^{125}I]T3 (A) and [^{125}I]T4 (B). Results are corrected for protein concentrations. Significances represent values obtained in fibroblasts of P4 compared to controls and other patients. Efflux of cellular T3 and T4 measured after 10 and 30 min after 30 min loading with [^{125}I] T3 (C) and [^{125}I]T4 (D) in wt or mutant MCT8-transfected JEG3 cells, shown as percentage of added ligand. Significances represent values of the F501del mutant *versus* other mutants and wt MCT8. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

Deiodinase activity

The dramatic reduction in T3 uptake in fibroblasts from patients P1-3 probably results in a decreased intracellular T3 concentration. In contrast, the even larger defect in T3 efflux from fibroblasts of patient P4 may result in a greater accumulation of intracellular T3. Therefore, we assessed intracellular TH status by measuring D2 and D3 activities. However, we were not able to obtain reliable results for D2 activity in lysates of fibroblasts.

Mean D3 activity in fibroblasts from patients P1-3 was decreased by 35% compared with controls (Fig. 7). In contrast, a significant 3.8-fold increase in D3 activity was observed in fibroblasts from patient P4. Subsequently, we aimed to assess T3 metabolism in intact fibroblasts. However, even after 72 h incubation with [^{125}I]T3 we could not detect degradation of T3 (data not shown).

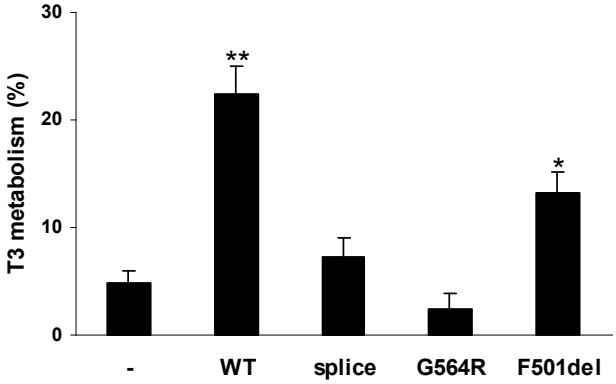

Fig. 6. Metabolism of T3 in intact JEG3 cells cotransfected with D3 and wt or mutant MCT8. Metabolism is shown as percentage of metabolites in the medium after 4 h incubation. Significances represent values obtained in (wt or mutant) MCT8 *versus* empty vector. * $P < 0.005$; ** $P < 0.001$

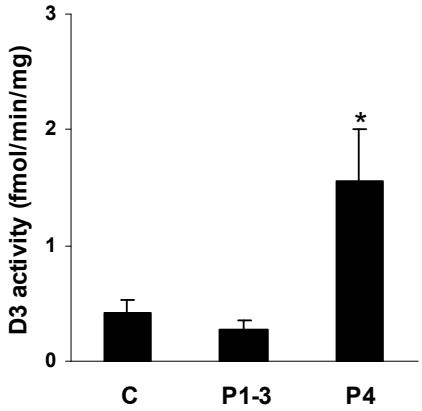

Fig. 7. Analysis of D3 enzymatic activity in lysates of fibroblasts from MCT8 patients. The means ± SE of triplicate experiments with fibroblasts from patients P1, P2 and P3 measured separately is denoted as P1-3. Significances represent values obtained in patient fibroblasts compared to control fibroblasts. * $P < 0.01$.

Analysis of T3-responsive genes in fibroblasts

We further investigated the intracellular T3 status by studying the expression of T3-responsive genes in the fibroblasts. As a first approach, we measured total RNA after a 6-h treatment with 10 nM T3. This resulted in a 38% increase in controls, a 27% increase in patients P1-3 and a 96% increase in patient P4 (Fig. 8A). We next examined the effect of 10 nM T3 on transcript levels of specific T3-responsive genes in the fibroblasts. There was a significantly higher induction of ZAKI 4α expression in fibroblasts of patient P4 than in controls and other patients (Fig. 8B). Although no significant effects of T3 on MCT4 and GLUT1 mRNA were observed, the expression of these genes tended to increase in cells from patient P4 (Fig. 8C,D).

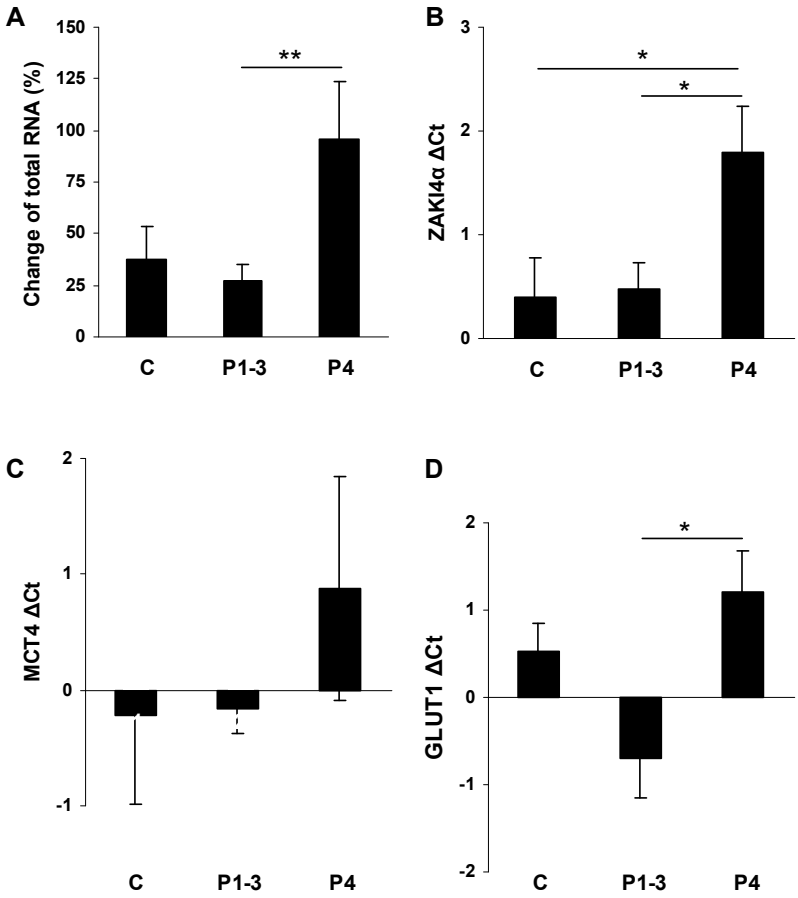

Fig. 8. Effects of incubation for 6 h with 10 nM T3 on total RNA content (A) and mRNA levels of the T3-responsive genes ZAKI 4α (B), MCT4 (C) and GLUT-1 (D). The changes of mRNA levels after T3 treatment are expressed by the ΔCt method (where ΔCt is the value obtained by subtracting the Ct value of the target mRNA from the Ct value of the house-keeping gene Cyclophilin A). The means ± SE of triplicate experiments in fibroblasts from patient P1, P2 and P3 measured separately is denoted as P1-3. Significances represent values obtained in fibroblasts of P4 compared to controls and other patients. * $P < 0.05$; ** $P < 0.01$.

DISCUSSION

We describe 3 new *MCT8* mutations in males with psychomotor retardation and abnormal TH levels. The mutation in patient P6 is the largest deletion in the *MCT8* region described until now. It is obvious that this deletion is devastating for the function of MCT8. The deletion includes the other genes *ZCCHC13* and *BMPKL2*. The possible contribution

of the deletion of these genes, which functions are currently unknown, to the clinical phenotype of patient P6 is not clear. We did not observe additional abnormalities in this patient that have not been detected in other MCT8 patients.

We observed a remarkable difference in phenotype of patients P4 and P5 *versus* patients P3 and P6 and other previously reported MCT8 patients. Although all patients have psychomotor retardation, the clinical features of patients P4 and P5 appear milder with less severe hypotonia and much better motor and communication skills. Patient P5 was even capable of reading, writing and talking. Compared to other patients, the serum TH levels are also less disturbed in patients P4 and P5. So far, no relationship has been observed between TH levels and the severity of the MCT8 syndrome (11). However, the less abnormal TH levels associated with the less severe clinical features in patients P4 and P5 may indicate that such a relationship exists. Our findings implicate that also slighty elevated T3 and normal rT3 concentrations in subjects with psychomotor retardation may be associated with MCT8 mutations. Furthermore, our findings suggest that T4 levels are already lowered at birth in MCT8 patients with a severe phenotype. It should be investigated whether neonatal T4 measurement may be used as a screening tool for earlier identification of this genetic defect.

We used fibroblasts from patients P1-4 as an *ex vivo* model to investigate the consequences of MCT8 mutations. Studying the effects of mutations on the mRNA level, the size of MCT8 mRNA in patients P3 and P4 was similar to wt-MCT8, whereas a smaller band was detected in patient P2, due to the deletion of 282 nt. In the MCT8 mRNA of patient P1, exons 3- 5 are deleted. Apparently, the donor splice site of intron 4, which is deleted in this MCT8 DNA, is needed for normal splicing of exon 5. The resultant MCT8 mRNA encodes a short out-of-frame protein.

Evidence was obtained for the existence of a MCT8 splice variant in patients P3 and P4 and in controls, which is ~0.3 kb smaller than wt-MCT8 mRNA and identical to the splice site mutant mRNA in patient P2. Interestingly, the intensity of this band was markedly increased in patient P4, suggesting that the F501del mutation gives rise to an enhanced alternative splicing. This corresponds to the location of the F501del mutation in a predicted exonic splicer enhancer (12). The pathophysiologic relevance of this finding is not clear, but it is known that alternative splicing variants may have (partial) dominant negative effects on the function of transporter proteins (13).

At the protein level, the splice site and the F501del mutant were clearly detected by IB. ICC showed a plasma membrane localization for the F501del mutant similar to wt-MCT8, indicating that the mutation in patients P4 and P5 does not hamper an adequate protein expression.

We noticed a severely diminished initial uptake of T3 and T4 in fibroblasts from all patients as compared to controls. This is in accordance with a previous report showing a markedly decreased T3 and T4 uptake in fibroblasts from two MCT8 patients (14). These

results indicate that MCT8 plays a major role in TH uptake in skin fibroblasts. Therefore, skin fibroblasts may be representative for cells which express MCT8 as the predominant TH transporter and may, thus, be a suitable tool for elucidating the cellular mechanisms in MCT8-expressing neurons involved in the pathogenesis of the MCT8 syndrome.

Cellular TH uptake increased with time in the fibroblasts of all patients, indicating the contribution of other TH transporters. Although T4 uptake was equally affected in all patients, T3 uptake was less diminished in patient P4 than in patients P1-3. When T3 uptake by fibroblasts from controls and patients P1-3 reached a plateau phase, it continued to increase in patient P4. As the equilibrium is the net result of influx and efflux, these results strongly suggested that T3 export is severely affected in P4. This was confirmed in efflux experiments, showing that the T3 efflux rate from fibroblasts of patients P4 was similar to that of patients P1-3, indicating the involvement of an endogenous export protein other than MCT8.

T3 and T4 transport by the MCT8 mutants was further tested in transfected JEG3 cells, providing the same cellular environment to all mutants. Differences in TH transport, thus, solely represent the specific characteristics of the transfected MCT8 mutants compared to wt-MCT8. The findings in the patients' fibroblasts were replicated in the transfected JEG3 cells, thereby substantiating that the affected T3 efflux in patient P4 results from the mutated MCT8 and not from an altered expression of other TH transporters in this patient. In addition, in transiently transfected cells, the F501del mutant afforded marked T3 metabolism compared to wt-MCT8, whereas both other mutants did not facilitate T3 metabolism.

Considering the different behaviour in cellular T3 transport in patient P4 compared to the other patients, it was highly interesting to study the intracellular TH status in the fibroblasts. Since D2 is negatively and D3 is positively regulated by TH (1), we measured the activity of these deiodinases. MCT8 KO mice are reported to have increased cerebral D2 activities (15, 16). In addition, there is an isolated report that D2 activity is increased in fibroblasts of MCT8 patients (14). However, we were not able to obtain reliable measurements of D2 activity, which may be due to methodological differences.

In lysates of fibroblasts, mean D3 activity was lower in patients P1-3 than in controls, whereas it was even increased in patient P4. It was not possible to detect T3 metabolism in intact skin fibroblasts, which is supported by the observation that D3 activity is much easier to detect in cell lysates than in intact cells (*Kester, M.H. and Visser, T.J., own observations*). MCT8 KO mice demonstrate diminished D3 activity in brain (16). Decreased D3 activity is thought to compensate for a decrease in cellular T3 supply. In contrast, the increased D3 activity in fibroblasts from patient P4 may represent an increased intracellular T3 availability due to more prominent decrease in the efflux than in the uptake of T3.

It is known that T3 influences transcriptional activity. Already more than 4 decades ago, Tata described an early acceleration of RNA synthesis after T3 administration in rats (17). Indeed, T3 treatment resulted in an induction of total RNA in fibroblasts. However, compared to the modest increase in controls, RNA was almost doubled in fibroblasts from patient P4, supporting the notion of a higher availability of intracellular T3. It is likely that abnormal gene expression profiles in brain resulting from altered intracellular T3 concentrations play an important role in the pathogenesis of the neurological abnormalities seen in MCT8 patients. We studied 3 genes, which are expressed in brain as well as in human skin fibroblasts, which are positively regulated by T3 (18). Although ZAKI 4α expression was up-regulated by T3 in all fibroblasts, the increase was significantly higher in patient P4 than in controls. Except for patient P4, we did not notice an increase in GLUT1 and MCT4 expression in control and patients' fibroblasts in response to T3 treatment.

The relatively short T3 incubation time, which was optimal for demonstrating differential gene regulation in patient P4, may be the explanation that GLUT1 and MCT4 expression did not increase in fibroblasts from controls and patients P1-3. Indeed, we (*Visser, W.E. et al., unpublished observations*) and others (18, 19) observed an increased expression of these genes when cells were treated with T3 for at least 24 h. The T3-mediated effects on the expression of these genes in normal brain development are currently unknown, but it is conceivable that aberrations in their regulation may have adverse effects on neurological development.

If our findings in skin fibroblasts can be extrapolated to MCT8-expressing neurons in MCT8 patients, our findings may be explained by assuming different mechanisms for the pathogenesis of the psychomotor retardation in patients P1-3 *versus* patients P4 and P5. The results in skin fibroblasts of patients P1-3, who fit the 'classical' MCT8 phenotype, are in line with the assumed function of MCT8 in neuronal T3 uptake. It is fully understandable that MCT8 mutations result in a diminished intracellular T3 concentration. Considering the crucial role of TH in normal brain development, it is conceivable that neurological defects will be the consequence of this neuronal T3 deprivation. The disturbed balance between T3 uptake and export, leads to increased intracellular T3 levels in patients P4 and P5. It is known that not only diminished, but also raised TH concentrations have harmful effects on brain development (20). It is likely that the increased D3 activity in fibroblasts of patient P4 is an adaptive response to lower toxic levels of T3.

It is thought that the initial event in the disturbed TH levels in MCT8 patients is the result of neuronal T3 deprivation (7). The decreased T3 supply to neuronally expressed D3 will result in a decreased T3 clearance. This gives rise to increased serum T3, which will stimulate renal and hepatic D1 activity and, thus, increase T3 production. This increased D1 activity may contribute to the lower T4 and rT3 serum levels. However, the serum T3 levels are also (slightly) increased in patients P4 and P5. In these patients,

the elevated intracellular T3 concentrations and increased D3 activity would result in an increased T3 clearance. Therefore, the current hypothesis concerning the disturbed serum TH levels in the MCT8 syndrome may not be correct. This is underscored by the observation that in MCT8 KO mice, which perfectly mimick the abnormal human serum TH levels, T3 clearance is not affected (15, 16). Further research is required to elucidate the initial events resulting in the disturbed TH levels in the MCT8 syndrome.

In conclusion, the data presented in this report suggest a novel mechanism involved in the pathophysiology of the neurological damage associated with human MCT8 mutations. We speculate that abnormal brain development in patients with MCT8 mutations may be the consequence of either decreased or increased intracellular T3 concentrations. Further research is required to expand insights in the pathophysiological mechanisms underlying this dramatic disease.

ACKNOWLEDGEMENTS

We thank dr. P. Rump (University Medical Center Groningen, The Netherlands) for providing information about patient P5.

SUPPLEMENTARY TABLES

Table S1. Synthetic oligonucleotides for identification of the deletion in patient P6.

Name	Primer (5'-3')
P6del1-fwd	GGGAGGTGTGTTGGTCATG
P6del1-rev	AGGTGAAGGGGAAAAAGGTG
P6del2-fwd	GCTCAGGGTTCCTTTTCCTAAC
P6del2-rev	AGGTCTCAGGTCTCCTCCATC

Table S2. Synthetic oligonucleotides of T3-responsive genes.

Gene	Sense primer (5'-3')	Antisense primer (5'-3')
ZAKI 4α	TCTTTACCAATCAGGAGGTTAAGGA	ACACTGCAAGGTCGATAAATTCTCAA
GLUT1	ACGGGTCGCCTCATGCT	GTCTGTACCCAGGTGGCG
MCT4	GATCGGCTACAGCGACACAG	GTGTTCAAGAGGTCACGGTAAC

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Chapter 6

Pathogenesis of psychomotor retardation by mutation of MCT8; insights from expression profiling of human fibroblasts

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ABSTRACT

Thyroid hormone (TH) is crucial for normal brain development. TH transporters control TH homeostasis in brain as evidenced by the complex endocrine and neurological phenotype of patients with mutations in monocarboxylate transporter 8 (MCT8).

We investigated the mechanisms of disease by analyzing gene expression profiles in fibroblasts from patients with MCT8 mutations. Studying MCT8 and its transcriptional context in different comprehensive spatiotemporal human brain transcriptome data sets revealed distinct region-specific MCT8 expression. Furthermore, MCT8 demonstrated a clear age-dependent decrease, suggesting its importance in early brain development. Performing comparative transcriptome analysis, we linked the genes differentially expressed (DE) in patient fibroblasts to the human brain transcriptome. DE genes in patient fibroblasts were strongly overrepresented among genes highly correlated with MCT8 expression in brain. Furthermore, using the same approach we identified which genes in the classical TH signaling pathway are affected in patients. Finally, we provide evidence that the TRα2 receptor variant is closely connected to MCT8.

The present study provides a molecular basis for understanding which pathways are likely affected in brains of patients with mutations in MCT8. Our data regarding a functional relationship between MCT8 and TRα2 suggest an unanticipated role for TRα2 in the (patho)physiology of TH signaling in brain. This study demonstrates how genome-wide expression data from patient-derived non-neuronal tissue related to the human brain transcriptome may be successfully employed to improve our understanding of neurological disease.

INTRODUCTION

Thyroid hormone (TH) is an important factor in for brain development and function (1). The transcriptional actions of the bioactive hormone T3 are mediated by binding to its nuclear receptors (TRα1 and TRβ, encoded by *THRA* and *THRB*, respectively) (1). Cellular action of TH requires transport across the plasma membrane, which is facilitated by plasma membrane transporters (2, 3).

Monocarboxylate transporter 8 (MCT8) is the most specific TH transporter known to date. Among different tissues it is significantly expressed in brain (4). Its biological relevance was demonstrated in humans with psychomotor retardation and disturbed serum TH levels caused by mutations in *MCT8* (5, 6).

The molecular mechanisms underlying the neurological abnormalities in patients with mutations in *MCT8* (for brevity hereafter called MCT8 patients) are unknown, because (i) knowledge of the normal spatiotemporal expression pattern of *MCT8* in human brain is largely lacking; (ii) genes downstream of MCT8 have not been identified; (iii) *Mct8 knockout* (KO) mice replicate the abnormal thyroid state of MCT8 patients, but lack neurological abnormalities and are therefore not a suitable model to unravel the pathogenesis of the psychomotor retardation in MCT8 patients (7, 8).

In an attempt to overcome these limitations, we first examined gene expression profiles of human fibroblasts from MCT8 patients and controls. Next, we assessed *MCT8* expression in brain using several comprehensive human brain transcriptome data sets. Finally, comparative analysis of the transcriptomes of fibroblasts from MCT8 patients and the human brain specified the deranged molecular signature of the disease. Furthermore, the data suggest a role of the TRα2 receptor variant in the (patho)physiology of TH signaling.

The present study illustrates how genome-wide expression data of patient-derived non-neuronal tissue related to the human brain transcriptome may be successfully employed to improve our understanding of neurological diseases.

PATIENTS AND METHODS

Cell culture

We obtained human skin fibroblasts from subjects with MCT8 mutations (patients P1-4) as described previously by punch biopsy after informed consent by the parents (9). Fibroblasts from 3 sex- and age-matched non-affected subjects were used as controls. Fibroblasts were cultured in DMEM/F12 medium (Invitrogen), supplemented with 9% FBS (heat-inactivated; Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 100 nM sodium selenite (Sigma). For the effects of T3 on gene expression in fibroblasts, culture

medium was replaced with DMEM/F12 plus 9% charcoal-treated FBS and 100 nM sodium selenite. After 24 h, medium was refreshed with the same medium containing 0, 1 or 10 nM T3, and the incubation was continued for 24h.

Microarray analysis

Microarray analysis was performed on skin fibroblasts from patients with MCT8 mutations, which have been described previously (9). Total RNA was isolated from fibroblasts grown in a 75 cm^2 flask using TRIzol reagent (Invitrogen, Carlsbad, CA) and further purified by the RNeasy isolation kit (Qiagen, Venlo, The Netherlands).

Purity and quality of isolated RNA were assessed by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA (3-5 µg) was used for subsequent production of biotinylated cRNA. Labeled cRNA was hybridized to the U133 Plus 2.0 GeneChip oligonucleotide microarray (Affymetrix, Santa Clara, CA) according to the protocol provided by the manufacturer.

To examine the quality of the various arrays, the R package affyQCreport for generating QC reports was run starting from the CEL files. All created plots, including the percentage of present calls, noise, background, and ratio of GAPDH 3′ to 5′ (<1.4) indicated a high quality of all samples and an overall comparability, except for one sample (C2_1) which was omitted from further analysis. Of the 54,674 probe sets, ~ 45% was called present in all samples. Raw intensities values of all samples were normalized by RMA normalization (Robust Multichip Analysis) (background correction and quantile normalization) using Partek version 6.4 (Partek Inc., St. Louis, MO). To visualize the clustering of the samples, PCA (Principal Component Analysis) was used. The normalized datafile was transposed and imported into OmniViz version 6.0.1 (Biowisdom, Ltd., Cambridge, UK) for further analysis.

For each probe set, the geometric mean of the hybridization intensities of all samples was calculated. The level of expression of each probe set was determined relative to this geometric mean and ^{2}log transformed. The geometric mean of the hybridization signal of all samples was used to ascribe equal weight to gene expression levels with similar relative distances to the geometric mean. Differentially expressed genes were identified using statistical analysis of microarrays (SAM analysis). Cut-offs values for significantly expressed genes were a false discovery rate (FDR) of ≤0.0003 (falsely called <1) and a fold change of 1.5. Complete data sets are available online as GEO entry GSE20538.

All samples assembled in data sets 1-3 were hybridized to Affymetrix U133 Plus 2.0 microarrays, which allowed direct comparison between all data sets. The obvious advantage of using the same array for different experiments is that it avoids loss of information which is often associated if different platforms are compared. Data set 1 consisted of 169 samples from 30 individuals representing gene expression profiles in 53 anatomically distinct human brain regions from at least three subjects. This unique data set allowed

us to investigate expression similarities and differences across different brain areas. The microarray data of data set 1 were obtained from Gene Logic Inc. (Gaithersburg, MD). Data set 2 consisted of 173 samples from 55 individuals taken from hippocampus, entorhinal cortex, superior-frontal gyrus and postcentral gyrus (10). Data set 3 consisted of 39 samples from as many individuals obtained from the dorsolateral prefrontal cortex (11). The broad age range (0.1-83 years) allowed us to specify age-related changes in gene expression. Raw data from these studies are available in the Gene Expression Omnibus as GSE11512 (data set 2) and GSE11882 (data set 3). Data set 4 was selected from samples from data set 1 which had high vs low levels of MCT8 (1.3 fold above or below the geometric mean, respectively). Furthermore, samples from subjects > 60 years of age were excluded to rule out age-related effects. Although many genes are represented by multiple probe sets and other probe sets are not fully annotated, for consistency we refer to probe sets as genes unless otherwise stated.

Functional annotation

Functional annotation was done using Ingenuity Pathway Analysis (Ingenuity, Mountain View, CA) and the web-based DAVID program (david.abcc.ncifcrf.gov; (12)).

Comparative analysis

A Pearson-based correlation metric was used to compare the probe sets of the seed genes (*MCT8*, *THRB*, *THRA1* and *THRA2*) with all other probe sets present on the chip. Per data set, probe sets which were not present in at least one sample were removed from the analysis to avoid unnecessary false-positive enrichment bias. Preliminary analysis indicated that an arbitrarily cut-off of $r \geq 0.40$ was sufficient to determine a stringent selection of interesting genes. Furthermore, for computational reasons this cut-off was optimal. Selected genes in the human brain data sets were intersected with DE genes in the fibroblasts of MCT8 patients. The ratio between expected vs. detected number of genes was calculated and a P value was calculated using a χ^2-test (or Fisher exact test if appropriate).

Overlap between the selected genes from the different brain data sets and the DE genes in MCT8 patient vs. control fibroblasts would strengthen the findings obtained from both gene expression profiling analyses. The fold enrichment and significance thereof were typically less in data set 3 than in the other data sets, suggesting that other factors also contribute to the clear age-dependent expression patterns of those genes. This approach provides the identities of genes which are likely involved in the abnormal brain development and function in MCT8 patients. Another advantage of our comparative analysis is the possibility to distinguish between genes likely involved the pathogenesis and those changing in a compensatory fashion. The genes identified to be correlated with *MCT8* (and with one of the TR isoforms) might more likely deranged

in brains from MCT8 patients, whilst other DE genes are more likely to reflect adaptive changes.

qPCR

cDNA was synthesized using 0.5 µg RNA and TaqMan RT reagent (Roche, Woerden, The Netherlands). SYBR Green I (Eurogentec, Maastricht, The Netherlands) was used as detector dye for qPCR of the DE genes AKR1C3, PTGDS, IGFBP6, PSG5, GRINA, TLR4, CCND2, HSD17B6, HES1 and TMEM16D. The primer sequences are presented in Table S11. mRNA levels are expressed relative to that of the house-keeping gene cyclophilin A. qPCR was performed in 18 out of 21 samples, because insufficient material was available.

Sequence database

Probe sets were blasted against the LIFESEQ Gold 5.1 database (Incyte Genomics) to validate the specificity of the probes.

RESULTS

Transcriptome analysis of fibroblasts from MCT8 patients and controls

To assess differences in gene expression levels per se and in different thyroid states, we performed microarray analysis on fibroblasts from MCT8 patients and controls under three different conditions. We hypothesized that T3 addition would amplify differences between patients and controls, because intracellular T3 availability is decreased in fibroblasts lacking functional MCT8. Unexpectedly, PCA analysis indicated that differences between patients and controls are much larger than differences induced by T3. The genes that differed in expression after T3 treatment between patients and controls are presented in Table S1. Validation of representative genes by qPCR is shown in Fig. S1.

As only a limited number of genes responded differently to T3 treatment in patients vs controls, we focused on the differentially expressed (DE) genes irrespective of thyroid state. Applying stringent selection criteria, we identified 2159 probe sets representing 1617 unique DE genes (Table S2). Hierarchical clustering of DE genes reveals large differences between patients and controls per se and only subtle effects of thyroid state (Fig. 1A). Well-known T3-target genes in brain such as *RELN*, *BDNF* and *PTGDS*, were markedly decreased in MCT8 patient fibroblasts and pathway analysis revealed an overrepresentation of genes in the TR/RXR pathway (data not shown). Together with our previous observations that TH uptake is impaired in fibroblasts from MCT8 patients (9), the present results suggest that fibroblasts are a suitable tool to study molecular derangements in MCT8 patients.

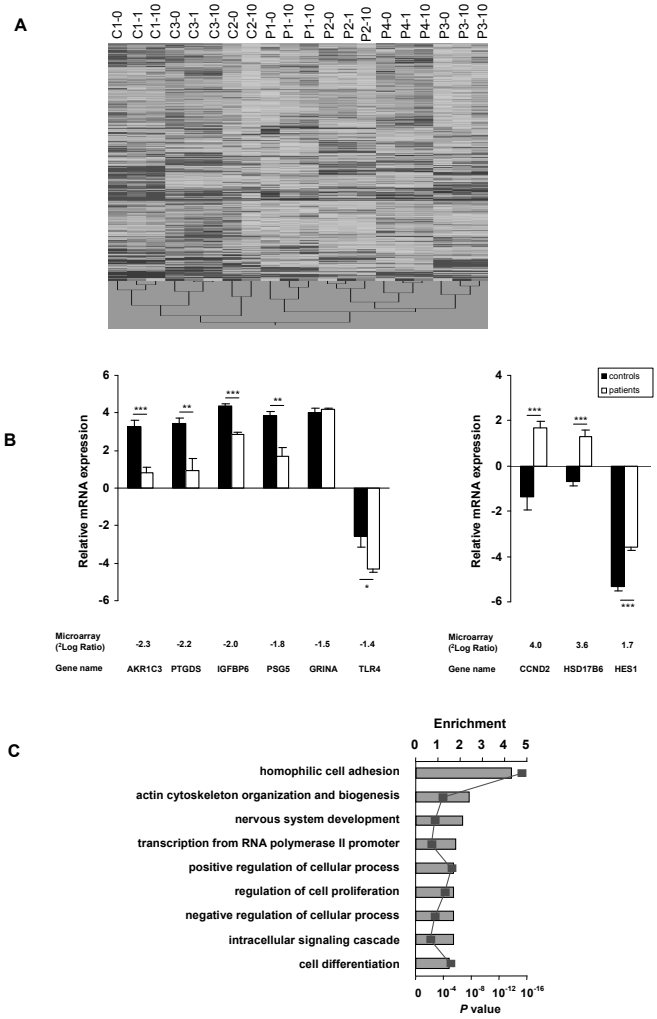

Fig. 1. Gene expression profiling in fibroblasts from patients with mutations in *MCT8* and controls. (A) Gene expression profiles of fibroblasts cultured in different thyroid states (0, 1, 10 nM T3) from patients (P) and controls (C). OmniViz Treescape showing the hierarchical clustering of genes (2159 probe sets) which matched the selection query. Gene expression levels: red, up-regulated genes compared to the geometric mean; green, down-regulated genes compared to the geometric mean. The color intensity correlates with the degree of change. (B) Verification of microarray results by qPCR. Six down-regulated (left panel) and three up-regulated (right panel) were selected from the microarray results. Results are shown as mRNA levels expressed relative to that of the house-keeping gene Cyclophilin A (ΔCt ± s.e.m.). The black bars represent controls and the white bars represent patients. Underneath the figure are the gene names and the values obtained by microarray analysis shown as ^{2}log ratio of the fold change in gene expression between patients and controls. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (C) GO enrichment analysis of DE genes in fibroblasts from MCT8 patients vs controls. Enriched GO terms for biological processes were selected from the DAVID functional annotation clustering module and corrected for multiple testing ($P < 0.01$; see Supplementary Fig. S2 and Table S3 for extensive lists). Enrichment is shown on the upper axis. P values are represented by the red squares.

Validation of DE genes by qPCR

Among the DE genes, 305 unique genes were at least twice represented with different probe sets on the microarray, strengthening the present findings. To validate the microarray results, we confirmed significant changes in expression of most of the selected genes by qPCR (Fig. 1B).

Gene ontology analysis of DE genes

Gene ontology (GO) enrichment analysis was used to functionally characterize the DE genes (12). Fig. 1C shows the most prominent biological processes identified, including specific GO categories related to cell adhesion and actin cytoskeleton processes.

Spatiotemporal MCT8 expression in human brain

Next, we assembled different microarray data sets generated from in total 381 human brain samples to explore spatial and age-dependent expression profiles of *MCT8* (for details see Supporting Information (SI) text).

Fig. 2A shows the expression pattern of *MCT8* in data set 1, revealing significant *MCT8* expression in distinct brain areas. As MCT8 appears crucial for brain development, we investigated whether *MCT8* expression changed with advancing age. Using data set 3, demonstrating distinct age-related gene clustering (Fig. S3A), *MCT8* is prominently ranked among the subset of genes with a clear age-dependent decrease in expression (Fig. S3B). *MCT8* expression rapidly declines after the first years of life (Fig. 2B). To assess the functional context of genes among which *MCT8* was expressed, we investigated which GO categories were overrepresented in genes correlated ($r \geq 0.4$) with *MCT8* in the different data sets. As might be anticipated, consistently enriched GO terms were related to neuron development, axonogenesis and cell adhesion (Table S4).

Comparative analysis of DE genes in fibroblasts with the human brain transcriptome

Then, we sought to integrate the data obtained in patients' vs control fibroblasts with the normal human brain transcriptome to define molecular pathways and specify candidate genes, which are possibly deranged in brains of MCT8 patients.

Our strategy entailed the following steps. First, to avoid potential bias, we removed from the analysis genes which were not expressed in at least one of the fibroblast samples. Second, for all remaining genes correlations with expression of *MCT8* in brain (or in principle any gene of interest) were calculated. Third, we selected genes which were correlated ($r \geq 0.4$) to *MCT8* expression. Based on the association-by-guilt principle, the resulting list was likely enriched in *MCT8*-related genes. Fourth, we intersected this list of selected genes in brain with the DE genes of fibroblasts from MCT8 patients. Fifth, the difference between the observed vs expected frequency by chance was calculated

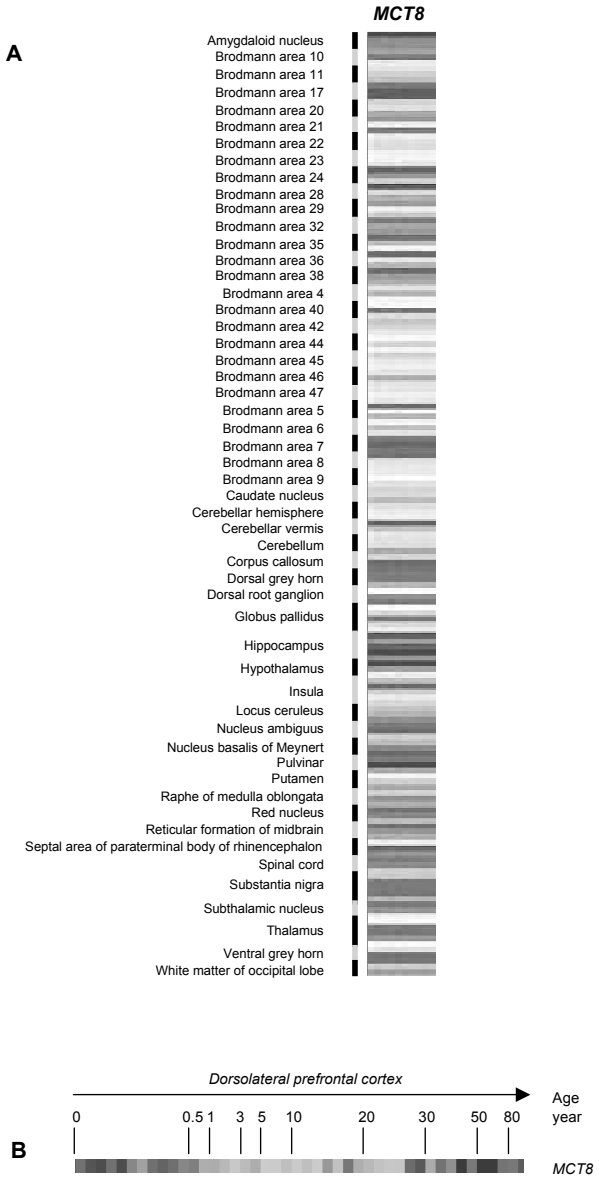

Fig. 2. Spatiotemporal expression of *MCT8* in human brain. (A) *MCT8* expression in 53 distinct brain regions from at least 3 different subjects (data set 1). High *MCT8* expression is observed in the amygdaloid nucleus, hippocampus, hypothalamus, caudate nucleus, nucleus basalis of Meynert and Brodmann areas 6, 11, 21-24, 28, 42 and 47. (B) Age-related *MCT8* expression in 39 samples of the dorsolateral prefrontal cortex from individuals ranging from 0.1-83 years of age (data set 3). For hierarchical clustering of all genes in data set 3 see Fig. S3.

red, increased expression compared to the geometric mean; green, decreased expression compared to the geometric mean. The color intensity correlates with the degree of change.

for the overlapping genes and a P value was calculated for the fold enrichment. This approach was performed in all data sets and was extended to the TRs.

Figure 3 illustrates this approach for *MCT8* in data set 1. Out of the 44,634 genes present, we selected 1172 genes which highly correlated with *MCT8* expression in brain (Fig. 3A,B). From the list of 1172 selected genes, 102 were identified among the DE genes in patients' vs control fibroblasts, representing a 2.1-fold enrichment ($P = 1.9 \times 10^{-12}$; Fig. 3C,D and Table S5). In contrast, the same approach with 1172 randomly selected genes did not result in an overrepresentation.

Thus, these findings indicate that genes which highly correlate with *MCT8* expression in human brain are more than twice as likely to be found among genes that differ between fibroblasts from MCT8 patients vs controls. This approach was validated using data set 2 (2.4-fold enrichment; $P = 4.2 \times 10^{-19}$; Table S5). Similarly, genes following the age-related pattern of *MCT8* expression in brain were overrepresented among the DE genes (data set 3; 1.5-fold enrichment; $P = 1.7 \times 10^{-4}$; Table S5).

Since *MCT8* expression varied highly among the different brain regions (Fig. 2A), background noise might obscure the number of identified genes likely associated with *MCT8*. Therefore, we selected the brain regions with the high vs low *MCT8* expression from data set 1 and excluded samples from subjects > 60 years of age to rule out age-related effects (data set 4). Theoretically, this data set should considerably increase the number and accuracy of *MCT8*-associated genes. Indeed, a total of 433 out of the 4152 selected transcripts were present among the DE genes, equaling a 3-fold enrichment ($P = 2.9 \times 10^{-67}$; Table S5). Again, categories involved in cell adhesion and actin cytoskeleton regulation were significantly overrepresented.

Then, we aimed to identify genes which are consistently correlated with *MCT8* among the different data sets and are DE in fibroblasts from MCT8 patients. Since the different data sets represent different brain areas, this approach precluded identification of brain region-specific genes. The search for *MCT8*-correlated genes in all four data sets resulted in 224 genes, of which 28 were DE in fibroblasts from MCT8 patients (2.7-fold enrichment; $P = 6.5 \times 10^{-7}$; Table 1). Interestingly, among these genes, two members of the plasticity-related gene family (*LPPR2* and *LPPR4*) are specifically expressed in neurons and have been implicated in axonal outgrowth (13).

Taken together, the approach of selecting genes in brain which correlate with *MCT8* expression and subsequent intersection with DE genes in MCT8 patient fibroblasts resulted in significant overrepresentations using all different data sets (Table 2).

Comparative transcriptome analysis links TRα1 and TRβ to MCT8

We tested the current paradigm which assumes that MCT8 transports T3 across the plasma membrane, ultimately to ensure nuclear availability of T3 for the TRs. Thus, we selected genes which expression correlated highly with both *MCT8* and one of the TR

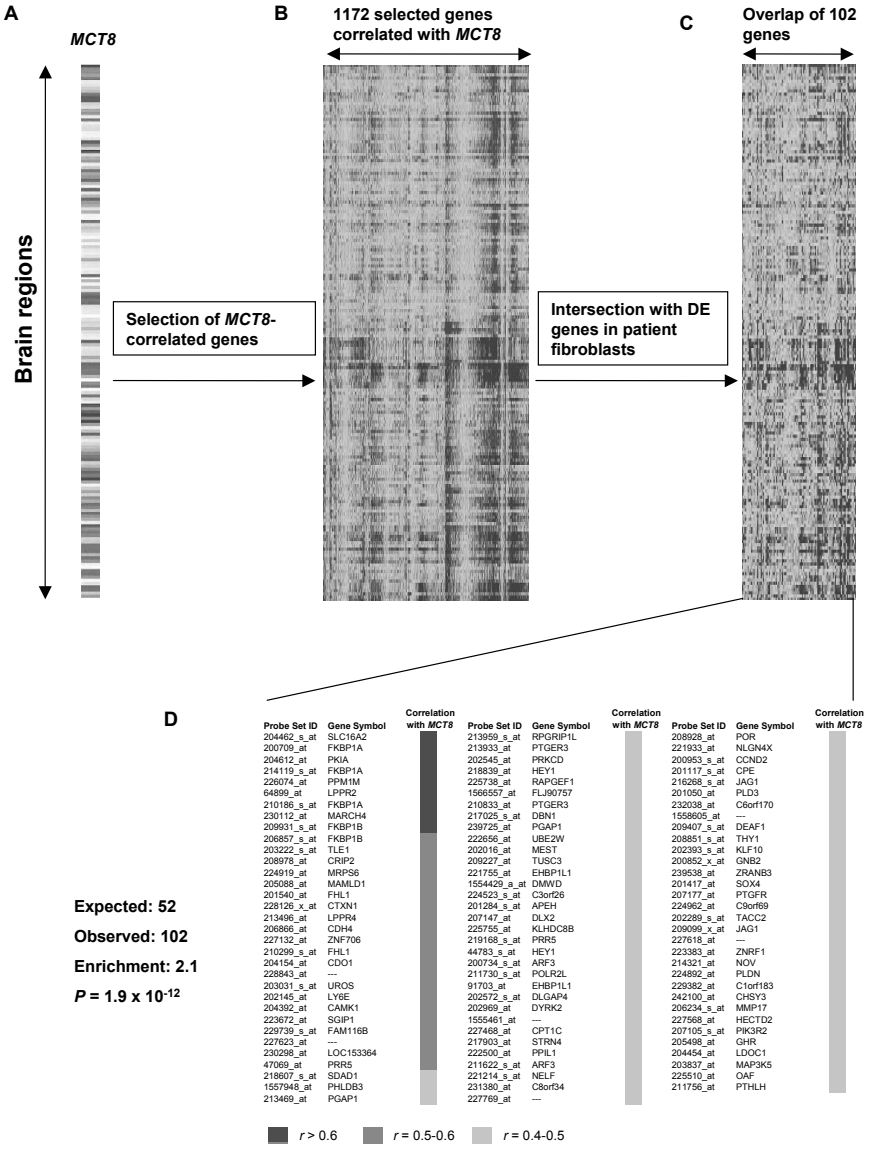

Fig. 3. Comparative analysis of DE genes in MCT8 patient fibroblasts and the human brain transcriptome. (A) Expression of *MCT8* in all 53 brain regions of data set 1 (identical to Fig. 2A). (B) Selection of genes whose expression correlates ($r \geq 0.40$) with *MCT8* expression across all brain regions (in total 1172 genes) are selected. (C) The 1172 selected genes shown in (B) are intersected with the 2159 DE genes in fibroblasts from MCT8 patients (obtained from Fig. 1A). The intersection results in 102 genes which are common to both selections. (D) Calculation of the representation of 1172 genes among the 2159 DE genes in fibroblasts from MCT8 patients indicates a strong and significant over-representation. The probe set IDs and gene symbols are shown for the 102 genes common to both selections and the degree of correlation with *MCT8* in brain (data set 1) is indicated.

Table 1. Genes consistently correlated with *MCT8* in normal human brain and DE in fibroblasts from *MCT8* patients.

Probe Set ID	Gene Symbol	Gene Title	Dataset			
			1	2	3	4
204462_s_at	SLC16A2	solute carrier family 16, member 2				
64899_at	LPPR2	lipid phosphate phosphatase-related protein type 2				
204612_at	PKIA	protein kinase (cAMP-dependent, catalytic) inhibitor alpha				
213496_at	LPPR4	plasticity related gene 1				
226074_at	PPM1M	protein phosphatase 1M (PP2C domain containing)				
227769_at	---	Transcribed locus				
210299_s_at	FHL1	four and a half LIM domains 1				
230298_at	LOC153364	similar to metallo-beta-lactamase superfamily protein				
228843_at	---	Full length insert cDNA clone YZ38E04				
231380_at	C8orf34	chromosome 8 open reading frame 34				
208928_at	POR	P450 (cytochrome) oxidoreductase				
200852_x_at	GNB2	guanine nucleotide binding protein (G protein), beta polypeptide 2				
206857_s_at	FKBP1B	FK506 binding protein 1B, 12.6 kDa				
47069_at	PRR5	proline rich 5 (renal)				
205088_at	MAMLD1	mastermind-like domain containing 1				
228126_x_at	CTXN1	cortexin 1				
239725_at	PGAP1	post-GPI attachment to proteins 1				
205110_s_at	FGF13	fibroblast growth factor 13				
209407_s_at	DEAF1	deformed epidermal autoregulatory factor 1 (Drosophila)				
201117_s_at	CPE	carboxypeptidase E				
213469_at	PGAP1	post-GPI attachment to proteins 1				
214321_at	NOV	nephroblastoma overexpressed gene				
221755_at	EHBP1L1	EH domain binding protein 1-like 1				
213933_at	PTGER3	prostaglandin E receptor 3 (subtype EP3)				
201540_at	FHL1	four and a half LIM domains 1				
1554429_a_at	DMWD	dystrophia myotonica, WD repeat containing				
44783_s_at	HEY1	hairy/enhancer-of-split related with YRPW motif 1				
207105_s_at	PIK3R2	phosphoinositide-3-kinase, regulatory subunit 2 (beta)				

isoforms. A clear clustering of different probe sets for each isoform was observed in all human brain transcriptome data sets (Fig. S4 and S5).

Since TRβ appears functional in fibroblasts (14), we started selecting genes which correlated with both *MCT8* and *THRB* expression in the human brain. These lists were

Table 2. Genes correlated with *MCT8* in normal human brain are overrepresented among DE genes of *MCT8* patients fibroblasts.

Data set	# selected genes in human brain transcriptome data sets	# overlapping genes (selected genes - DE genes in *MCT8* patients fibroblasts	Enrichment	*P* value
1	1172	102	2.1	1.9×10^{-12}
2	1207	121	2.4	4.2×10^{-19}
3	6418	413	1.5	1.7×10^{-4}
4	4152	433	2.9	2.9×10^{-67}
1+2+3+4	224	28	2.7	6.5×10^{-7}

Table 3. Genes correlated with *MCT8* and *THRB* in normal human brain are overrepresented among DE genes of *MCT8* patients fibroblasts.

Data set	# selected genes in human brain transcriptome data sets	# overlapping genes (selected genes - DE genes in *MCT8* patients fibroblasts	Enrichment	*P* value
1	713	58	1.9	2.3×10^{-6}
2	147	15	2.3	1.5×10^{-3}
3	0			
4	448	48	2.2	5.5×10^{-7}

Table 4. Genes correlated with *MCT8* and *THRA2* in normal human brain are overrepresented among DE genes of *MCT8* patients fibroblasts.

Data set	# selected genes in human brain transcriptome data sets	# overlapping genes (selected genes - DE genes in *MCT8* patients fibroblasts	Enrichment	*P* value
1	155	19	3.0	3.0×10^{-6}
2	170	22	3.1	3.1×10^{-7}
3	3530	264	1.9	9.7×10^{-15}
4	2555	332	3.5	1.6×10^{-84}

intersected with the DE genes in patient fibroblasts resulting in a ~2-fold enrichment (Table 3 and Table S6). An exception was data set 3, in which no genes passed the selection criteria, which is explained by the observation that THRB does not exhibit an age-related expression pattern similar to *MCT8* (Fig. S5). Examining those genes likely downstream of *MCT8* and TRβ revealed several known T3-responsive genes (e.g. *CCND2* and *RELN*). Furthermore, this approach suggested also many interesting candidates for

further investigation such as the LIM homeobox gene *LHX2* and *THY1* and a hypothetical protein encoded by the locus FLJ90757 (15).

Next, we intersected genes correlated with both *MCT8* and *THRA1* expression in human brain with the DE genes in patient fibroblasts. As this query resulted in only a few genes in the data sets 1-3, enrichment did not reach significance (data not shown). This could be explained if *MCT8* and TRα1 co-expression is not brain-wide, but restricted to a few distinct brain areas. Indeed, in data set 4, genes which correlated with *MCT8* and *THRA1* expression were highly over-represented among the DE genes (3.7-fold enrichment; $P = 1.6 \times 10^{-11}$ and Table S7). In addition, we performed a similar approach using a probe set which specifically detects *THRA1* mRNA with an extended 3'-UTR (Fig. S4) also resulting in a strong enrichment (11-fold; $P = 4.4 \times 10^{-23}$; Table S7).

Taken together, our results specify genes in a functional pathway putatively downstream of *MCT8* and each of the T3-binding TRs, which are dysregulated in MCT8 patients.

TRα2 appears functionally linked to MCT8

The *THRA1* and *THRA2* mRNAs encoding TRα1 and TRα2, respectively, result from differential splicing of the *THRA* gene (Fig. S4). Scrutinizing the different probe sets, we found that two probe sets specifically target the mRNA coding for TRα2 (designated as *THRA2*; Fig. S4). Blasting the sequence of these probes in an independent sequence database verified this finding. In addition, the intensities of the probe sets for *THRA2* were 3-10-fold higher ($P < 1 \times 10^{-37}$ in data sets 1-3) than for *THRA1*, which is in perfect agreement with previous findings of higher *THRA2* than *THRA1* expression in brain (16, 17). In contrast to TRα1 and TRβ, T3 does not bind to TRα2, leaving it an "orphan receptor" (18).

The first striking observation was that *THRA2* expression closely followed *MCT8* expression in human brain (Fig. S5 and Table S8). To test the hypothesis that MCT8 and TRα2 are functionally linked, we selected genes correlating with both *MCT8* and *THRA2* expression in brain. After intersection of these lists with the DE genes in patients' fibroblasts, strong and consistent enrichments (Table 5 and Table S9) were found, which is illustrated in Fig. 4. We first separately identified 4152 and 4877 genes which highly correlated with *MCT8* and *THRA2* expression, respectively. Intersection of these lists with the DE genes in patients' fibroblasts yielded 433 and 552 overlapping genes for *MCT8* and *THRA2*, respectively (Fig. 4A,B). Importantly, the large majority of genes were identical between these two lists (Fig. 4C and Table S8). Survey of this list uncovered genes important for cerebral function (e.g. *NPDC1*, *PEA-15* and *ZNF706*), but also numerous genes whose function in brain is as yet unknown (e.g. *JOPS2* and *CTXN*1). The number of DE genes that overlapped with the genes in brain selected on the *MCT8/THRA2* combination was much larger than on all other possible combinations (Fig. S6).

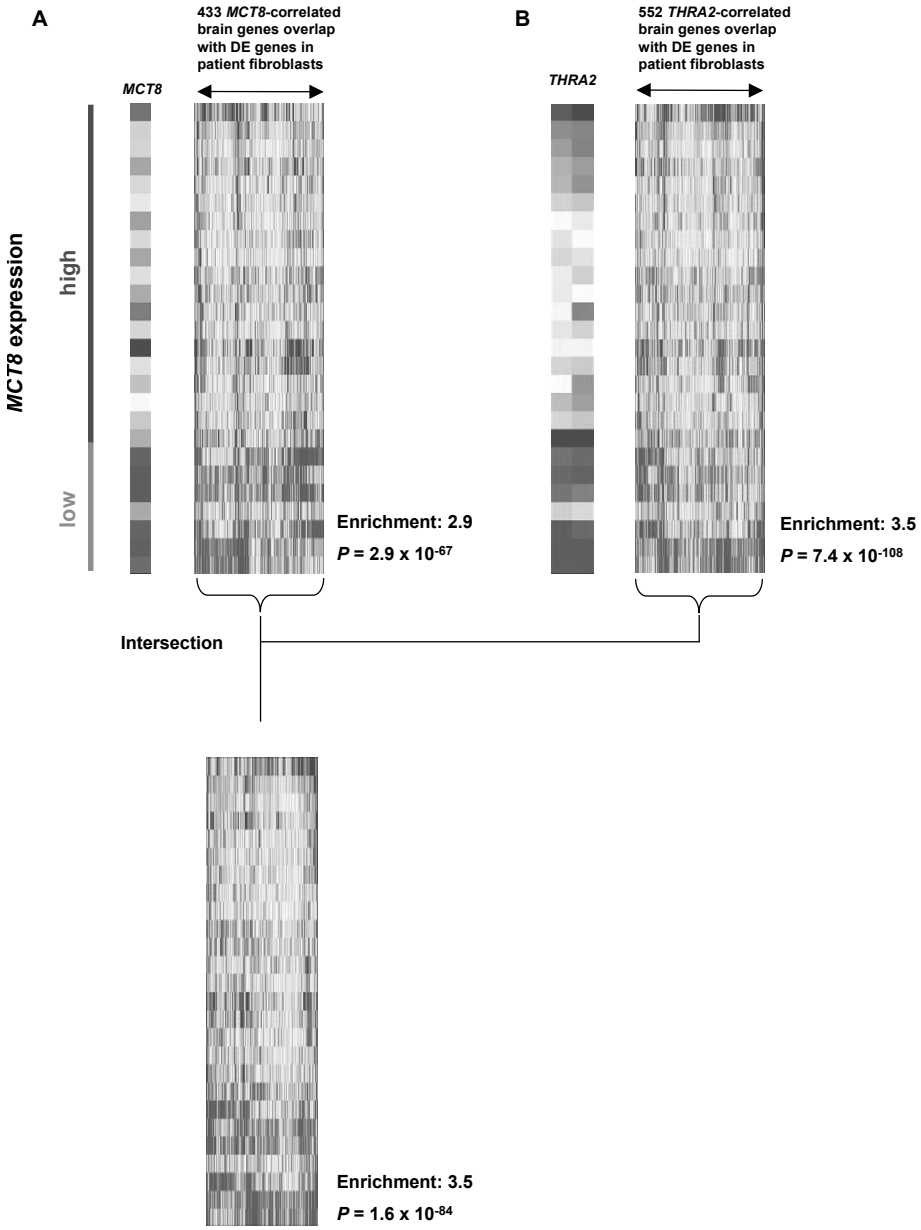

Fig. 4. Comparative analysis of DE genes in MCT8 patient fibroblasts and the human brain transcriptome (data set 4) suggests a role for TRα2. (A) Selection of genes whose expression correlates ($r \geq 0.40$) with *MCT8* expression in the human brain (data set 4) resulted in 4152 genes. Intersection with the 2159 DE genes in fibroblasts from MCT8 patients yielded 433 overlapping genes. (B) Selection of genes whose expression correlates ($r \geq 0.40$) with expression of *THRA2*, encoding the orphan receptor TRα2, in the human brain (data set 4) resulted in 4877 genes. Intersection with the 2159 DE genes in fibroblasts from MCT8 patients yielded 552 overlapping genes. (C) Intersection of the genes obtained from the analysis in (A) and (B), resulted in 332 identical genes.

As these observations point to an unanticipated role of TRa2 in (patho)physiology of TH signaling, we aimed to substantiate the robustness of our strategy. Therefore, genes whose expression changed only after T3 treatment in control fibroblasts were retrieved (Table S1, Fig. S7). This list of genes was searched for correlation with the TR isoforms in brain. As might be expected, the list of T3-responsive genes was not enriched for genes highly correlated with *THRA2* in brain. In contrast, a significant enrichment was noted for genes correlated with *THRA1* or *THRB* in multiple human brain data sets (Table S10). Furthermore, comparative analysis of T3-responsive genes in fibroblasts identified previously (14), yielded a preferential enrichment in *THRB*-correlated genes. In addition, several well-known T3-responsive genes identified in mouse brain (*HR*, *NGRN/RC3*, *BTEB* and *BDNF*) all markedly correlated with *THRA1* or *THRB* in human brain. Thus, T3-responsive genes were mapped to the T3-binding TRs in human brain, substantiating the validity of our approach. Taken together, these findings strongly suggest that MCT8 and TRa2 are closely linked and may act together in controlling specific gene sets.

DISCUSSION

Understanding the neurological phenotype in MCT8 patients is currently hampered as knowledge of *MCT8* expression in human brain is limited. A few studies reported MCT8 staining in some parts of the brain, but a systematic analysis of *MCT8* expression in human brain has not been performed yet (19-21). In the present study, we explored MCT8 mRNA expression across multiple human brain regions, using genome-wide expression profiles. The present results indicate that *MCT8* is significantly expressed in the limbic system, cortical regions involved in language function and areas important for motor control. Although confirmatory studies are needed before anatomical *MCT8* expression may be linked to the disease phenotype, the affected cognitive functions in patients are comprehensive if MCT8 is normally expressed in these regions in unaffected subjects. Species-specific cerebral patterning of MCT8 may partially explain the differences between humans and mice lacking functional MCT8 (8, 22). Furthermore, we noted that *MCT8* has a strong age-dependent expression pattern in humans, which provides a basis for the hypothesis that treatment during the first years of life may improve the neurological development of MCT8 patients (23).

In the present study, we analyzed and compared the transcriptome of fibroblasts from MCT8 patients with the transcriptome of normal human brain. Microarray studies have advanced the understanding of numerous processes in normal and diseased brain (24-28). Although conventional microarray analysis has yielded novel insights, important information may be missed. Therefore, integrating data from different sources may truly further our understanding (28). Recently, several studies have improved the understand-

ing of the complexity of human brain by the application of network analysis on gene expression profiles in different brain regions demonstrating intrinsic organizational transcription patterns (29-32). An intuitive disadvantage of analyzing gene expression in brain is that cell-type specific gene expression levels are missed. However, Oldham et al. clearly demonstrated the existence of specific modules comprised of co-expressed genes, which enabled to distinguish cell-specific gene expression signatures in bulk brain tissue (31). Correlative analysis of gene expression in brain more precisely defined specific functional units than based on classical morphological markers (33).

In many neurological disorders, especially rare syndromes, diseased tissue is not available for analysis. Therefore, genetically engineered mice are frequently used as models for disease. However, this approach is often limited by species differences in brain function. Alternatively, peripheral cells obtained from patients with neurological disease have been used to seek for abnormalities, which is limited by the uncertainty to which extent differences reflect abnormalities in brain (34, 35). Obviously, the absence of neurological abnormalities in the *Mct8* KO mice precludes their use as models for the human brain phenotype (7, 8). We aimed to overcome these difficulties by integrating and comparing the DE genes in patient cells with the human brain transcriptome. To our knowledge, the present study is the first linking a human model of neurological disease (MCT8 patients) with different large human brain transcriptome data sets from as many as 381 samples.

The current working hypothesis assumes that in MCT8 patients transport of T3 across the blood-brain barrier and/or transport of T3 into central neurons is hampered. From this it is commonly speculated that T3-target genes important for brain development are dysregulated. However, proof of this concept is currently lacking and the molecular mechanisms underlying the neurological phenotype are elusive. By using patient-derived fibroblasts, we now demonstrate that numerous genes are dysregulated in patients. We first focused on the direct effects of T3 addition to the cultured fibroblasts. Different T3 exposure levels resulted in distinct clustering of responsive genes with modest differences between patients and controls.

The modest response to T3 is partially related to the strict cut-off values used to exclude false-positives. Approximately half of the genes responding to T3 treatment in the present study were reported previously as T3 responsive in fibroblasts (e.g. *AKR1C1-3* and *RCAN2/ZAKI-4*) or in other cells and tissues, supporting the validity of our findings (9, 14, 36-39). Furthermore, 24 h T3 treatment may be insufficient to induce large changes in T3-responsive genes in fibroblasts. It is likely that fibroblasts from MCT8 patients are in a chronic hypothyroid state, which was also suggested by the enrichment of the TR/RXR pathway among DE genes irrespective of the cultured thyroid state Interestingly, we observed reduced levels of several genes which are known to be T3-responsive in brain such as *RELN*, *BDNF* and *PTGDS*. Other classical genes which are regulated by T3

in brain, such as *MBP*, *NGRN/RC3* and *HR* were very low or not expressed in fibroblasts, precluding T3-dependent regulation.

The comparative analysis with genome-wide expression data in the human brain enabled us to explore the relevance of DE genes in patient fibroblasts for brain. The list of down-regulated genes included genes that cause mental retardation when disrupted such as the *SLC6A8*, *GAMT*, *MECP2* and *NLGN4X* (40). Among the up-regulated genes in MCT8 patients, the Notch-ligand *JAG1* and Notch-signaling targets *HES1* and *HEY1* are of particular interest, because these genes are important transcriptional repressors of neurogenesis (41). Interestingly, many of these genes (e.g. *HES1*, *HEY1*, *JAG1*, *GAMT* and *NLGN4X*) are strongly correlated to *MCT8* expression in one or more of the studied brain transcriptome data sets. This specifies them as genes likely affected in brains of MCT8 patients.

An over-representation of genes related to cell adhesion and actin skeleton processes was noted among the DE genes. As these processes are intimately linked and act together in synapse formation, dysregulation of these molecules likely affects brain development (42). Furthermore, this approach identified a number of DE genes which are highly correlated with both MCT8 and the classic TRs in brain, providing evidence for the hypothesis that deficiency in MCT8 results in insufficient T3 supply to the TRs and consequent dysregulation of T3-responsive genes. Furthermore, this method suggests the concrete identities of putative T3-target genes (e.g. *CTXN1* and *DEAF1*) which likely underlie the neurological abnormalities as examples for future studies.

The most unexpected observation was the strong link between MCT8 and the TRα2 receptor variant. In contrast to TRα1 and TRβ, it has been established that TRα2 does not bind T3 (17, 43). TRα2 is able to inhibit effects mediated by either TRα1 or TRβ, but it is unknown whether this is a biologically relevant mechanism (43, 44). There are several indications to believe that TRα2 has physiological relevance beyond this antagonistic mechanism of action. First, TRα2 has a ligand-binding domain. Although it does not bind T3, this does not preclude binding of other ligands to TRα2 which could thus be regarded as an orphan receptor. Second, expression of TRα2 is roughly 10-fold higher than TRα1 in brain, whereas this ratio is lower in other tissues (16). The divergent expression pattern between cerebral TRα1 and TRα2 may hint at a specific role for TRα2 in brain.

The present results add several lines of evidence suggesting a direct role of TRα2 in TH signaling in brain. First, the expression patterns of *THRA2* and *MCT8* were highly similar in the human brain transcriptome. Second, the functional link between MCT8 and TRα2 was suggested by our comparative analysis which demonstrated that much more DE genes in patient fibroblasts were among the selection of genes in human brain correlated with the combination *MCT8/THRA2* than with the combinations *MCT8/THRA1* or *MCT8/THRB*. The scenario in which TRα2 antagonizes TRα1 or TRβ function does not

explain the present results. Rather less than more DE genes would be expected to overlap with the selected genes in brain correlating with the combination *MCT8/THRA2*.

We like to emphasize that T3-responsive genes among the DE genes in patient fibroblasts clearly overlapped with the selected genes in brain correlating with the combination *MCT8/THRA1* or *MCT8/THRB*, but not with *MCT8/THRA2*. These data are perfectly in line with the current dogma of T3 binding to the classic TRs and provide further confidence in our analysis method. What could be the functional relationship between MCT8 and TRα2? It may be speculated that MCT8 transports a ligand across the plasma membrane, which subsequently binds to TRα2, thereby controlling gene transcription. Taken together, our findings indicate a novel role for TRα2 in TH signaling in the brain, independent of the actions mediated by the T3-receptors.

In conclusion, the present study demonstrates for the first time which genes are differentially expressed in cells derived from patients with mutations in *MCT8*. Furthermore, we analyzed expression of *MCT8* and its transcriptional context in several comprehensive data sets of the human brain transcriptome. Comparative analysis of the transcriptome of cells from MCT8 patients with the human brain transcriptome enabled us to identify genes which are putatively dysregulated in brains of MCT8 patients. Our approach indicated a functional relationship between MCT8 and TRα2. This may have not only implications for the understanding of the disease, but also suggests an important role for the "orphan receptor" TRα2 and its relevance for TH signaling in brain. Finally, this study exemplifies how genome-wide expression data from patient-derived non-neuronal tissue related to the human brain transcriptome may be successfully employed to improve our understanding of neurological diseases.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY TABLES AND FIGURES

Supplementary Figures: see next pages.

Supplementary Tables S1-S12 are available upon request.

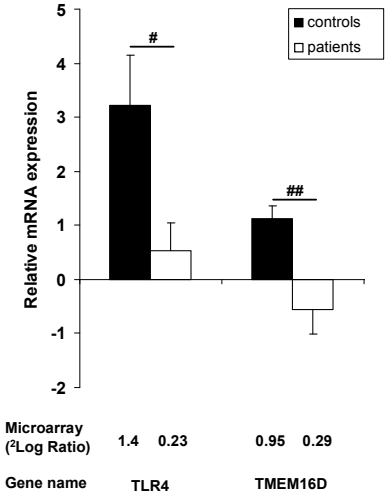

Fig. S1. Verification of microarray results by qPCR. Genes which responded differently to T3 treatment were confirmed by qPCR. Results are shown as mRNA levels expressed relative to that of the house-keeping gene Cyclophilin A (ΔCt ± s.e.m.). The black bars represent controls and the white bars represent patients. Underneath the figure are the gene names and the values obtained by microarray analysis shown as 2log ratio of the fold change in gene expression between patients and controls. # $P = 0.07$; ## $P = 0.08$

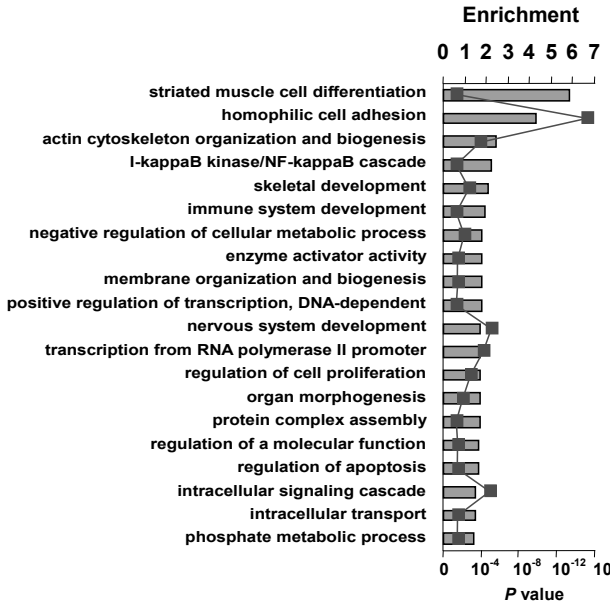

Fig. S2. GO enrichment analysis of DE genes in fibroblasts from *MCT8* patients *vs* controls. Enriched GO terms for biological processes are selected from the DAVID functional annotation clustering module and corrected for multiple testing ($P < 0.01$). See Table S3 for extensive lists. Enrichment is shown on the upper axis. *P* values are represented by the red squares.

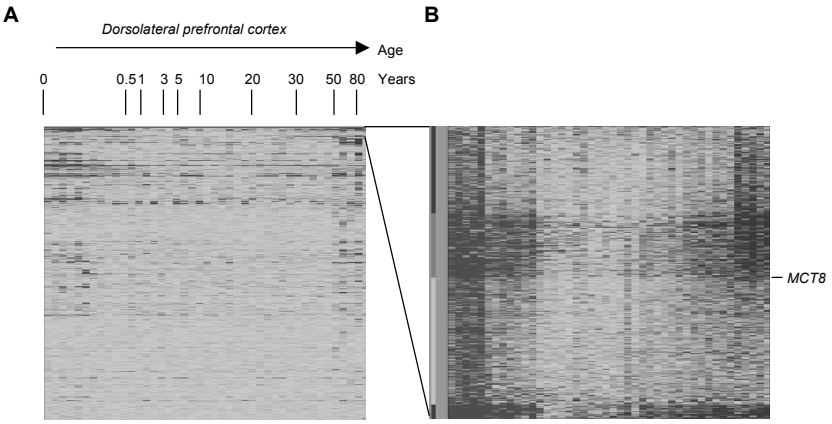

Fig. S3. OmniViz Treescape shows the hierarchical clustering of all Affymetrix probe sets in data set 3. Distinct age-related gene sets are clustered (Fig. S3A). MCT8 is among the 4 clusters showing the most pronounced high-to-low transition of expression (Fig. S3B). MCT8 was categorized among the most significantly downregulated genes with advancing age (3.1-fold decrease in 10 individuals aged > 30 yr *vs* 10 individuals aged < 0.5 yr).

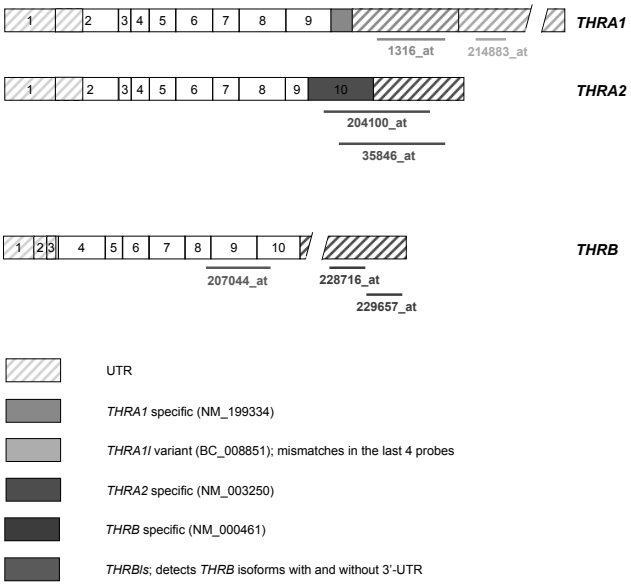

Fig. S4. Specific probe sets for different isoforms of the thyroid hormone receptor. All individual probes of the probe sets were aligned with the BLAST programm of the NCBI. Results were verified in an independent sequence database (LifeSeq Gold 5.1). Probe set ID 1316_at was specific for *THRA1*, encoding TRα1. Probe set ID 214483 specifically detects a *THRA1* variant, which we designated as *THRA1I*. This variant is characterized by a extended 3'-UTR. Analysis of this probe set was complicated by mismatches in the last 4 probes. Probe set IDs 204100_at and 35846_at specifically recognized *THRA2*, encoding TRα2. The probe sets for *THRA2* did not align to THRA1 and vice versa. Probe sets IDs 228716_at and 229657_at are located in 3'-UTR and specific for *THRB*, which encodes TRβ. Probe set ID 207044_at is located in exons 8, 9 and 10 of the *THRB* gene and, therefore, recognize variants with different 3'-UTRs lengths.

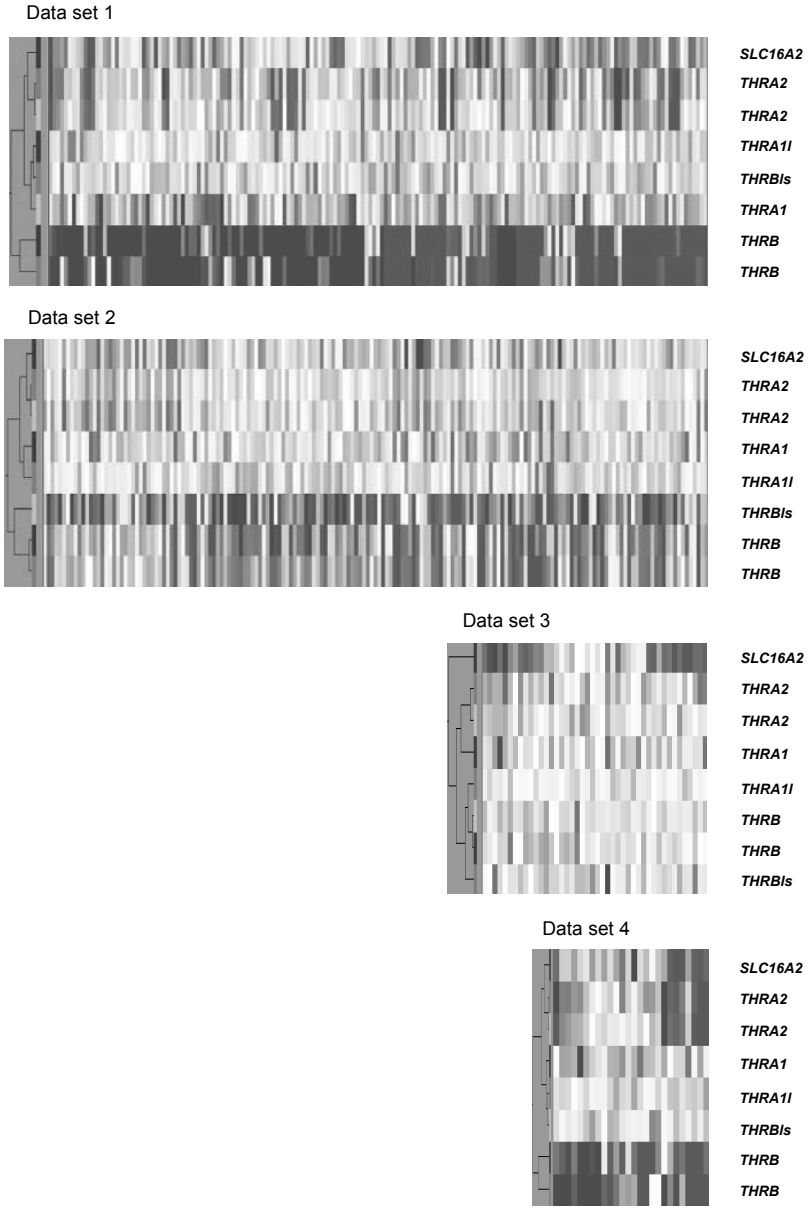

Fig. S5. Hierarchical clustering of *SLC16A2* (*MCT8*) and the various TR isoforms. *MCT8* strongly clusters with *THRA2* in all data sets.

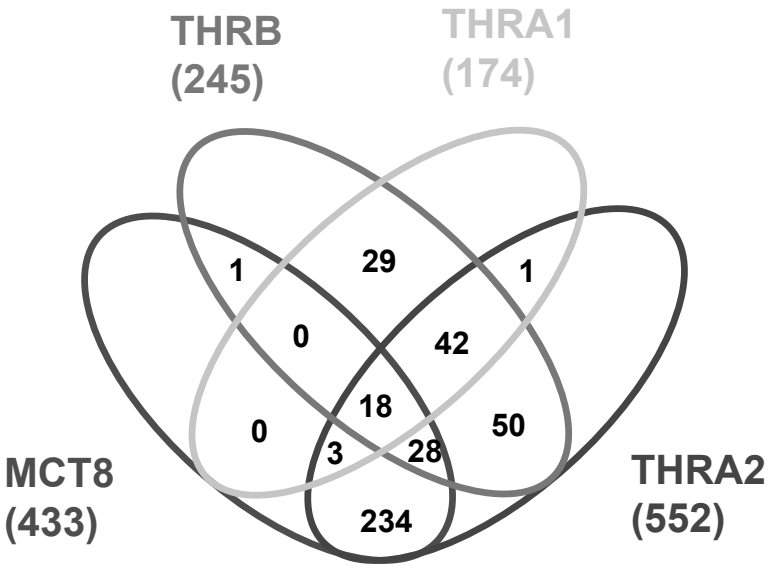

Fig. S6. Venn diagrams representing the overlap after intersection of genes correlated with all combinations of *MCT8*, *THRA1*, *THRA2* and *THRB* in data set 4 and DE genes in fibroblasts from *MCT8* patients. Numbers in similar colors represent similar genes, indicating that an additional correlation filter does not reduce the number of genes.

Fig. S7. Hierarchical clustering of T3-responsive genes in fibroblasts from 3 control subjects. These genes were used for the comparative analysis to search for overlap with genes correlated with the TR isoforms in the human brain transcriptome.

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Chapter 7

Study of the transport of thyroid hormone by transporters of the SLC10 family

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ABSTRACT

Transport of (sulfated) iodothyronines across the plasma membrane is required for their intracellular metabolism. Rat Na^+/taurocholate cotransporting polypeptide (Ntcp; Slc10a1) has been identified as an important transporter protein. We demonstrate that among the 7 members of the solute carrier family SLC10, only human SLC10A1 mediates sodium-dependent transport of the iodothyronine T4 and iodothyronine sulfates T3S and T4S. In contrast to SLC10A2-7, cells co-expressing SLC10A1 and the deiodinase D1 demonstrate a dramatic increase in T3S and T4S metabolism. The SLC10A1 substrates taurocholate, DHEAS and E3S inhibit T3S and T4S transport. Furthermore, co-transfection of SLC10A1 with CRYM, a well-known intracellular iodothyronine-binding protein, results in an enhanced intracellular accumulation of T3S and T4S, indicating that CRYM binds iodothyronine sulfates.

The present findings indicate that the liver-specific transporter SLC10A1 transports (sulfated) iodothyronines, thereby increasing their intracellular availability. Therefore, SLC10A1 may fulfill a critical step in providing liver D1 with iodothyronine sulfates for rapid degradation.

INTRODUCTION

Thyroid hormone (TH) is essential for numerous metabolic processes. A principal regulatory role to achieve appropriate intracellular TH concentrations is reserved for the 3 deiodinating enzymes. The deiodinases D1 and D2 activate TH by converting the prohormone T4 to T3, whereas D3 has an inactivating function by deiodination of T4 and T3 to transcriptionally inactive rT3 and 3,3'-T2, respectively (1). Although deiodination is regarded as the major pathway for TH metabolism, a number of alternate pathways have physiological relevance (2). Sulfoconjugation is catalyzed by sulfotransferases, which transfer a sulfate group to the phenolic ring of iodothyronines. Although the thyromimetic activity of iodothyronine sulfates is limited, these compounds may have biological relevance (3, 4). It is known that sulfation accelerates degradation of the iodothyronines by D1 (5). On the other hand, biologically inactive sulfated TH may serve as a pool contributing to the availability of TH, because, for example, bioactive T3 may be recovered from T3S by sulfatases in selective tissues (2).

Deiodination of iodothyronines and their sulfates takes place intracellularly. Therefore, transport of (sulfated) iodothyronines across the plasma membrane, which is facilitated by transporter proteins, is required (6). However, only a few proteins are known to be active and specific iodothyronine transporters (6). Previously, we found that *Xenopus laevis* oocytes injected with cRNA coding for rat Na^+/taurocholate cotransporting polypeptide (Ntcp), currently known as Slc10a1, facilitated uptake of iodothyronines and their sulfates (7). In addition to the known human ileal apical sodium-dependent bile acid transporter (ASBT; SLC10A2), five new members of the SLC10 family were identified recently (8-10). However, SLC10A3, 4, 5 and 7 are orphan transporters for which no substrates have been identified.

For several TH transporters, it has been shown that different members of the same transporter family, facilitate TH transport, such as monocarboxylate transporter (MCT) 8 and MCT10 of the MCT family, and multiple members of the organic anion transporting polypeptide (OATP) family, including OATP1A2, 1C1, 1B1 and 1B3 (11-14). Therefore, in the present study we investigate in transfected HEK293 and COS1 cells which members of the SLC10 family, in addition to SLC10A1, may transport (sulfated) iodothyronines, whether or not in addition to already known substrates.

MATERIALS AND METHODS

Materials

[^{125}I]T3, [^{125}I]T4, [^{125}I]T3S and [^{125}I]T4S were synthesized as described (15). [^{3}H]taurocholate ([^{3}H]TC) and [^{3}H]estrone-1-sulfate ([^{3}H]E1S) were purchased from Perkin Elmer (Boston,

MA). Unlabeled iodothyronines were obtained from Henning (Berlin, Germany). All other compounds were purchased from Sigma (St. Louis, MO).

Cloning

Human (h) SLC10A1 (kindly provided by prof. dr. P.J. Meier, Zurich, Switzerland), hSLC10A2 (obtained from Open Biosystems, Huntsville, AL) and hSLC10A3 (kindly provided by dr. T. Abe, Japan) were subcloned in pcDNA3 (Invitrogen, Carlsbad, CA). The coding regions of rat (r) Slc10a4 and hSLC10A5-7 were cloned in pcDNA5 (Invitrogen, Carlsbad, CA) as described (9, 10, 16, 17). rSlc10a4 was used as the hSLC10A4 clone was not available at the time of our experiments. Human μ-crystallin (hCRYM) was cloned in the pSG5 vector (Stratagene, La Jolla, CA) (12) and rD1 in pcDNA3 (18).

Transport studies

For transport experiments equal numbers of HEK293 and COS1 cells were seeded in 6-well culture dishes in DMEM/F12 medium supplemented with 9% heat-inactivated FBS and 100 nM sodium selenite. This results in very similar amounts of cellular protein as determined by a standard Bradford assay. At 75% confluency, cells were transfected with 500 ng pcDNA3.hSLC10A1-3, pcDNA5.rSlc10a4, or pcDNA5.hSLC10A5-7 alone or co-transfected with 500 ng pSG5.hCRYM. 48 h after transfection, cells were washed with incubation medium (Dulbecco's PBS containing 0.1% D-glucose with or without 0.1% BSA). Uptake was tested by incubation of the cells for 5-30 min at 37°C with 1 nM ($1x10^5$ cpm) [^{125}I]T3, [^{125}I]T4, [^{125}I]T3S or [^{125}I]T4S in 1.5 ml incubation medium, in the absence or presence of taurocholate (TC), dehydroepiandrosterone sulfate (DHEAS) or estrone-3-sulfate (E3S). After incubation, cells were washed with incubation medium, lyzed with 0.1 M NaOH, and counted in a gamma counter. Uptake of [^{3}H]TC and [^{3}H]E1S by the SLC10 carriers was tested in a similar manner by incubation of cells for 30 min with 1 μM [^{3}H]TC or 0.1 μM [^{3}H]E1S in 1.5 ml incubation medium. Cells were lyzed with 0.1% SDS and subsequently counted for radioactivity.

For measurement of efflux, cells were loaded for 30 min with 1.5 ml incubation medium containing 1 nM ($1x10^5$ cpm) [^{125}I]T3S or [^{125}I]T4S. After removal of the medium, cells were briefly washed and incubated for 5-30 min with incubation medium (containing 1% BSA) without ligand. Finally, medium was removed, and cells were washed with incubation medium, lyzed with 0.1 M NaOH and counted in a gamma counter.

To evaluate Na^+-dependence of transport, 1 nM [^{125}I]T4, [^{125}I]T3S, [^{125}I]T4S or 1 μM [^{3}H]TC were incubated with the cells in a medium containing 142.9 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 20 mM HEPES and 0.1% BSA (pH 7.4) or in medium where Na^+ was replaced with an equimolar amount of choline.

Metabolism studies

COS1 cells were cultured in 24-well culture dishes and transfected with 100 ng pcDNA3.rD1 and 100 ng pcDNA3, pcDNA3.hSLC10A1-3, pcDNA5.rSlc10a4 or pcDNA5.hSLC10A5-7. After 48 h transfection, cells were incubated for 24 h at 37°C with 1 nM ($5x10^5$ cpm) [^{125}I]T3S or [^{125}I]T4S in 0.25 ml DMEM/F12. After incubation, medium was harvested and analyzed by HPLC as described previously (18).

Statistical analysis

Results are presented as the means and ranges of duplicate determinations in a representative experiment (n=2-4). Statistical significance was determined using the Student's *t* test.

RESULTS

Previously, HEK293 cells as well as COS1 cells have been used to study various members of the SLC10 family (9, 10, 16, 17, 19). Therefore, we used both cell lines in our experiments. We first assessed uptake of (sulfated) iodothyronines in HEK293 cells. No induction of T3 uptake was observed in cells transfected with any of the SLC10 transporters (Fig. 1A and *data not shown*), whereas an insignificant increase in T4 uptake was seen if SLC10A1 was expressed (Fig. 1B). T3S and T4S uptake was largely induced by SLC10A1 in contrast to the other SLC10 transporters (Fig. 1C,D and *data not shown*). Figure 1E shows a clear time-dependent increase in the uptake of T3S and T4S in cells expressing SLC10A1. As transporters may facilitate not only substrate uptake but also efflux, we investigated whether SLC10A1 mediates T3S and T4S export. No difference in efflux of T3S and T4S was observed between cells expressing low *versus* high levels of SLC10A1 (Fig. 1F,G), suggesting that SLC10A1 does not facilitate the efflux of these substrates. Re-uptake of T3S and T4S does not play a major role, as 1% BSA in the efflux medium efficiently blocks uptake of sulfated iodothyronines (*data not shown*). We confirmed that SLC10A1 transports TC in a Na^+-dependent manner (*data not shown*). As anticipated, uptake of T4 (*data not shown*), T3S (Fig. 1H) and T4S (Fig. 1I) is also a Na^+-dependent process.

Because HEK293 cells demonstrate a considerable endogenous transport activity of (sulfated) iodothyronines, we also tested transfected COS1 cells for uptake of different ligands. To confirm that COS1 cells transfected with cDNA coding for each of the SLC10 family members are appropriate to study transport function, uptake was studied of TC, a known substrate for SLC10A1 and 2. Fig. 2 shows a 69-fold and 6-fold increase of TC uptake in cells transfected with SLC10A1 and 2, respectively, whereas the other SLC10 members did not demonstrate any transport activity. Furthermore, E1S was only transported by SLC10A1 and 6 (*data not shown*). These results agree with the existing

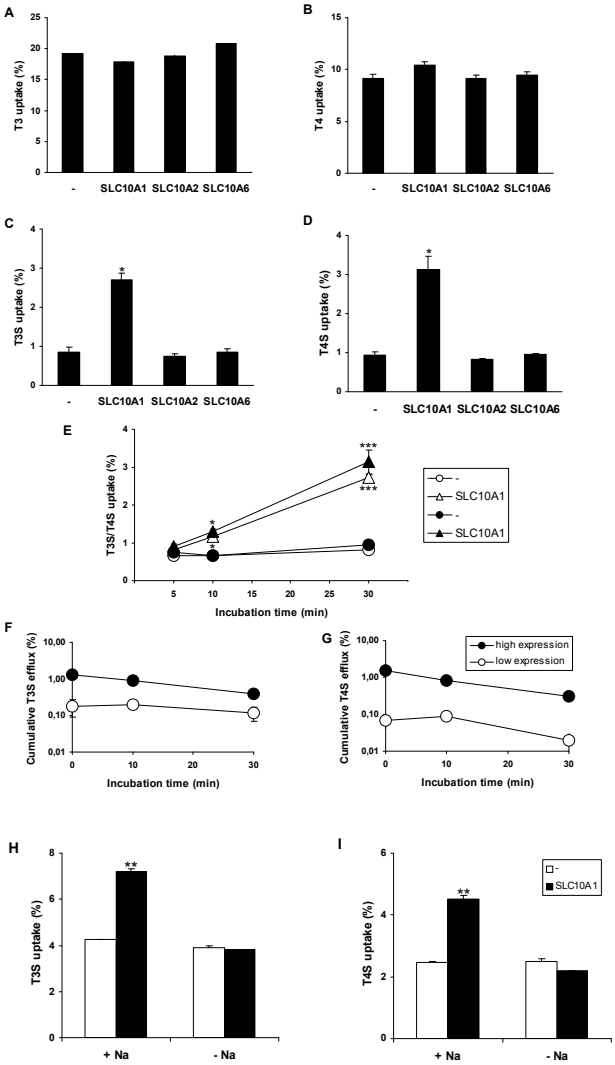

Fig. 1. Uptake of (sulfated) iodothyronines in HEK293 cells transfected with hCRYM and hSLC10A1, hSLC10A2 and hSLC10A6. Uptake was measured after 30 min incubation with 1 nM T3 (A), T4 (B), T3S (C) or T4S (D) in D-PBS containing 0.1% glucose and 0.1% BSA. (E) Uptake of T3S or T4S in HEK293 cells co-transfected with hCRYM and empty vector (circles) or hSLC10A1 (triangles). Uptake was measured after 5, 10 and 30 min incubation with 1 nM T3S (empty symbols) or T4S (filled symbols) in D-PBS containing 0.1% glucose and 0.1% BSA. 1% uptake represents 10 pmol of incubated substrate. Cellular protein amounts to ~3 mg in all wells. Effects of high (500 ng - filled circles) versus low (100 ng - open circles) expression of SLC10A1 on the efflux of T3S (F) and T4S (G). Cells were incubated for 30 min with 1 nM T3S or T4S in D-PBS containing 0.1% glucose and 0.1% BSA. After brief washing, cells were incubated (10-30 min) with efflux medium, containing 1% BSA, and subsequently processed as described in Materials and Methods. Results (mean ± range) are corrected for uptake in control-transfected cells. Treated as a first order process, the plot of log cellular T3S or T4S *vs* time is linear, providing efflux rate constants which are independent of the amount of SLC10A1 expressed. Na^+-dependence of the SLC10A1-mediated uptake of T3S (H) and T4S (I). Cells were incubated for 30 min with 1 nM T3S or T4S in sodium chloride medium or sodium-free choline medium. Significances represent mean ± range obtained in cells expressing a SLC10 transporter *versus* empty vector. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

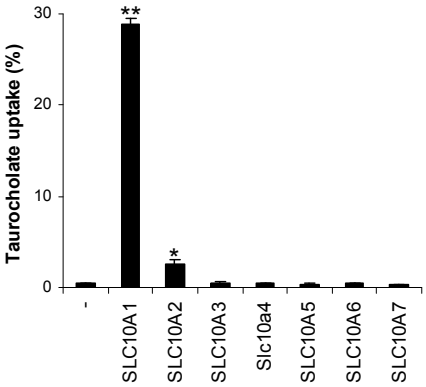

Fig. 2. Uptake of 1 µM TC in COS1 cells transfected with cDNA coding for each of the SLC10 family members. Cells transfected with empty vector were used as controls. Uptake was measured after 30 min TC incubation in D-PBS containing 0.1% glucose and 0.1% BSA. 1% uptake represents uptake of 10 nmol taurocholate. Cellular protein amounts to ~3 mg in all wells. * $P < 0.05$; ** $P < 0.001$.

evidence, indicating that this cell expression system is appropriate to study transporter characteristics. Because functional expression can only be demonstrated for SLC10A1, 2 and 6, transport studies are only shown for these transporters.

Then, we examined the uptake of the iodothyronines T3 and T4 by transfected COS cells. Fig. 3A,B clearly shows that only SLC10A1 is modestly capable of transporting T4, whereas T3 is not transported by any of the SLC10 proteins. In contrast to the unconjugated iodothyronines, the T3S and T4S cellular uptake is largely stimulated if SLC10A1 is expressed (Fig. 3C,D). The induction of uptake is more pronounced if cells are co-transfected with CRYM, a cytosolic binding protein with high affinity for iodothyronines. In contrast to SLC10A1, SLC10A2-7 do not transport the tested iodothyronine sulfates (Fig. 3C,D and *data not shown*).

To assess whether SLC10A1-mediated transport of iodothyronine sulfates facilitates their intracellular metabolism by D1, cells were co-transfected with SLC10A1-7 and D1. Subsequently, cells were incubated for 24 h with T3S or T4S and medium radioactivity was analyzed. Intracellular T3S and T4S metabolism dramatically increased in cells co-expressing SLC10A1 and D1 (Fig. 3E). In contrast, in cells co-expressing any of the other SLC10 proteins with D1, negligible metabolite production was seen. In the analyzed medium, only the substrate (T3S or T4S) or I^- was observed, but no intermediate metabolites.

In an attempt to calculate kinetic parameters for T3S and T4S uptake by SLC10A1, we incubated cells transfected with SLC10A1 with 0.01-5 µM of each substrate. No clear saturation was observed for T3S and T4S uptake at concentrations of up to 5 µM (*data*

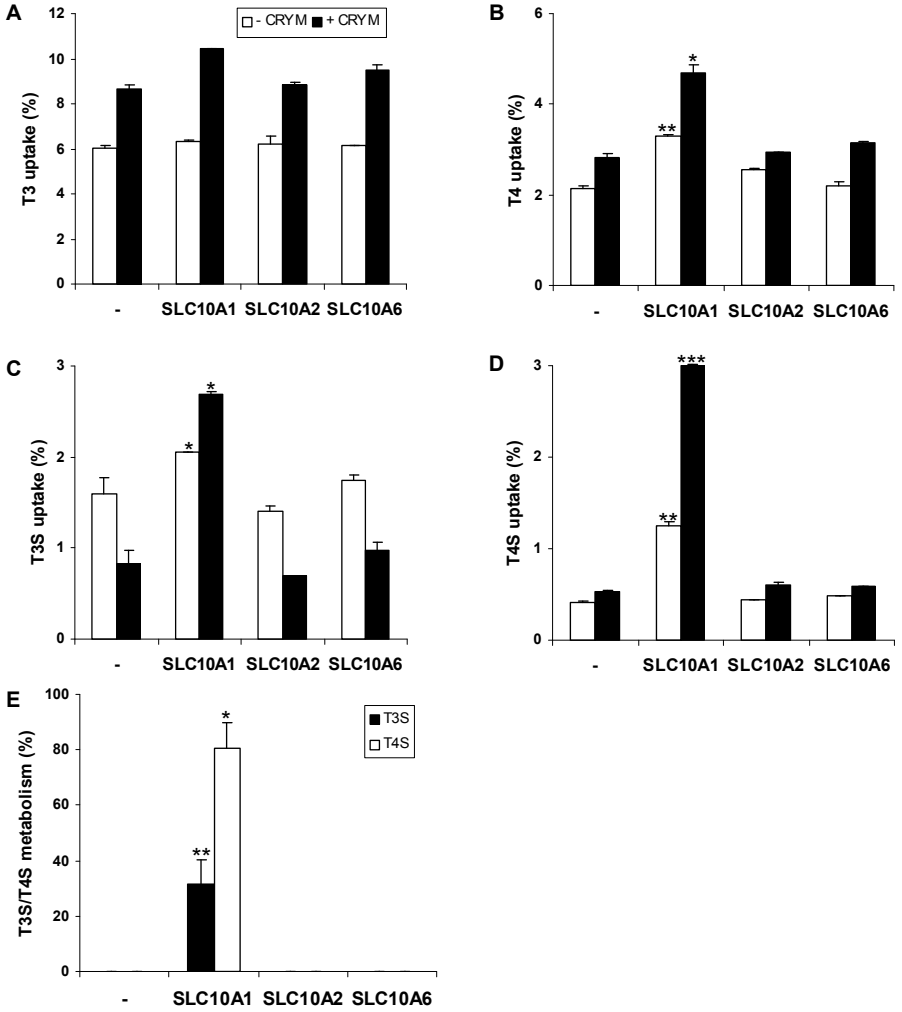

Fig 3. Uptake and metabolism of iodothyronines in COS1 cells transfected with cDNA coding for hSLC10A1, hSLC10A2 and hSLC10A6. Uptake was measured after 30 min incubation with 1 nM T3 (A), T4 (B), T3S (C) or T4S (D) in D-PBS containing 0.1% glucose and 0.1% BSA. Significances represent mean ± range obtained in cells expressing a SLC10 transporter *versus* empty vector, co-transfected with (grey bars) or without (white bars) CRYM. 1% uptake represents uptake of 10 pmol of incubated substrate. Cellular protein amounts to ~3 mg in all wells. (E) Metabolism of T3S and T4S in intact COS1 cells co-transfected with cDNA coding for hSLC10A1, hSLC10A2 and hSLC10A6. Cells transfected with D1 alone were used as controls. Metabolism is shown as percentage of I- production in the medium after 24 h incubation. Significances represent mean ± SEM obtained in cells expressing a SLC10 transporter versus empty vector. * $P < 0.05$; ** $P < 0.01$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

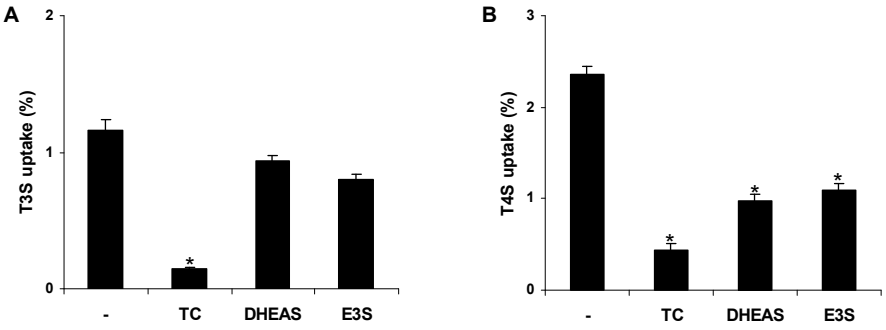

Fig. 4. Influence of 50 µM concentrations of TC, DHEAS and E3S on the uptake of T3S (A) and T4S (B) in HEK293 cells transfected with SLC10A1. Cells were incubated for 10 min with 1 nM T3S or T4S in D-PBS containing 0.1% glucose in the presence of each of the inhibitors. Results are corrected for uptake in control-transfected cells. 1% uptake represents uptake of 10 pmol of incubated substrate. Cellular protein amounts to ~3 mg in all wells. * $P < 0.05$.

not shown). It was not possible to study the effects of higher concentrations of T3S and T4S due to the limited availability of these compounds caused by the low solubility of the iodothyronines used for their synthesis.

The previous experiments indicate that of all SLC10 transporters only SLC10A1 specifically transports T3S and T4S. Because SLC10A1 also facilitates uptake of bile acids and steroid sulfates, we studied the inhibitory effects of TC, DHEAS and E3S on SLC10A1-mediated transport of T3S and T4S. As the tested compounds may interfere with T3S or T4S binding to BSA, we used incubation medium without BSA to directly test the interactions with SLC10A1. At 50 µM concentrations, SLC10A1-mediated T3S and T4S transport is reduced to ~20% by TC, whereas weaker inhibitory effects are observed by DHEAS and E3S (Fig. 4).

Finally, we pursued the interesting observation that co-transfection of CRYM, which has affinity for the iodothyronines T4, T3, 3,5-T2 and, less so, *r*T3 (20, 21), enhanced net cellular uptake of iodothyronine sulfates (Fig. 3). Therefore, we tested the hypothesis that CRYM binds intracellular T3S and T4S, resulting in a diminished T3S and T4S efflux. This mechanism would explain the increased accumulation of the iodothyronine sulfates in cells co-expressing SLC10A1 and CRYM. We studied efflux of T3S and T4S after 30 min incubation with these ligands in cells transfected with SLC10A1 alone or in combination with CRYM. Fig. 5 demonstrates that in the absence of CRYM, T3S and T4S are rapidly released from the cells. In contrast, T3S and T4S efflux was diminished significantly in cells co-expressing SLC10A1 and CRYM. HPLC analyis of the cell contents detected solely the incubated substrates T3S or T4S, but no metabolites (*data not shown*). This indicates that CRYM binds intracellular T3S and T4S and, thus, largely prevents their efflux.

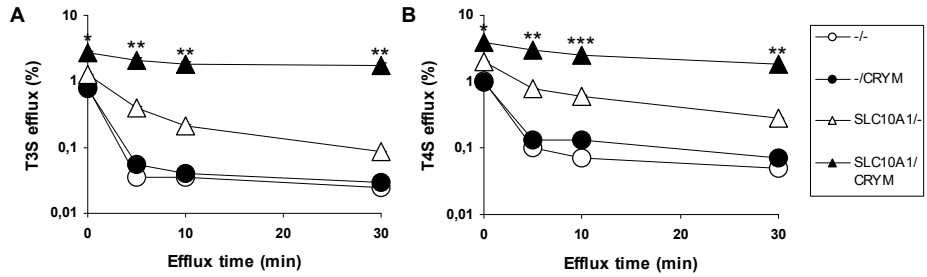

Fig. 5. Effects of CRYM on the efflux of iodothyronine sulfates. COS1 cells were transfected with pcDNA3 or pcDNA3.hSLC10A1 with or without pSG5.hCRYM. Cells were incubated for 30 min with T3S (A) or T4S (B). After brief washing, cells were incubated (5-30 min) with efflux medium, containing 1% BSA, and subsequently processed as described in Materials and Methods. Significances represent values obtained in cells expressing hSLC10A1 with hCRYM *versus* hSLC10A1 without hCRYM. 1% uptake represents uptake of 10 pmol of incubated substrate. Cellular protein amounts to ~3 mg in all wells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

DISCUSSION

NTCP (SLC10A1) and ASBT (SLC10A2), which were the SLC10 family members discovered initially, are both known as bile acid transporters (8). Of the 5 recently identified SLC10 proteins, only the sodium-dependent organic anion transporter (SOAT; SLC10A6) has been shown to transport steroid sulfates but not bile acids, whereas SLC10A3-7 remained orphan carriers (8-10). The hypothesis to test which members of the SLC10 family transport iodothyronies is based on the observation that rSlc10a1 transports (sulfated) iodothyronines and that in different transporter families several members, with varying degrees of similarity, transport iodothyronines (7, 22). Therefore, we investigated which members of this SLC10 transporter family are capable of mediating transport of (sulfated) iodothyronines.

We directly compared the TC transport by all SLC10 proteins. As expected, only SLC10A1 and 2 facilitated TC uptake. This finding confirms earlier reports that only these two SLC10 proteins transport TC (8, 9). In cells expressing SLC10A1, TC uptake is much higher than in cells transfected with SLC10A2. This is also in agreement with earlier observations, which suggested a higher apparent V_{max} for TC transport by SLC10A1 compared to SLC10A2 (19).

In subsequent experiments the uptake was studied of the iodothyronines T3 and T4 as well as the iodothyronine sulfates T3S and T4S by cells transfected with cDNA of each of the SLC10 members. Our findings clearly demonstrate the absence of any uptake of the tested iodothyronine derivatives by any of the SLC10 proteins, except for SLC10A1. These

negative findings were substantiated by metabolism studies, showing no stimulation of T3S and T4S conversion in cells co-expressing SLC10A2-7 and D1 compared to controls. These data are remarkable as at least some SLC10 proteins appeared to be likely transporters for the tested substrates. For example, despite a marked amino acid similarity (62.8%) between SLC10A1 and 2, which even have a common substrate (TC), no transport was oberved of the tested iodothyronine derivatives by SLC10A2 (8). Furthermore, no transport was seen of iodothyronine sulfates by SLC10A6, in spite of the preferential transport of steroid sulfates and the 62.6% amino acid similarity to SLC10A1 (8). These data suggest a high degree of substrate specificity in this transporter family. Closely agreeing results were obtained for SLC10A1-7 in different cell lines (HEK293, COS1) and for SLC10A1 and 3 in *Xenopus* oocytes (Friesema *et al.*, unpublished observations). However, it cannot be excluded that other factors like ancillary proteins are needed for proper function of the transporters which did not demonstrate transport activity in the present study. It should be noted that substrates are only known for SLC10A1, 2 and 6. Thus, the absence of TH transport has only been demonstrated for SLC10A2 and 6. Functional expression of SLC10A3, 4, 5 and 7 could not be established due to the lack of physiological substrates.

Among the SLC10 family, our findings indicate that only SLC10A1 transports the iodothyronine T4 and iodothyronine sulfates T3S and T4S. As expected, this transport was Na^+-dependent, like that of other substrates such as TC and E3S (8). Our data suggest that a negative charge is important for iodothyronines to be transported by SLC10A1. The phenolic hydroxyl group of T4 dissociates (becomes negatively charged) above pH 6.5, whereas that of T3 only above pH 8.5. The highly negative charge of the sulfate group substantiates the importance of ionisation for substrate recognition or binding to SLC10A1. If SLC10A1 and D1 are co-expressed, a dramatic increase of T3S and T4S metabolism is observed. This indicates that SLC10A1 increases the intracellular availability of T3S and T4S. It was not possible to calculate kinetic parameters for T3S and T4S transport.

The fold increase in uptake induced by hSLC10A1 was larger for iodothyronine sulfates than for the native iodothyronines (due to lower background rather than larger absolute increase). Our findings suggest that hSLC10A1 plays an important role in the uptake of iodothyronine sulfates into the liver *in vivo* where they are rapidly degraded by D1 (5). This is in line with the observation that SLC10A1 mainly facilitates uptake, but not efflux. In our T3S and T4S metabolism assays, we observed only I^- as a metabolite, which underlines that these compounds and their intermediates (T2S and rT3S) are converted very rapidly. Therefore, SLC10A1 may fulfill a critical step in providing liver D1 with iodothyronine sulfates for rapid degradation, thereby contributing to the low circulating levels of iodothyronine sulfates in adulthood.

An opposite situation may exist in fetal life. It is known that serum iodothyronine sulfate levels are much higher in the fetus than in adults, which may serve as an additional regulatory mechanism for optimizing tissue-specific TH bioavailability (23, 24). Several factors, like low D1 activity in fetal life, may contribute to these increased levels of fetal iodothyronine sulfates. However, mouse and rat Slc10a1 expression dramatically increases from low levels before birth to high levels at birth (25, 26). In human fetal lever, the expression of SLC10A1 is almost undetectable (27). The low expression of human SLC10A1 during fetal development may result in a low uptake of iodothyronine sulfates into the liver. This will diminish the supply of iodothyronine sulfates to hepatic D1, resulting in a decrease of clearance of these compounds. This indicates that the metabolism of iodothyronine sulfates is not only regulated by D1 expression but also depends on the level of transporter proteins. This is supported by recent observations in which some members of the OATP family are also capable of transporting T3S and T4S (14, 28). The contribution of the different transporters (SLC10A1 *versus* OATPs) to T3S and T4S transport requires further studies.

The SLC10A1 substrates TC, DHEAS and E3S were able to inhibit T3S and T4S transport, of which TC exerted the most potent inhibitory effects. Recently, bile acids were shown to induce TH-mediated metabolism via a signalling pathway involving D2 (29). Our observations that iodothyronine sulfates are transported by the predominant hepatic bile acid transporter SLC10A1 may add a new dimension to the concept connecting bile acids and TH in the control of energy expenditure. When serum bile acids concentrations increase, for example after a meal (30), uptake of iodothyronine sulfates by SLC10A1 will diminish, thereby preventing hepatic clearance of iodothyronine sulfates. As a consequence, increased serum concentrations of iodothyronine sulfates may be available for desulfation in extrahepatic tissues, resulting in increased bioactive TH concentrations and, thus, increased metabolism in these tissues. These effects would be most effective in cells possessing both a T3S/T4S transporter and a sulfatase. It should be stressed that the physiological importance remains speculative, as serum concentrations of iodothyronine sulfates are generally low in adults (23, 31). Therefore, the degree of contribution to biologically active TH via desulfation of iodothyronine sulfates remains uncertain.

We performed several experiments in which we studied the effect of co-transfection with CRYM on the uptake and efflux of iodothyronine sulfates. Our observations that CRYM prevents the efflux of the tested substrates may suggest that CRYM binds iodothyronine sulfates. This is substantiated by the finding that in these experiments cellular T3S and T4S remained intact. Originally, CRYM was reported to bind to native iodothyronines in the presence (T3>T4>rT3) and also the absence (3,5-T2) of NADPH (20, 21). Subsequent studies mainly focused on the role of CRYM in the intracellular binding of T3 (32-34). Our findings may implicate an extension of the role of CRYM to function as a binding protein for iodothyronine sulfates. If so, one would speculate that it may

direct the iodothyronine sulfates to intracellular targets, for example D1. However, it should be noted that kinetic properties of CRYM were not directly studied. Therefore, additional studies are required to elucidate the *in vivo* relevance of this apparent novel function of CRYM.

In conclusion, we provide evidence that also human SLC10A1 transports iodothyronine sulfates, in contrast with SLC10A2-7. In addition, our results suggest that CRYM is capable of binding iodothyronine sulfates. The present paper supports the notion that TH regulates metabolism via different pathways. Further studies are required to investigate to which extent our findings are relevant for physiology.

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Chapter 8

Transport of thyroxine and 3,3′,5-triiodothyronine in human umbilical vein endothelial cells

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ABSTRACT

The prerequisite for the uptake of thyroid hormone (TH) in peripheral tissues is the exit of TH from the bloodstream. The first step in this process is transport across the endothelium. Little is known about this important step in TH physiology. Therefore, we aimed to characterize the TH transport processes across the endothelium using human umbilical vein endothelial cells (HUVECs) as a model.

Transport studies showed rapid uptake of 1 nM [^{125}I]T3 and [^{125}I]T4 in these cells. The apparent Km value for [^{125}I]T3 uptake was ~ 1 μM, and the IC_{50} for T4 inhibition of T3 uptake was ~3 μM. The aromatic amino acids Phe, Tyr and Trp, and the L-type amino acid transporter specific ligand BCH did not inhibit [^{125}I]T3 or [^{125}I]T4 uptake. Verapamil was capable to reversibly reduce transport of [^{125}I]T3 and [^{125}I]T4. HUVECs incubated with the affinity label BrAcT3 resulted in a labeling of multiple proteins, which are probably protein disulfide isomerase (PDI) related.

Extrapolating our findings to the endothelial lining of blood vessels, they suggest that T3 and T4 uptake is mediated by the same transport system. Because TH transport characteristics do not correspond to known TH transporters, further studies are required to identify the TH transporter protein(s) at the molecular level. Possible candidates may be widely expressed Na^+-independent transporter proteins.

INTRODUCTION

The thyroid gland generates predominantly the prohormone T4 and a small fraction of the biological active hormone T3, which are collectively called thyroid hormone (TH), and subsequently secretes these molecules into the bloodstream. As metabolism and action of TH in the target organs take place intracellularly, translocation of TH across the plasma membrane is required. It has become increasingly clear that this process is facilitated by TH transporter proteins (1, 2).

The prerequisite for the uptake of TH in peripheral tissues is the exit of TH from the bloodstream. The first step in this process is transport across the endothelium, the cell layer lining all blood vessels. The endothelium is the opposite of just a barrier between blood and tissues, but is actively involved in many (patho)physiological processes (3). Considering the interaction of the endothelium with a variety of hormones, it is not surprising that associations between serum TH levels and endothelial function have been observed in health and disease (4-6). So far, only a few studies have been carried out investigating the presence and function of thyroid hormone receptors (TRs), which mediate actions of T3, and the type 3 deiodinase (D3), which degrades T3 and T4, in endothelial cells (7-9). Even less is known about TH transport across the endothelium. For example, although it is known that TH transporters like organic anion transporter polypeptide 1C1 (OATP1C1) and OATP1A2 are expressed in brain capillaries (10, 11), there is only one report, to our knowledge, directly studying the transport of T4 across the capillary endothelium (12). In the present study, we explored the characteristics of TH transport processes across the endothelium, using the human umbilical vein endothelial cell (HUVEC) line as a model for vascular endothelial cells.

MATERIALS AND METHODS

Materials

[3′-^{125}I]T3 and [3′,5′-^{125}I]T4 were prepared as previously described (13). BrAc[^{125}I]T3 was synthesized as described (14). Nonradioactive iodothyronines and BrAcT3 were obtained from Henning (Berlin, Germany). 2-aminobicyclo-(2,2,1)-heptane-2carboxylic acid (BCH), aromatic amino acids and verapamil were purchased from Sigma (St. Louis, MO)

Cell culture

HUVECs were cultured in dishes (28 cm^2; Nuncbrand, Fisher Scientific, Landsmeer, The Netherlands) for non-radiolabeled BrAcT3 incubation experiments and in six-well culture dishes (9.6 cm^2; Corning, Schiphol, The Netherlands) for all other experiments

in EBM2 medium (Lonza) supplemented with the EBM2 bullet kit (Lonza) and 9% heat-inactivated FBS.

TH transport experiments

At confluency, cells were washed with incubation medium (Dulbecco's PBS containing 0.1% D-glucose and 0.1% BSA). Uptake of TH was tested by incubation (5-180 min) of the cells at 37 C with 1 nM (2 x 10^5 cpm) [^{125}I]T3 or [^{125}I]T4 in 1.5 ml incubation medium. After incubation, cells were washed with the medium, lyzed with 0.1 M NaOH and counted in a gamma counter. For measurement of TH efflux, cells were loaded for 30 min with incubation medium containing 1 nM (2 x 10^5 cpm) [^{125}I]T3 or [^{125}I]T4, briefly washed with incubation medium and subsequently incubated with efflux medium (Dulbecco's PBS containing 0.1% D-glucose and 1% BSA). After incubation with the efflux medium, cells were washed with incubation medium, lyzed with 0.1 M NaOH and counted in a gamma counter. The uptake and efflux of the [^{125}I]T3 or [^{125}I]T4 were performed in the presence or absence of nonradioactive iodothyronines, BCH, Phe, Tyr, Trp or verapamil at concentrations indicated in the text.

To allow evaluation of Na^+ dependence of the [^{125}I]T3 or [^{125}I]T4 uptake, the cells were incubated in a buffer containing 142.9 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 20 mM HEPES and 0.1% BSA (pH 7.4) or in medium where Na^+ was replaced with a equimolar amount of choline.

Affinity-labeling with BrAc[^{125}I]T3

Affinity-labeling was carried out as described elsewhere (14). At confluency, BrAc[^{125}I]T3 (2 x 10^5 cpm/well) was added and cells were incubated for 4 h at 37 C in EBM2 medium. Cells were washed with cold PBS and lysed in 200 µl 100 mM phosphate (pH 7.2), 2 mM EDTA (PE) buffer. Treatment of lysates with bicarbonate buffer (pH 8 or 11) to discriminate between integral and adherent membrane proteins was done as described previously (15). SDS-PAGE loading buffer (4 x) was added with a final concentration of 10 mM DTT. Samples were analyzed by SDS-PAGE (10% gels, Pierce, The Netherlands). Distribution of radioactivity on blots was analyzed by phosphorimaging (Typhoon 9200).

Affinity-labeling with non-radioactive BrAcT3

At confluency, cells were incubated for 1 h at 37 C with different concentrations (0.5 nM – 10 µM) non-radiolabeled BrAcT3 in incubation medium. After incubation, cells were washed twice with cold PBS and harvested in PE buffer.

Western blotting

The procedure of SDS-PAGE with lysates of HUVECs was performed as reported recently, except for the use of 10% acrylamide gels (Pierce) (14, 16). A home-made T3 antibody

was used for Western blotting of lysates after incubation with unlabeled BrAcT3 incubation. The T3 antibody has been raised in rabbits using T3 coupled through the alanine side chain to BSA (17). Therefore, the antibody recognizes proteins modified by BrAcT3. A specific polyclonal PDI antibody (Cell Signaling Technology, Danvers, MA) was used for probing blots of non-treated HUVEC lysates, following manufacturer's protocol. The monocarboxylate transporter 8 (MCT8)-specific antibody 1306 was used for detection of MCT8 protein.

qPCR

Total RNA was isolated from 1 x 10^6 cells according to standard methods (Roche Diagnostics). We used standard primers crossing introns and probes for PCR of MCT8, MCT10, OATP1A2, OATP1C1, D2 and D3, which are available upon request. mRNA levels are expressed relative to mRNA levels of the house-keeping gene Cyclophilin.

Deiodinase activity

D1, D2 and D3 activities in cell lysates were determined by standard methods, as described previously (18).

Statistical analysis

All results are the mean of at least duplicate determinations from representative experiments. Values are expressed as means ± SE. Statistical significance was determined using the Student's *t* test for unpaired observations.

RESULTS

We first assessed the time course of the T3 and T4 uptake in HUVECs. Uptake of T3 and T4 both increased with time, although cellular uptake of T4 tended to be lower than T3 (Fig. 1).

Next we investigated the effects of increasing concentrations of unlabeled iodothyronines on the uptake of [^{125}I]T3 and [^{125}I]T4. A dose-dependent reduction of [^{125}I]T3 uptake was observed after the addition unlabeled T3 or T4. Figure 2A shows two uptake processes. One process is not saturated for T3 or T4 concentrations up to 100 µM. The saturable T3 uptake process shows an apparent Km value of ~1 µM. The IC_{50} value for T4 inhibition of T3 uptake was ~3 µM. As the absolute T4 uptake was much lower than T3 uptake (Fig. 1), adequate assessment of kinetic parameters for T4 uptake was not possible.

To characterize the uptake system in HUVECs in more detail, we measured uptake in the presence of 1 mM aromatic amino acids. T3 or T4 uptake was not significantly af-

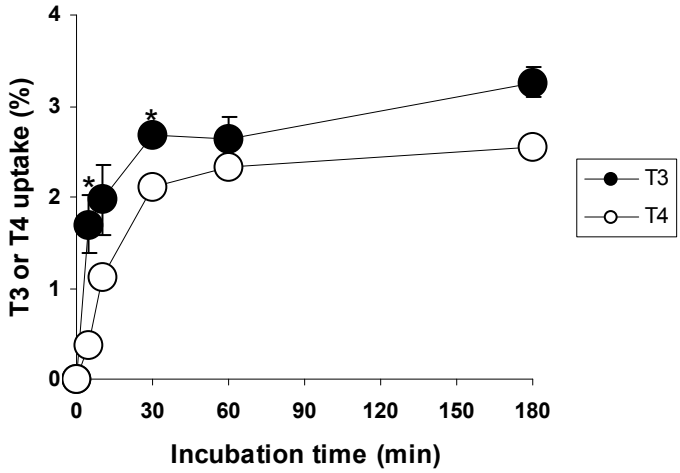

Fig. 1. Time course (min) of uptake of 1 nM [^{125}I]T3 (●) and [^{125}I]T4 (●) in cultured HUVECs. Uptake was measured in D-PBS containing 0.1% glucose and 0.1% BSA. Significances represent differences between T3 and T4 uptake; * $P < 0.05$.

fected by any of the aromatic amino acids. In addition, no inhibition of T3 and T4 uptake was seen in the presence of 1 mM of the L-type transporter specific substrate BCH (Fig. 2B,C).

To investigate the sodium dependence of TH transport, we performed uptake experiments with medium containing equimolar substitution of Na^+ by choline. Replacement of Na^+ by choline reduced [^{125}I]T3 and [^{125}I]T4 uptake by 17% and 25 % ($P < 0.01$ and $P < 0.05$), respectively (Fig. 3), indicating that the TH transport system has a minor sodium dependency.

It has been shown that, depending on the cell-type studied, T3 uptake, efflux or both may be sensitive to the calcium channel blocker verapamil. As shown in Fig. 4 (A and B), incubation with 0.1 mM verapamil resulted in a marked inhibition of [^{125}I]T3 and [^{125}I]T4 uptake (by 53%; $P < 0.001$ and 44%; $P < 0.05$, respectively, after 30 min incubation).

HUVECs export [^{125}I]T3 and [^{125}I]T4 rapidly, as about 65% of cellular TH is released within 10 min (Fig. 4, C and D). The rapid phase of [^{125}I]T3 efflux is not blocked by 0.1 mM verapamil, whereas the slower terminal component of the export process is inhibited if verapamil is added to the efflux medium, resulting in 1.8 fold increased cellular T3 content at 30 min (Fig. 4C; $P < 0.05$). The effects of verapamil on [^{125}I]T4 export are similar to the results obtained after T3 incubation (Fig. 4D).

We next attempted to characterize the protein(s) possibly involved in T3 transport by incubation of intact HUVECs with BrAc[^{125}I]T3, which is recognized as a good affinity-

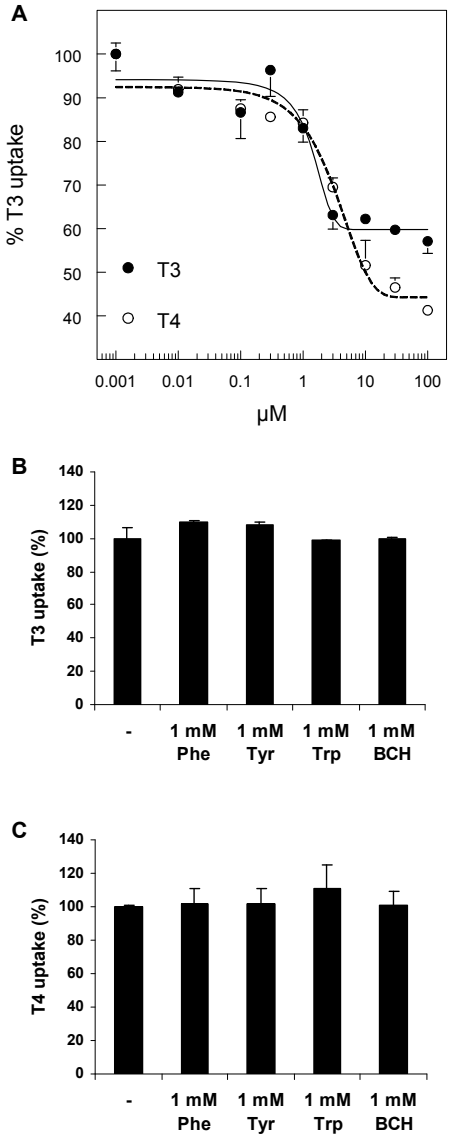

Fig. 2. Effects of increasing concentrations (1 nM to 10 µM) unlabeled iodothyronines on the uptake of [^{125}I]T3 (A) in cultured HUVECs. Effects of 1 mM unlabeled aromatic amino acids (Phe, Tyr, Trp) and BCH on the uptake of [^{125}I]T3 (B) and [^{125}I]T4 (C). Cells were incubated for 10 min. Net uptake in the presence of a competitor is expressed as a percentage of control uptake (1 nM [^{125}I]T3 or [^{125}I]T4).

label for a number of proteins interacting with TH, for example type 1 deiodinase (D1), protein disulfide isomerase (PDI) and the TH transporter MCT8 (14, 15). We noticed a clear labeling of two proteins of ~ 51 and ~ 58 kDa (Fig. 5A). Subsequently, we incubated

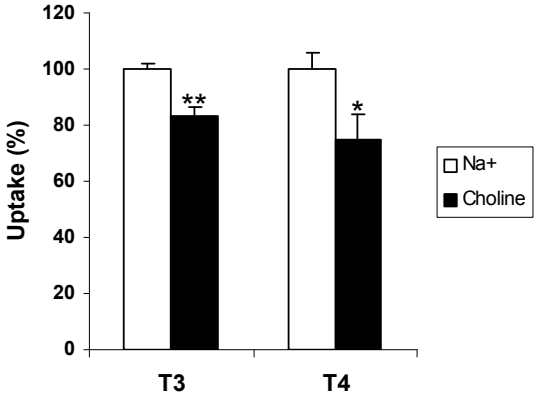

Fig. 3. Na^+ dependence of the uptake of [^{125}I]T3 and [^{125}I]T4 in cultured HUVECs. Cells were incubated with 1 nM [^{125}I]T3 or [^{125}I]T4 for 10 min in sodium chloride medium (gray bars) or in sodium-free choline (black bars) medium. Net uptake in the presence of choline is expressed as a percentage of control (sodium chloride) uptake. * $P < 0.05$; ** $P < 0.01$.

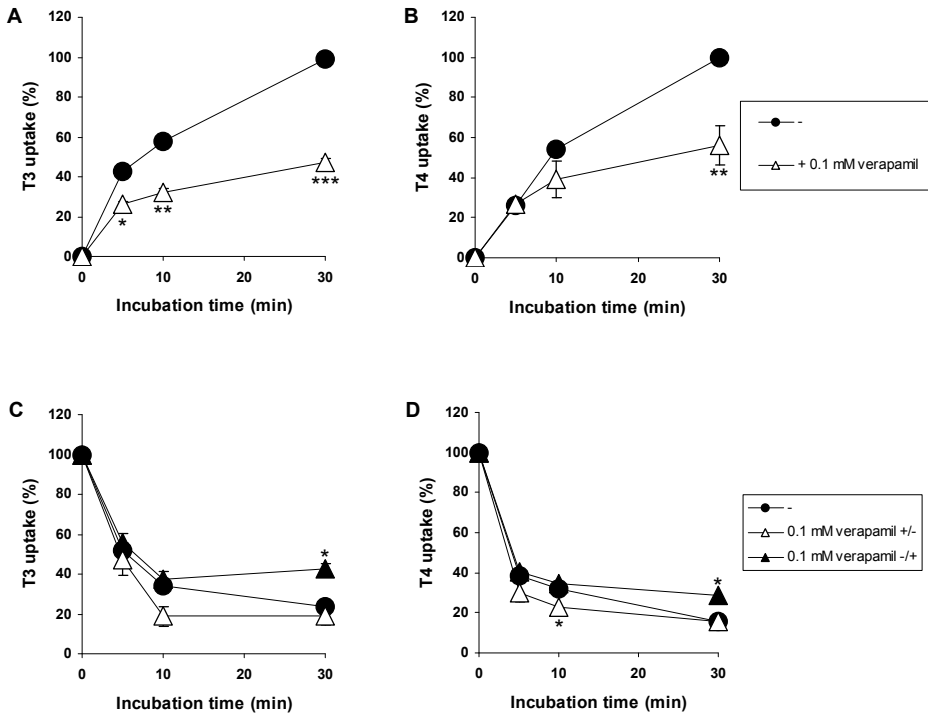

Fig 4. Effects of the Ca^{2+} channel blocker verapamil on T3 and T4 transport. Time course of the effects of 0.1 mM verapamil on the uptake of 1 nM [^{125}I]T3 (A) and [^{125}I]T4 (B) in cultured HUVECs. The efflux of 1 nM [^{125}I]T3 (C) and [^{125}I]T4 (D) in the presence of 0.1 mM verapamil during the 30-min preincubation period (+/-, open triangle) or during the efflux period only (-/+, filled triangle). Significances represent values compared to controls. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

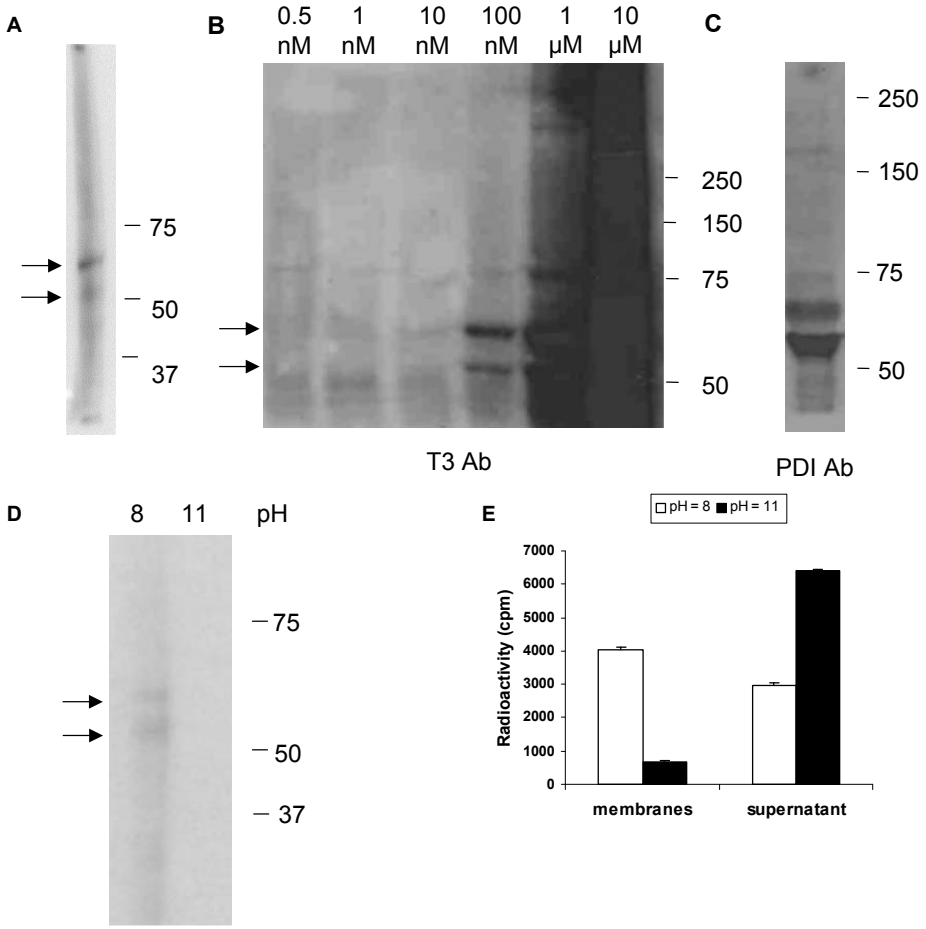

Fig. 5. (A) Affinity labeling of HUVECs. Intact cells were incubated for 4 h at 37 C with BrAc[^{125}I]T3. Subsequently, cells were harvested and cell lysates were separated by SDS-PAGE. Radioactivity was measured by phosphor imaging. (B) Expression of proteins, which are associated with non-radiolabeled BrAcT3. Lysates of cells were separated by SDS-PAGE and analyzed by Western blotting. BrAcT3-associated proteins were detected with a T3-specific antibody. (C) Western blot on a lysate of HUVECs probed with a specific PDI antibody. (D) Influence of treatment of cell lysates at pH 8 or 11 on the distribution of BrAc[^{125}I] T3-labeled proteins. Membranes were isolated and membrane proteins were separated by SDS-PAGE. (E) Total radioactivity of BrAc[^{125}I]T3-labeled proteins in subcellular fractions after treatment of cell lysates at pH 8 or 11. * $P < 0.01$

intact HUVECs with increasing concentrations of (non-radioactive) BrAcT3. When we performed Western blots with a T3 antibody on lysates of these incubated cells, we observed different bands of ~ 51 kDa, ~ 58 kDa, ~ 73 kDa and a band with a molecular mass higher than 250 kDa (Fig. 5B). The intensity of these bands, especially the two low molecular mass bands, increased with higher concentrations of BrAcT3. To investigate whether these bands were PDI, we performed Western blotting on lysates of HUVECs

with a PDI-specific antibody. Figure 5C shows bands with a molecular mass exactly corresponding to the bands modified by BrAcT3, as seen in Figure 5B. This strongly suggests that the BrAcT3-labeled bands are PDI-related. This notion was supported by experiments showing that the BrAc[^{125}I]T3-labeled protein was associated with the membranes if lysates were treated at pH 8, whereas it was released from the membranes if the lysates were treated at pH 11 (Fig. 5D). The latter is known to release luminal proteins from the endoplasmic reticulum like PDI. Indeed, counting radioactivity in the fractions, indicated that most of the BrAc[^{125}I]T3-labeled protein was released in the supernatant, arguing against the labeling of a plasma membrane protein (Fig. 5E).

We further investigated the expression of known active and specific TH transporters with a wide tissue distribution in human tissues. qPCR revealed no MCT10 mRNA expression, whereas MCT8 mRNA was modestly expressed. However, no MCT8 protein was detected in HUVEC lysates by Western blot analysis (data not shown). Furthermore, gene expression levels of OATP1A2 and OATP1C1, which are expressed in brain capillaries, were very low (data not shown).

Finally, we examined whether HUVECs had deiodinating enzyme activity for iodothyronines. We did not observe any D1, D2 or D3 activity in cell lysates, which corresponds to the low mRNA levels of these genes (data not shown).

DISCUSSION

The importance of investigating TH transport processes across the endothelium is obvious, as these cells are the first barrier to cross if circulating TH leaves the blood stream. It is surprising that little is known about this physiologically important process.

Our results show that T3 and T4 are rapidly taken up by HUVECs, resulting in an equilibrium within 30 min. This might suggest that HUVECs do not need high intracellular TH levels for metabolism and action by themselves, but rather serve as a passage for supplying TH to the tissues. This hypothesis is supported by our observation that no T3 or T4 metabolism by D1, D2 or D3 is detected. In addition, it has been suggested that TR expression levels in HUVECs are too low to assume a physiological role for TH in the classical genomic pathway (8). Studies in humans have shown that TH effects on vascular reactivity are exerted at the level of the endothelium (6), but it is not known to which extent these are mediated by genomic or non-genomic mechanisms.

Within the range of tested T3 and T4 concentrations, we observed a saturable as well as a non-saturable component. The latter may reflect iodothyronine partitioning in the cell membrane and/or a transporter system with Km values in the mM range. The saturable process shows a dose-dependent reduction in [^{125}I]T3 uptake if unlabeled T3 is added with an apparent Km of ~1 μM. Slightly higher concentrations of T4 (~3 μM) are

needed to inhibit 50% of [^{125}I]T3 uptake. Detailed kinetic analysis of [^{125}IT4 uptake was not possible due to its low magnitude. The observation that [^{125}I]T3 uptake is inhibited by similar T3 and T4 concentrations may suggest that T3 and T4 uptake is mediated by the same transporter.

The observation that TH uptake is not inhibited by any of the aromatic amino acids indicates that a carrier transporting aromatic amino acids together with TH is unlikely to be responsible for the TH transport in HUVECs. Taken together, these data seem to preclude an important role for the known specific TH transporters MCT8 and MCT10, because aromatic amino acids interact with both TH transporters, especially MCT10 (19). This corresponds to the observation that MCT8 and MCT10 expression is low in HUVECs. The transporters OATP1A2 and OATP1C1, which are possibly expressed in endothelium of brain capillaries, are not likely to play an important role in HUVECs as they are minimally expressed. As BCH, which is regarded as a prototypic ligand for the L-type amino acid transporter, did not affect uptake of T3 and T4, it is likely that this transporter system, although expressed in HUVECs, does not play an important role in the uptake of these iodothyronines in HUVECs. This does, however, not preclude a role for this protein for the transport of other iodothyronines, like T2, because *Xenopus laevis* oocytes injected with LAT1 cRNA preferentially transported T2 over T3 and T4 (20).

In contrast to other tissues, like liver and pituitary, TH transport in HUVECs is only minimally dependent on the presence of Na^+ (21, 22).

It has been shown that TH uptake as well as efflux in different cell lines is verapamil-sensitive (23-27). Cellular presence of multidrug resistance P-glycoproteins (MDRs) does only partially explain this phenomenon, as observations in different cell lines suggest the existence of a 90-100 kDa protein, which is responsible for the verapamil-sensitive T3 efflux and not is compatible with the molecular mass of MDRs (24).

Our experiments indicate that not only T3 uptake but also T3 efflux is reduced if cells are exposed to verapamil. This interaction is reversible because 30 min preincubation with verapamil and normal efflux medium did not affect T3 efflux, whereas verapamil in the efflux medium decreased efflux. The observation that verapamil affects T3 efflux only after 30 min following addition to the efflux medium, suggests that it takes time for verapamil to reach sufficiently high intracellular concentrations to compete with intracellular T3.

The TH efflux curves were bi-phasic, showing a rapid initial phase and a slower terminal component, processes which have been described previously (23). The rapid phase may represent a rapidly exchanging component perhaps due to nonspecific binding of TH to the membrane and/or the existence of a readily-available sub-membrane TH pool. The slower phase may represent release of intracellular TH, which takes time to be trafficked to the TH transporter protein.

Although not studied in detail, it has been proposed that Ca^{2+} channel blockers may reduce cardiac thyrotoxic symptoms (28). Further studies are required to investigate the mechanisms behind the intriguing relationships between transport of TH and verapamil.

It has been shown that uptake of T3 is reduced at lower verapamil concentrations than uptake of T4 in rat cardiomyocytes (29). In contrast, T3 as well as T4 transport is similarly inhibited by verapamil in HUVECs. From this, it appears that a single transporter may be responsible for T3 and T4 transport. This is underscored by our other inhibition studies suggesting that the same transport system mediates both T3 and T4 uptake.

Theoretically, labeling of proteins with the affinity label BrAcT3 is a valuable technique for identification of TH transporters. Indeed, the important TH transporter MCT8 is labeled by BrAcT3 (14). However, other proteins may be labeled as well. Our affinity labeling experiments in HUVECs strongly suggest that BrAcT3 did not react with a TH transporter, but with PDI-related proteins. PDI is an ER associated chaperone protein, which is known to be well labeled by BrAcT3 (15). PDI is usually an abundant protein and different isoforms have been detected by mass spectrometry analysis in HUVECs, which is in accordance with our findings (30).

In the present study we investigated TH transport processes in endothelial cells as a model for the endothelial lining of blood vessels by studying HUVECs. It is difficult to assess to which extent transport through the interendothelial space contributes to the total amount of TH transported across the endothelium. Therefore, extrapolation of findings in these cells to the endothelium *in vivo* may have limitations. However, HUVECs are commonly used to study transport and metabolism processes of, among others, amino acids and glucose (3). Therefore, it is generally accepted that these cells may serve as a model for transport processes of the endothelium.

To date, characteristics of TH transport across the endothelium of the systemic circulation are largely unknown. Transport across the endothelial cell would entail basolateral uptake, transcellular trafficking and apical efflux of the hormone. This process may need to be repeated in other cell types (e.g. vascular smooth muscle cells) before the target cell is reached. Cellular TH uptake and efflux may be facilitated by different transporters. The present results add to the understanding of these processes and may reveal insights in the systemic and local regulation of TH, thereby providing a basis for identification of these transporter proteins. Possible candidates may be widely expressed Na^+-independent transporter proteins.

In conclusion, we have provided evidence that there is rapid transport of T3 and T4 in HUVECs, which is probably mediated via the same transporter. As the transport characteristics do not correspond with the widely expressed transporters MCT8 and MCT10, the TH transporter protein(s) in HUVECs need to be discovered at the molecular level. Further studies are required to identify the proteins responsible for transport of iodothyronines across the endothelium.

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Chapter 9

Thyroid state in a large cohort of patients with mental retardation: the TOP-R (Thyroid Origin of Psychomotor Retardation) study

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Submitted

Chapter 10

Novel monocarboxylate transporter 8 (*MCT8*) mutations in a cohort of patients with unexplained mental retardation

W. Edward Visser, Frank E. Visser, Willem Frans M. Arts and Theo J. Visser

Submitted

Chapter 11

Mutations in the type 2 (*DIO2*) and type 3 (*DIO3*) deiodinases in patients with unexplained mental retardation

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Chapter 12

Physiological thyroid hormone levels regulate numerous skeletal muscle transcripts

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ABSTRACT

Context: Skeletal muscle is an important target tissue for thyroid hormone (TH). It is currently unknown which genes are regulated by physiological TH levels.

Objective: We examined the effects of L-thyroxine on human skeletal muscle transcriptome.

Design: Microarray analysis of transcript levels was performed using skeletal muscle biopsies from patients under euthyroid and hypothyroid conditions.

Setting: The study was conducted in a university hospital laboratory.

Patients: We studied skeletal muscle obtained from 10 thyroidectomized patients with differentiated thyroid carcinoma on and after 4 weeks off L-thyroxine replacement.

Mean outcome measures: Gene expression changes were measured using microarrays. Results were analyzed using dedicated statistical methods.

Results: We detected 607 differentially expressed genes on L-thyroxine treatment, of which approximately 60% were positively and approximately 40% were negatively regulated. Representative genes were validated by quantitative PCR. Genes involved in energy and fuel metabolism were overrepresented among the up-regulated genes, of which a large number were newly associated with thyroid state. L-thyroxine therapy induced a large down-regulation of the primary transcripts of the non-coding microRNA pair miR-206/miR-133b.

Conclusion: We demonstrated that physiological levels of TH regulate a myriad of genes in human skeletal muscle. The identification of novel putatively TH-responsive genes may provide the molecular basis of clinical effects in subjects with different TH status. The observation that TH regulates microRNAs reveals a new layer of complexity by which TH influences cellular processes.

INTRODUCTION

Skeletal muscle is an important target tissue for thyroid hormone (TH) (1). In humans, skeletal muscle is a major contributor to the basal metabolic rate. As TH is a potent stimulator of body metabolism, it likely controls basal metabolic rate by modulating energy processes in skeletal muscle. However, the physiological relevance of the processes underlying TH-dependent changes in the control of energy expenditure is currently unclear (2).

The major biologically active TH is T3, which is produced from the prohormone T4 by the deiodinating enzymes D1 and D2. The genomic effects of T3 are mediated by its nuclear receptors (TH receptors), which are transcription factors with a ligand-modulated activity. Consequently, T3-induced alterations in the transcriptional complex will result in changes in gene transcription.

Transcriptome analysis of tissues or cells with different TH status are helpful to identify which genes are regulated by T3. Unbiased approaches using microarray technologies have identified large numbers of novel T3-regulated genes, mostly in animal tissues like liver and brain (3-6). The effects of TH on gene expression profiles in human tissues have been studied less intensively (7, 8). To what extent physiological TH levels influence gene transcript levels in human skeletal muscle is currently unknown.

In the present study, we compared the skeletal muscle transcriptome in thyroidectomized patients treated for differentiated thyroid carcinoma (DTC) off and on L-thyroxine replacement. This allowed us to identify hundreds of genes, involved in various cellular processes, which are presumably regulated by TH.

MATERIALS AND METHODS

Patients

Patients were recruited from the outpatient clinic of the Department of Endocrinology of Leiden University Medical Center, which is a tertiary referral center for differentiated thyroid cancer (DTC). Patients were included who had been diagnosed with DTC and had received initial therapy consisting of near-total thyroidectomy and radioiodine ablation therapy. Additional therapies were allowed, as long as they resulted in cure. Cure was documented by the absence of measurable serum thyroglobulin (Tg) as well as by a negative total-body scintigraphy with 4 mCi ^{131}I after withdrawal of L-thyroxine treatment. Patients who had diabetes mellitus or other endocrine diseases or had a BMI >30 kg/m^2 were excluded. Patients who used any drugs known to influence TH metabolism were also excluded. The ethics committee of Leiden University Medical Center approved the study. Written informed consent was obtained from all subjects.

Patients with DTC undergoing TSH-stimulated ^{131}I scintigraphy were asked to participate in the study. Four weeks after L-thyroxine withdrawal and 8 weeks after subsequent L-thyroxine replacement, patients were admitted to the clinical research unit. All subjects fasted from the preceding evening (6 p.m.) until the time of biopsy (8 a.m.). Body length (m) and weight (kg) were measured.

Patients were studied in a semirecumbent position. A catheter was inserted in a dorsal hand vein to collect blood samples for measurement of serum TSH, fT4 and T3. Muscle biopsies were taken from the quadriceps muscle (vastus lateralis) under local anesthesia (Lidocaine 20 mg/ml; Fresenius, Kabi, Den Bosch, The Netherlands) as described earlier (9). Biopsy specimens were quickly washed in HEPES-buffered saline to remove blood, inspected for fat or fascia content, dried on gauze swabs, and subsequently stored in liquid nitrogen until analysis. Serum samples were handled immediately and stored at –20 C. Two patients were excluded from analysis, because insufficient tissue was available from both time points.

Serum analyses

Serum TSH, fT4 and T3 levels of the patients were determined as described previously (10).

RNA isolation and microarray analysis

Total RNA was isolated from ~50 mg tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) and further purified by the High Pure RNA isolation kit (Roche, Woerden, The Netherlands). The amount of total RNA per mg tissue was similar in both thyroid states.

Purity and quality of isolated RNA were assessed by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All samples showed a RIN>8 and were all used for subsequent labeling. RNA (3-5 µg) from all samples at both time points was used for subsequent production of biotinylated cRNA. Labeled cRNA was hybridized to the U133 Plus 2.0 GeneChip oligonucleotide microarray (Affymetrix, Santa Clara, CA) according to the protocol provided by the manufacturer.

To examine the quality of the various arrays, the R package affyQCreport for generating QC reports was run starting from the CEL files. All created plots, including the percentage of present calls, noise, background, and ratio of GAPDH 3′ to 5′ (<1.4) indicated a high quality of samples and an overall comparability. Raw intensities values of all samples were normalized by RMA normalization (Robust Multichip Analysis) (background correction and quantile normalization) using Partek version 6.4 (Partek Inc., St. Louis, MO). To visualize the clustering of the samples, PCA (Principal Component Analysis) was used. The normalized datafile was transposed and imported into OmniViz version 6.0.1 (Biowisdom, Ltd., Cambridge, UK) for further analysis.

For each probe set, the geometric mean of the hybridization intensities of all samples was calculated. The level of expression of each probe set was determined relative to this geometric mean and ^{2}log transformed. The geometric mean of the hybridization signal of all samples was used to ascribe equal weight to gene expression levels with similar relative distances to the geometric mean. Differentially expressed genes were identified using statistical analysis of microarrays (SAM analysis). Cut-offs values for significantly expressed genes were a false discovery rate (FDR) of ≤0.05 and a fold change of 1.5. Functional annotation of the SAM results was done using Ingenuity Pathway Analysis (Ingenuity, Mountain View, CA) and EASE software (http://david.abcc.ncifcrf.gov/ease). EASE (Expression Analysis Systematic Explorer) calculates significant over-representation of GO-classified biological processes by comparing the number of genes in a gene list for a given biological process to the number of genes for that biological process printed on the array. The results are shown of EASE analysis of biological processes, which are significantly ($P < 0.05$) enriched after multiple testing.

qPCR

cDNA was synthesized using 0.5 µg RNA and TaqMan RT reagent (Roche, Woerden, The Netherlands). SYBR Green I (Eurogentec, Maastricht, The Netherlands) was used as detector dye for qPCR of the differentially expressed genes SLC2A5, IL32, MYLK4, ITGB1BP3, SCN4B, UCP3, DKK2, CALML6, CRIM1, FEZ2, primary transcript of miR-206 (pri-miR-206), pri-miR-133b, pri-miR-1-1, pri-miR-133a-2, pri-miR-1-2 and pri-miR-133a-1. The primer sequences are presented in Table S1. mRNA levels are expressed relative to that of the house-keeping gene cyclophilin A. qPCR was performed in 6 out of 10 patients at both time points, because insufficient material was available from the other biopsies.

RESULTS

Study population

We studied muscle samples from 10 patients with DTC. Muscle biopsies were performed when overt biochemical hypothyroidism was achieved by withdrawal of L-thyroxine substitution in thyroidectomized patients as well as when euthyroidism was restored after restarting L-thyroxine replacement therapy. Baseline characteristics are summarized in Table S2. Table 1 shows the serum TH concentrations in subjects off and on L-thyroxine treatment.

Global view on gene expression levels

To assess the effects of L-thyroxine replacement therapy on global gene expression in muscle, we hybridized cRNA from skeletal muscle, obtained off and on L-thyroxine

Table 1. Thyroid hormone parameters off and on L-thyroxine replacement

L-thyroxine treatment	off	on
TSH (0.4-4.8 mU/L)	139.7 ± 11.1	0.6 ± 0.2[a]
fT4 (10-24 pmol/L)	1.3 ± 0.2	25.5 ± 1.1[a]
T3 (1.4-2.5 nmol/L)	0.1 ± 0.1	4.8 ± 0.24[a]

Data (means ± SEM) were compared using paired t-test.
[a] $P < 0.001$

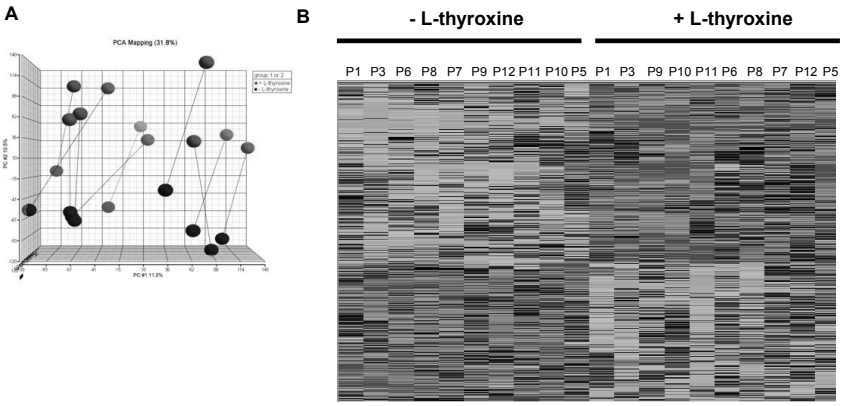

Fig. 1. Regulation of genes expressed in skeletal muscle on *versus* off L-thyroxine treatment (euthyroidism *versus* hypothyroidism, respectively). PCA of all probe sets and hierarchical clustering of 931 probe sets, which have a differential expression. (A) PCA analysis separates samples on their response to L-thyroxine treatment on the y-axis: *red*, off L-thyroxine treatment; *blue*, on L-thyroxine treatment. (B) OmniViz Treescape showing the hierarchical clustering of Affymetrix probe sets which matched the selection query. Gene expression levels: *red*, up-regulated genes compared to the geometric mean; *green*, down-regulated genes compared to the geometric mean. The color intensity correlates with the degree of change.

treatment, to Affymetrix Human Genome U133 Plus 2.0 oligonucleotide GeneChips. Of the 54674 probe sets, approximately 40% was called present in all samples. PCA analysis showed a high degree of clustering of patients off and on L-thyroxine treatment (Fig. 1A). We selected probe sets for analysis, which showed a >1.5-fold change in gene expression on L-thyroxine treatment. At a FDR of 0.05, we identified 931 such probe sets representing 607 unique genes and 54 nonannotated Affymetrix IDs. Fig. 1B shows the selected probe sets as a hierarchical clustering, clearly indicating that treatment exerts both positive and negative effects on gene regulation. Among the 607 known genes, 349 were up-regulated, whereas 258 transcripts were down-regulated. The 144 genes that differed 2-fold in expression are presented in Table 2.

Table 2. Transcripts that differed 2-fold (^{2}Log Ratio >1 or <1) in expression

Up-regulated genes

^{2}Log Ratio	Molecules	Description
3.955	SLC2A5	solute carrier family 2 (facilitated glucose/fructose transporter), member 5
3.414	IL32	interleukin 32
2.897	MYLK4	myosin light chain kinase 4
2.775	ITGB1BP3	integrin beta 1 binding protein 3
2.626	SCN4B	sodium channel, voltage-gated, type IV, beta
2.549	UCP3	uncoupling protein 3 (mitochondrial, proton carrier)
2.223	LOC121952	hypothetical protein LOC121952
2.195	TP53INP2	tumor protein p53 inducible nuclear protein 2
2.155	B3GNT5	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5
1.928	C9ORF135	chromosome 9 open reading frame 135
1.913	DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4
1.902	SERPINA5	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5
1.9	TRIM7	tripartite motif-containing 7
1.888	THRSP	thyroid hormone responsive (SPOT14 homolog, rat)
1.806	RRAD	Ras-related associated with diabetes
1.771	GBE1	glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme)
1.749	CCDC80	coiled-coil domain containing 80
1.742	COQ10A	coenzyme Q10 homolog A (S. cerevisiae)
1.722	ANGPTL2	angiopoietin-like 2
1.696	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
1.623	MXRA5	matrix-remodelling associated 5
1.55	PHKG1	phosphorylase kinase, gamma 1 (muscle)
1.542	MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)
1.538	NOV	nephroblastoma overexpressed gene
1.537	SLC16A3	solute carrier family 16, member 3 (monocarboxylic acid transporter 4)
1.536	C6ORF32	chromosome 6 open reading frame 32
1.535	FAM55C	family with sequence similarity 55, member C
1.533	ATPGD1	KIAA1394 protein
1.513	PAQR9	progestin and adipoQ receptor family member IX
1.51	PEBP4	phosphatidylethanolamine-binding protein 4
1.495	CTSC	cathepsin C
1.488	KIF22	kinesin family member 22
1.436	UCP2	uncoupling protein 2 (mitochondrial, proton carrier)
1.422	CD38	CD38 molecule
1.395	FNDC1	fibronectin type III domain containing 1
1.383	NRP1	neuropilin 1
1.382	HK2	hexokinase 2
1.381	C3ORF43	chromosome 3 open reading frame 43
1.374	FBXO32	F-box protein 32
1.372	THBD	thrombomodulin
1.352	LOC283737	hypothetical protein LOC283737
1.334	SOX4	SRY (sex determining region Y)-box 4
1.319	DYRK1B	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B
1.306	MLZE	melanoma-derived leucine zipper, extra-nuclear factor
1.303	DCLK1	doublecortin-like kinase 1
1.301	RET	ret proto-oncogene
1.294	SLC25A25	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25
1.292	CXCR7	chemokine (C-X-C motif) receptor 7
1.259	IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta

1.258	PREB	prolactin regulatory element binding
1.203	LOC165186	similar to RIKEN cDNA 4632412N22 gene
1.201	SLC16A9	solute carrier family 16, member 9 (monocarboxylic acid transporter 9)
1.199	MAP2K3	mitogen-activated protein kinase kinase 3
1.199	RP5-1022	P6.2 hypothetical protein KIAA1434
1.197	POPDC2	popeye domain containing 2
1.192	AGXT2L1	alanine-glyoxylate aminotransferase 2-like 1
1.188	ARRDC3	arrestin domain containing 3
1.175	AGTRL1	angiotensin II receptor-like 1
1.169	TMEM108	transmembrane protein 108
1.168	TMEM135	transmembrane protein 135
1.142	MLLT11	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 11
1.136	PRPH2	peripherin 2 (retinal degeneration, slow)
1.131	CTHRC1	collagen triple helix repeat containing 1
1.123	KLF9	Kruppel-like factor 9
1.12	SLC25A33	solute carrier family 25, member 33
1.117	GYS1	glycogen synthase 1 (muscle)
1.105	ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1
1.102	SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
1.099	COL1A2	collagen, type I, alpha 2
1.098	ANKRD23	ankyrin repeat domain 23
1.096	COL15A1	collagen, type XV, alpha 1
1.092	CD93	CD93 molecule
1.089	FRAS1	Fraser syndrome 1
1.08	FNDC5	fibronectin type III domain containing 5
1.073	IGF1	insulin-like growth factor 1 (somatomedin C)
1.071	ADCY7	adenylate cyclase 7
1.057	SLC38A1	solute carrier family 38, member 1
1.055	GREM1	gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)
1.051	TMEM38A	transmembrane protein 38A
1.048	SNED1	sushi, nidogen and EGF-like domains 1
1.045	FABP3	fatty acid binding protein 3
1.039	CD9	CD9 molecule
1.038	COL4A4	collagen, type IV, alpha 4
1.033	NNT	nicotinamide nucleotide transhydrogenase
1.028	COL1A1	collagen, type I, alpha 1
1.021	PHKA1	phosphorylase kinase, alpha 1 (muscle)
1.021	ST3GAL6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6
1.012	PFKFB1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1
1.009	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10
1.008	LOC286167	hypothetical LOC286167
1.007	WDR62	WD repeat domain 62
1.004	TRAF3IP1	TNF receptor-associated factor 3 interacting protein 1
1.002	TPCN1	two pore segment channel 1

Down-regulated genes

^{2}Log Ratio	Molecules	Description
-3.374	C1ORF168	chromosome 1 open reading frame 168
-2.025	DKK2	dickkopf homolog 2 (Xenopus laevis)
-1.948	CALML6	calmodulin-like 6
-1.897	ABCC12	ATP-binding cassette, sub-family C (CFTR/MRP), member 12

-1.881	C11ORF75	chromosome 11 open reading frame 75
-1.811	FEZ2	fasciculation and elongation protein zeta 2 (zygin II)
-1.718	LOC100008588	18S ribosomal RNA
-1.657	CRIM1	cysteine rich transmembrane BMP regulator 1 (chordin-like)
-1.551	C21ORF7	chromosome 21 open reading frame 7
-1.543	CDCA7	cell division cycle associated 7
-1.492	NANOS1	nanos homolog 1 (Drosophila)
-1.465	ST3GAL3	ST3 beta-galactoside alpha-2,3-sialyltransferase 3
-1.451	TMEM46	shisa homolog 2 (Xenopus laevis)
-1.406	GATM	glycine amidinotransferase (L-arginine:glycine amidinotransferase)
-1.4	DDIT4L	DNA-damage-inducible transcript 4-like
-1.329	HCN1	hyperpolarization activated cyclic nucleotide-gated potassium channel 1
-1.325	NAV2	neuron navigator 2
-1.321	FAM129A	family with sequence similarity 129, member A
-1.308	ACTN3	actinin, alpha 3
-1.297	SMYD2	SET and MYND domain containing 2
-1.295	BTD	biotinidase
-1.289	LOC730057	hypothetical LOC730057
-1.281	PTP4A1	protein tyrosine phosphatase type IVA, member 1
-1.273	LOC196541	hypothetical protein LOC196541
-1.236	CCDC110	coiled-coil domain containing 110
-1.229	C1ORF105	chromosome 1 open reading frame 105
-1.216	PMP22	peripheral myelin protein 22
-1.204	SPOCK1	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1
-1.201	ARMCX2	armadillo repeat containing, X-linked 2
-1.201	ATP2C1	ATPase, Ca++ transporting, type 2C, member 1
-1.192	MTUS1	mitochondrial tumor suppressor 1
-1.183	PDLIM5	PDZ and LIM domain 5
-1.174	SH3RF2	SH3 domain containing ring finger 2
-1.169	C15ORF41	chromosome 15 open reading frame 41
-1.145	ARID5B	AT rich interactive domain 5B (MRF1-like)
-1.14	TMEM70	transmembrane protein 70
-1.135	PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2
-1.131	LRRC3B	leucine rich repeat containing 3B
-1.127	PLP2	proteolipid protein 2 (colonic epithelium-enriched)
-1.111	NFIL3	nuclear factor, interleukin 3 regulated
-1.108	AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)
-1.102	SVIL	supervillin
-1.101	MAGED2	melanoma antigen family D, 2
-1.1	NEDD1	neural precursor cell expressed, developmentally down-regulated 1
-1.085	SLC16A10	solute carrier family 16, member 10 (aromatic amino acid transporter)
-1.074	SCN3B	sodium channel, voltage-gated, type III, beta
-1.066	EFR3A	EFR3 homolog A (S. cerevisiae)
-1.062	OTUD1	OTU domain containing 1
-1.044	CYR61	cysteine-rich, angiogenic inducer, 61
-1.033	PBX1	pre-B-cell leukemia homeobox 1
-1.008	MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)
-1.002	MYH3	myosin, heavy chain 3, skeletal muscle, embryonic

Validation of selected genes identified by microarray

Among the probe sets, which significantly differed in expression, 166 unique genes were at least twice represented with different probe sets on the chip, which strengthens the present findings. To confirm the microarray results by an independent technique, qPCR was used to analyze representative genes whose expression was either increased (6 genes) or decreased (4 genes). The results obtained by qPCR correlated well with the microarray data (Fig. 2A,B).

Gene ontology analysis

Next we examined whether the 607 differentially expressed genes are associated with similar biological processes. GO enrichment analysis using EASE revealed that 32 biological processes were significantly overrepresented. Because several GO terms overlap, based on similar groups of genes, we reduced the number of biological processes to 15 (Fig. 3A). Subsequently, the up-regulated and down-regulated genes were analyzed separately, to investigate whether different biological processes were overrepresented. Among the up-regulated genes, 14 biological processes were overrepresented (Fig. 3B),

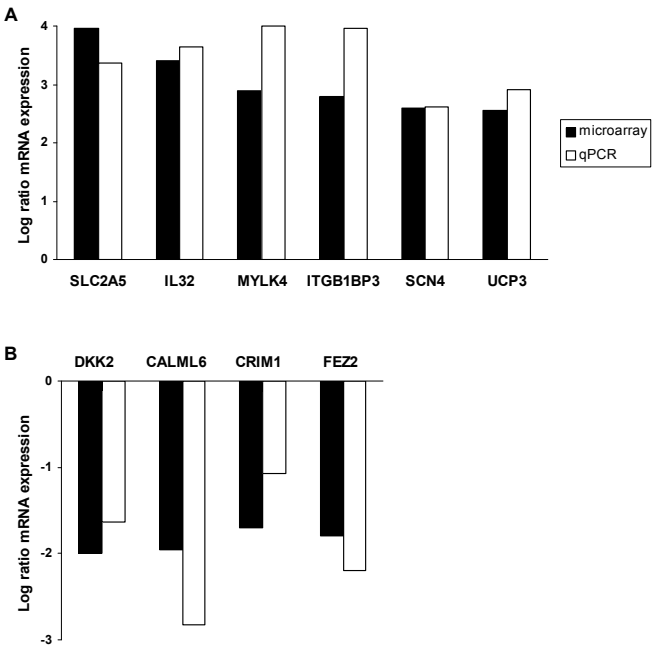

Fig. 2. Verification of microarray results by qPCR. Six up-regulated (A) and four down-regulated (B) genes were selected from the microarray results. Results are shown as ^{2}log ratio of the fold change in gene expression between euthyroidism (on L-thyroxine treatment) and hypothyroidism (off L-thyroxine treatment).

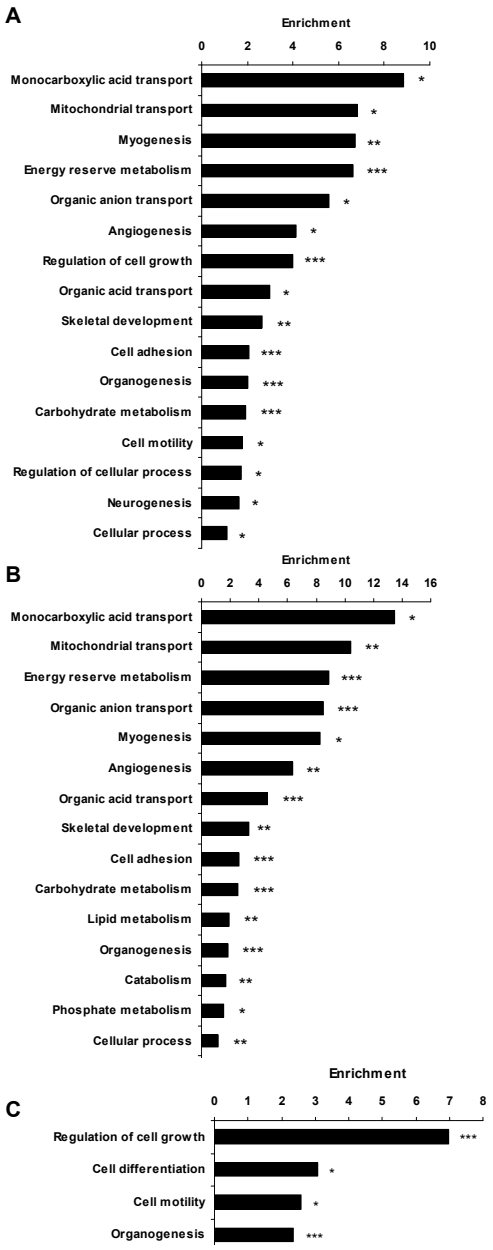

Fig. 3. Gene ontology (GO) enrichment analysis of differentially expressed genes in muscle off and on L-thyroxine treatment. Enriched GO terms for GO biological processes within all 607 selected genes (A), within the up-regulated genes (B) and within the down-regulated genes (C). Several GO terms are excluded, based on similarity of represented genes. Enriched categories are those identified as significantly enriched by the EASE score (x-axis), which is a modified Fisher exact probability test. * P <0.05; ** P <0.01; *** P <0.001.

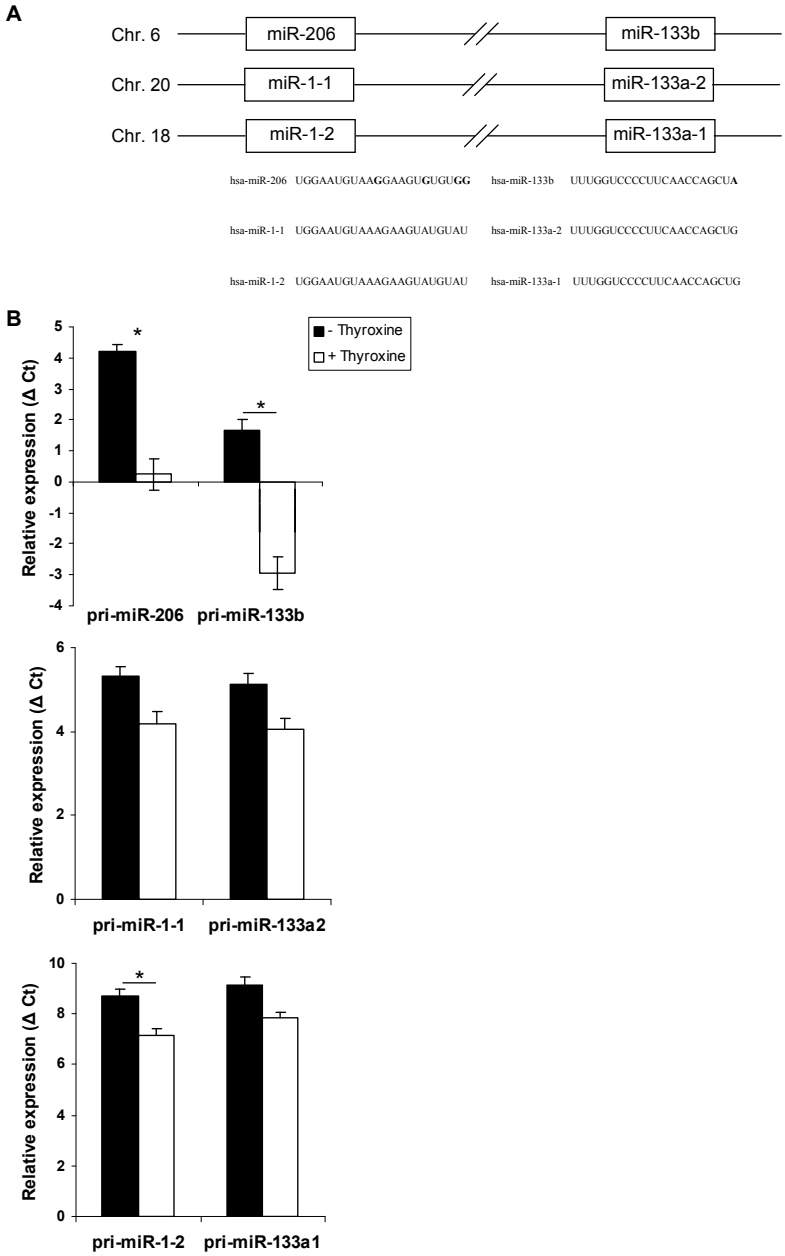

Fig. 4. (A) The bicistronic miR-206/miR-133b, miR-1-1/miR-133a-2 and miR-1-2/miR-133a-1 clusters. The genomic structure of the microRNA pairs is shown (*left panel*). The mature sequences are depicted in the *right panel* (sequence differences in bold). (B) qPCR of primary transcripts of microRNA pairs miR-206 and miR-133b (*upper panel*), miR-1-1 and miR-133a-2 (*middle panel*) and miR-1-2 and miR-133a-1 (*lower panel*) off (black bars) and on (white bars) L-thyroxine treatment. mRNA levels are expressed relative to that of the house-keeping gene cyclophilin A. * P <0.005

in contrast to the down-regulated genes, in which only 4 biological processes were identified (Fig. 3C). The biological processes overrepresented in the group of up-regulated genes are mostly related to fuel metabolism and energy expenditure. Table S3 shows the differentially expressed genes which are represented by the GO categories monocarboxylic acid transport, mitochondrial transport, energy reserve metabolism, organic anion transport, carbohydrate metabolism, lipid metabolism and phosphate metabolism.

Of the 64 genes involved, 10 have been identified earlier by microarray analysis of muscle from healthy subjects treated with T3 (7). A literature search in the PubMed database combined with Google Scholar, using the combination of the key words ''thyroid hormone" and each of the remaining 54 ''gene symbols", yielded only 11 genes, which have previously been reported to be transcriptionally regulated by TH in at least one tissue or cell type. Thus, we identified 43 novel genes implicated in energy and fuel metabolism homeostasis, which are regulated by TH.

Regulation of pri-miR-206 and pri-miR-133b

A considerable number (~6%) of the differentially expressed probe sets had nonannotated Affymetrix IDs. Although they do not represent known protein-encoding genes, some show substantial regulation, suggesting that these transcripts have physiological relevance. Affymetrix ID 240244_at showed a 8.4-fold down-regulation (^{2}log change of –2.9). Interestingly, this represents the gene for miR-133b, which is a short noncoding RNA molecule (microRNA or miR). Fig. 4B (*upper panel*) demonstrates the validation of this finding by qPCR. miR-133b forms a bicistronic pri-miRNA with pri-miR-206. Thus, transcriptional regulation of the pri-miR-133b/pri-miR-206 pair would also affect pri-miR-206 expression. As expected, we observed a marked down-regulation of pri-miR-206 on L-thyroxine treatment (Fig. 4B, *upper panel*), which correlated well with the effects on pri-miR-133b (Pearson correlation 0.89; $P < 0.005$). The closely homologous miR-1-1/miR-133a-2 and miR-1-2/miR-133a-1 pairs (Fig. 4A) (not present on the chip) demonstrated much less, if any, transcriptional regulation by L-thyroxine therapy (Fig. 4B, *middle and lower panel*).

Because miRs may regulate gene expression not only by inhibiting mRNA translation, but also by promoting mRNA degradation, we investigated whether targets of miR-133b were up-regulated. Depending on the prediction programs used, we found a significant overlap of miR-133b target genes with the differentially expressed genes (data not shown). However, the proportion of up-regulated genes among the target genes of miR-133b was not significantly enlarged. Similar results were obtained for miR-206 (data not shown).

DISCUSSION

In recent years, microarray studies have largely extended the number of TH target genes, indicating the broad variety of cellular processes, which are regulated by TH. Gene expression profiles from different animal tissues (*e.g.* brain, liver and muscle) in various TH states have been analyzed (3-6, 11-14). Similar approaches have been performed on human tissues (7, 8), although these are limited because of the availability and accessibility of tissues. Little is known about the effects of TH on the genome-wide regulation of gene expression in skeletal muscle, despite evidence that TH is an important modulator of skeletal muscle (1).

One of the most striking findings revealed by microarray studies in animals is that a large number of genes are regulated negatively by TH (3, 4, 6). We demonstrate for the first time that also in humans a large proportion of genes (~ 43%) is significantly down-regulated by L-thyroxine treatment. While the mechanisms of the TH-dependent positive gene regulation are well understood, the processes via which TH reduces gene expression are much less clear (15). Possibly, ligand-bound TRs directly or indirectly affect other transcription factors, which in turn repress gene expression.

Next, we analyzed whether biological processes were overrepresented in the differentially expressed genes. This analysis was carried out using EASE software, which calculates the overrepresentation of functionally annotated genes for every possible GO term with respect to the genes in the data set. GO categories related to energy expenditure and metabolism were highly enriched in the set of up-regulated genes, in keeping with the well-known function of TH to induce energy processes (2). About 70% of the genes involved in energy and metabolism were newly identified. The identification of these genes may help to understand via which pathways TH influences fuel metabolism. In addition, it is remarkable that a number of classical T3-responsive genes were not detected. Previously, a number of genes have been implicated in T3-dependent energy expenditure in skeletal muscle, including sarco/endoplasmic-reticulum calcium ATPase 1 (SERCA1), SERCA2, malic enzyme, phosphoenolpyruvate carboxykinase (PEPCK), myosin heavy chain I (MHC1), beta-adrenergic receptor (βAR), glucose transporter 4 (GLUT4) and uncoupling protein 3 (UCP3). Although probe sets for all these genes were present on the chip, only SERCA2 and UCP3 were differentially expressed (1, 16). This may reflect methodological differences as the T3-responsiveness of these genes has previously been studied *in vitro* and/or in rodents (see references in Ref. 1). For example, muscle samples were obtained after fasting, whereas it has been shown that fasting decreases GLUT4 expression (17). In any case, our findings reiterate that observations in animals and *in vitro* studies do not necessarily reflect the human situation.

Insulin sensitivity is disturbed during hypothyroidism, but it improves during L-thyroxine replacement therapy (18). Impaired insulin sensitivity, among others charac-

terized by increased glucose concentrations, results from impaired insulin-dependent glucose uptake and decreased glycogen synthesis in skeletal muscle (19). It has been proposed that changes in glucose transporters, like GLUT4, may underlie these observations (20). However, this was not supported by our findings, because GLUT4 was minimally detectable in our experiment. In contrast, we observed a significant induction of genes critically involved in glycogenesis (hexokinase 2, HK2; glycogen synthase, GYS1; glycogen branching enzyme). Indeed, reduced insulin sensitivity has been associated with decreased HK2 and GYS1 expression and activity (21-23). Therefore, stimulation of these enzymes may be the molecular basis of the positive effects of L-thyroxine replacement on glucose metabolism (18). The fructose transporter SLC2A5 (GLUT5) was most potently stimulated (~15-fold) in keeping with previous reports (7, 24). Although physiological blood fructose levels are much lower than serum glucose concentrations, fructose may account for a significant amount of glycogen formation (25). Thus, the TH-mediated increased GLUT5 expression may result in increased intracellular fructose and hence contribute to glycogen formation. Obviously, the significance of this finding remains to be clarified.

Our study has certain limitations that need to be taken into account when considering the results. Firstly, it should be noted that the changes in gene expression may not necessarily reflect direct effects of TH. Gene transcription may also be indirectly dependent on TH, if the hormone modulates intermediate signaling molecules, which influence the muscle transcriptome. Furthermore, other variables such as increased exercise during L-thyroxine treatment may contribute to the observed effects. Secondly, skeletal muscle has a heterogenous fiber composition. TH may have differential and even opposite effects on gene transcripts in different fiber types (1). In addition, completely different cell types, such as fibroblasts, endothelial cells, smooth muscle cells, blood cells, which are present in the biopsies, may obscure gene transcript changes. Therefore, gene expression changes may become undetectable, if the sum of gene expression in different fibers or cell types does not result in a significant change. Thirdly, genes that rapidly and transiently change during L-thyroxine treatment may have been missed by the present approach. Fourthly, although GO analysis is a valuable method in analyzing data sets, it should be carefully used. For example, among the list of down-regulated genes, the categorie "cell differentiation" was overrepresented. Some of these genes are truly inducers of differentiation, suggesting that TH can reduce differentiation. However, genes, which are inhibitors of differentiation, were also linked to this GO term. The down-regulation of these differentiation inhibitors, will result in increased differentiation. Finally, in search strategy for the identification of novel target genes, we used the gene symbols as search terms. Genes which are represented by alternative gene names are not detected in our search.

In addition, several strengths should be mentioned. Firstly, the variability was limited as each subject was its own control. Secondly, we applied strict conditions (i.e. 1.5-fold change at a FDR ≤0.05), which substantiates the robustness of our findings. Thirdly, the present study has some advantages over an earlier microarray study, in which the effects of T3 on skeletal muscle in healthy subjects were examined (7). We analyzed transcriptome profiles in human skeletal muscle in thyroidectomized patients off and on L-thyroxine treatment, probably more closely reflecting gene regulation at physiological TH levels. Furthermore, because of the development of microarray technologies, our chip contained more than twice as many probes (~55,000 *vs*. ~24,000). Apart from the biological variability inherent to these studies, these technical and methodological differences may explain that the number of genes changed by TH treatment identified in both studies was limited to 43 (Table S4).

In the present study we studied changes in gene expression on L-thyroxine treatment. However, TH may affect cellular processes via other routes. This is indicated by the changes in ubiquitin-related genes, ultimately involved in protein degradation, and in alteration of numerous kinase and phosphatase transcripts, involved in post-transcriptional modification. Interestingly, we detected significant regulation of the primary transcripts of the noncoding RNAs miR-206 and miR-133b. miRs inhibit the translation or promote the degradation of large numbers of target mRNAs by annealing to (almost) complementary sequences therein (26). Thus, regulation of one miR may affect numerous target genes. The bicistronic transcripts miR-1-1/miR-133a-2, miR-1-2/miR-133a-1 and miR-206/miR-133b are highly expressed in muscle (27). These miRs are key regulators in muscle differentiation and proliferation (26). Interestingly, it has been shown that the myocyte enhancer factor-2 transcription factor activates transcription of miR-1-1/miR-133a-2 and miR-1-2/miR-133a-1, but not of miR-206/miR-133b (28, 29).

Although we did not show that the levels of mature miR-206 and miR-133b decreased, our data may suggest that TH is an important modifier of the miR-206/miR-133b pair. Little is known about the regulation of miRs by TH. To our knowledge, only one report described effects of TH on miR regulation by showing that miR-208 *null* mice fail to induce β-myosin heavy chain in heart in hypothyrodism (30). We are currently elaborating on the potential of TH to regulate miRs, as this field adds an additional layer of complexity by which TH may regulate cellular processes.

In conclusion, we have demonstrated that L-thyroxine treatment exerts large transcriptional effects in skeletal muscle. The identification of putative T3-target genes may provide a molecular explanation for clinical effects of L-thyroxine therapy. The observation that TH regulates non-coding RNAs markedly broadens the scope by which TH influences cellular processes.

SUPPLEMENTARY TABLES

Table S1. Synthetic oligonucleotides for qPCR

Gene	Sense primer (5′-3′)	Antisense primer (5′-3′)
SLC2A5	TCTTCGGAATCTCCTTGCAAA	CGTCTTTCTTCTGAATCAGCAGGTA
IL32	GCCTTGGCTCCTTGAACTTTT	TTCAGCTTCTTCATGTCATCAGAGA
MYLK4	CAGGATTCAAGATCTGGACATAATGA	TTGACGGGCATCCTTTCC
ITGB1BP3	GCCCAACTGCTGCGTGAT	AGCACGTCCCACTGTTTGAAG
SCN4B	CAGCAGTGACGCATTCAAGATT	TCATCGTCTTTCAACGTCACCTT
UCP3	CGACAGAAAATACAGCGGGACTA	TGTTGGGCAAAGTTCCTTTCC
DKK2	GGGCAGGCCTACCCTTGTA	TTCTCCGACACACCATGCA
CALML6	TGTCACCACCCTGCAGAATC	GCCGACAGGCGCTCTGT
CRIM1	GCGACTCCCTCACCGAGTAC	TTCATTGCATGGTTTAAAACCAA
FEZ2	TGTGTTAATGATGAACCCCTCTTC	GGTCCGGTGATTCCTGCAT
miR-206	AATGTAAGGAAGTGTGTGGTTTCG	CCTGTGCTCCGGGTGGTA
miR-133b	GCAGTCACCTCAGAAGAAAGATG	TCTCCAAGGACTGGGCATTGC
miR-1-1	GATACATACTTCTTTACATTCCATAGCTTAGC	CAGACTGCCTGCTTGGGAAA
miR-133a-2	CCAAATCCATTGGACAGTCGAT	GATCTGGGAGCCAAATGCTTT
miR-1-2	TTTTGGTGTTAGTATTGCCAAAGG	CGTGAATGACCGTCATATGGTAA
miR-133a-1	TTGTCCCGTAGTAATCAATGCATAG	CGCCTCTTCAATGGATTTGG

Table S2. Baseline characteristics of the patients

Age (yrs)	46.5 ± 3.1
Sex (F/M)	6/4
BMI (*kg/m2)*	28.0 ± 1.4
L-Thyroxine dose (µg/day)	195 ± 13.5

Data are presented as means ± SEM

Table S3. Differentially expressed genes which represent the GO categories: monocarboxylic acid transport, mitochondrial transport, energy reserve metabolism, organic anion transport, carbohydrate metabolism, lipid metabolism and phosphate metabolism.

10 genes identified by Clement *et al*. Genome Research 2002.			**43 genes newly identified in the present paper**	
Molecules	*Log Ratio*	*Tissue*	*Molecules*	*Log Ratio*
CDK2AP1	0.756	muscle	ABCC1	1.105
DDR1	0.67	muscle	ACOT11	0.666
EPM2A	0.676	muscle	ADIPOR2	0.705
GBE1	1.771	muscle	AGPAT3	0.63
IMPA2	0.628	muscle	ALPK3	0.619
OSBPL11	0.631	muscle	B3GNT5	2.155
PHKB	0.619	muscle	C4ORF18	0.596
PPP1R2	0.752	muscle	CES3	0.819
SLC25A4	0.6	muscle	DMPK	0.742
SLC2A5	3.955	muscle	DYRK1B	1.319
			FBLN2	0.989
11 genes identified by literature search			GYS1	1.117
Molecules	*Log Ratio*	*Tissue*	IKBKB	1.259
ACLY	0.667	other	MLZE	1.306
ACSL1	0.785	other	MXRA5	1.623
GPD1	0.588	other	NDUFB8	0.593
HK2	1.382	muscle	PFKFB1	1.012
IRAK1	0.645	other	PHKA1	1.021
MAP2K3	1.199	other	PHKG1	1.55
MYOD1	0.659	muscle	PITPNA	0.612
PDK2	0.671	other	PKM2	0.799
SLC16A3	1.537	muscle	PLCD3	0.7
THRSP	1.888	other	PPP3CB	0.687
UCP2	1.436	muscle	PPP6C	0.616
			PRKAG2	0.953
			PRKAG3	0.754
			PTPN20B	0.889
			PYGM	0.599
			RBP1	0.625
			RET	1.301
			SCYL2	0.596
			SEL1L2	0.792
			SGK1	0.76
			SLC16A1	0.922
			SLC16A7	0.7
			SLC1A4	1.102
			SLC1A7	0.623
			SLC25A15	0.811
			ST3GAL6	1.021
			SYNJ2	0.733
			TIE1	0.769
			TPP1	0.681
			WNK2	0.585

Table S4. Overlapping genes with those reported by Clément et al. (Genome Research 2002).

	Clément *et al*.	**Present experiment**
Name	*Fold change*	*Fold change*
ARPC2	2.68	1.69
ASPN	1.76	1.86
BCL6	2.04	-1.55
CD9	2.55	2.05
CDK2AP1	2.33	1.69
COL15A1	2.00	2.14
COL3A1	2.02	3.24
CXCR7	1.32	2.45
CYCS	3.03	1.51
DDR1	1.80	1.59
DNMT3A	2.05	1.86
EIF4G3	1.88	1.52
EPM2A	2.25	1.60
EXTL1	1.68	1.57
FNDC5	2.46	2.11
FRAS1	1.78	2.13
GBE1	2.82	3.41
IMPA2	2.04	1.55
ITGB6	1.74	1.51
KBTBD2	2.18	1.86
KIF21A	1.50	1.71
MAFB	2.06	2.91
MLXIPL	1.79	1.56
MRPL19	2.13	1.66
OSBPL11	2.06	1.55
PDLIM3	1.90	-0.34
PHKB	2.00	1.54
PIK3R1	2.01	-0.72
PPP1R2	2.44	1.68
QKI	2.01	1.52
RP5-1022P6.2	1.90	2.30
SAR1B	2.00	1.51
SBK1	1.55	1.64
SLC25A33	2.32	2.17
SLC25A4	2.66	1.53
SLC2A5	2.66	15.51
SMAD3	2.44	1.57
SPARC	2.11	1.74
THSD4	1.50	1.66
TNCRNA	2.16	1.96
TNFAIP2	1.74	1.64
UCP3	2.30	5.85
WIPI1	2.27	1.58

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Chapter 13

Thyroid hormone signaling is suppressed in progeria and normal aging

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Chapter 14

General discussion

Part of this chapter is based on

Thyroid hormone transporters: the knowns and the unknowns
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''There can be little question that the thyroid hormones must ultimately enter the tissue cells (…)'' (1). This statement formulated some 50 years ago is still a basic principle for understanding thyroid hormone (TH) action. However, the concept about the underlying mechanism for tissue uptake of TH has dramatically changed. While Robins and Rall were convinced that TH traversed the plasma membrane via diffusion, it is currently beyond doubt that transporter proteins are required for plasma membrane transport of TH (2, 3).

The well-known effects of TH on development and metabolism are ultimately the result of changes in signaling pathways at the cellular level. Many actions of TH are mediated by changes in gene transcription, when the bioactive hormone T3 binds to its receptors (TRs) which function as transcription factors (4). Deiodinating enzymes regulate intracellular concentrations of T3. The type 2 deiodinase (D2) converts the precursor T4 to T3, while the type 3 deiodinase degrades T4 to rT3 and T3 to 3,3′-T2 (5).

The prerequisite for the intracellular processes of deiodination and genomic actions of TH is the translocation of the hormone across the plasma membrane. Since the main focus of this thesis concerned TH transport and TH transporters protein, we first describe established concepts of TH transport (the knowns). Next, we discuss several gaps in our knowledge of established TH transporters (the unknowns). Both sections will be biased towards the TH transporter monocarboxylate transporter 8 (MCT8). Then, we reflect on some issues beyond the current dogmas, which may stimulate future research (the guesses). We shortly reflect on TH metabolism and action which we studied in various tissues. Finally, the TOP-R study is put in perspective and future directions of research with this cohort are presented.

TRANSPORTERS: THE KNOWNS

TH transport processes

It has been known for a long time that TH accumulates in tissues. These early observations were well explained by assuming that high-affinity intracellular binding proteins determined intracellular TH accumulation (1). Based on the chemical properties of the molecule, it was rationalized that transfer of TH across the plasma membrane occurred via diffusion (6). Though it was recognized that the side chain was hydrophilic, it was assumed that the hydrophobic aromatic part of T3 and T4 interacts with the lipid bilayer, presumably constituting the first step to enter the cell. Subsequent studies were dedicated to characterize the cell surface binding properties and binding proteins for TH (for a historical overview, see Ref. (2)). Important progress was made by reports showing that TH uptake was saturable, energy- and Na^+-dependent in certain cell types (2). From this it was deduced that transporter proteins were responsible for the transport of TH

across the plasma membrane (2). This initiated experiments aimed to investigate kinetic properties of transport processes by *in vitro* and *in vivo* studies (2).

Only a decade ago, the first TH transporter was identified at the molecular level (7). In the following years, it was demonstrated that iodothyronine derivatives are ligands for several types of transporters, including the Na^+/taurocholate cotransporting polypeptide (NTCP, SLC10A1) (8, 9), multidrug resistance-associated proteins (10), the heterodimeric L-type amino acid transporters LAT1 and LAT2 (reviewed in 11,(11)), various members of the organic anion-transporting polypeptide (OATP) family (reviewed in (12, 13)), and the monocarboxylate transporters MCT8 and MCT10 (reviewed in (14)). Most of these transporters accept a variety of ligands, with notable exceptions for MCT8 (SLC16A2) and to a lesser extent MCT10 (SLC16A10) and OATP1C1 (SLCO1C1).

Function and expression of MCT8

Based on homology, MCT8 belongs to the MCT family. Most members of this family are orphan transporters (MCT5,7,9,11-14), whereas MCT1-4 transport simple monocarboxylates, such as lactate and pyruvate (15). MCT6 is involved in transport of compounds with a carboxyl group, such as the diuretics bumetanide and nateglinide (16).

The *MCT8* gene is located on human chromosome Xq13.2. The 6 exons encode a protein with 12 predicted transmembrane domains (TMDs); both N and C terminus have a cytoplasmic localization.

A major breakthrough was accomplished when rat (r) Mct8 was identified as a specific transporter of TH (17). Functional characterization of rMct8 in *Xenopus laevis* oocytes revealed that uptake of the iodothyronines T4, T3, rT3 and 3,3'-T2 was strongly stimulated if rMct8 was expressed (17). Neither sulfated and sulfamated iodothyronines nor amino acids (Leu, Phe, Tyr, Trp) and lactate were transported by rMct8. This specificity was substantiated by inhibition studies, demonstrating strong effects of iodothyronine derivatives on rMct8-mediated T3 and T4 uptake, in contrast with several other compounds.

Subsequent studies of human MCT8 showed that its TH transport characteristics are highly similar to rMct8 (18). Importantly, it was demonstrated that MCT8 markedly enhances intracellular deiodination of various iodothyronines. Furthermore, coexpression of MCT8 with the cytoplasmic TH binding protein CRYM resulted in a strong accumulation of intracellular TH. These data strongly supported the concept that MCT8 facilitates transport of TH across the plasma membrane and, thus, provides intracellular compartments including cytosol and endoplasmic reticulum with TH. In contrast to other members of the MCT family, we established in **chapter 1** that expression and function of MCT8 appears to be independent of the ancillary proteins basigin, embigin or neuroplastin (19). MCT8 rather appears to form homodimers as evidenced by *in vitro* studies (19, 20).

In different species, MCT8 expression has been detected in numerous tissues, including brain, heart, liver, kidney, adrenal and thyroid gland (21, 22). Because mutations in *MCT8* result in a neurological phenotype (see below), MCT8 expression in mouse brain has been investigated in further detail. Intense staining of *Mct8* was observed in the choroid plexus (23-26). Prominent *Mct8* expression was also noted in different cortical layers, parts of the limbic system (amygdala, hippocampus and olfactory bulb) and hypothalamus (23, 25). Furthermore, MCT8 is expressed in microvessels of mice and humans (25, 26). This is in agreement with functional studies suggesting that Mct8 is important for transport of T3 across the blood-brain barrier (24, 27, 28). In humans, MCT8 expression has been documented in cortex, hippocampus and circumventricular organs (25, 26, 29, 30). Furthermore, MCT8 is present in different parts of the human hypothalamus and folliculostellate cells of the anterior pituitary (29, 30). Taken together, these data demonstrate that MCT8 has a distinct expression pattern in brain.

Knowledge about MCT8 regulation is limited, although several studies have incidentally reported on changes in MCT8 expression under different conditions. With advancing age, *MCT8* expression alters as evidenced by changes in *MCT8* expression during brain development of mice, in livers of old rats and in human placenta with advancing gestation (23, 31, 32). Elevated *MCT8* expression was found in placenta associated with severe intrauterine growth restriction, in femurs of hypothyroid mice, and in liver and skeletal muscle of critically ill patients and rabbits (32-34).

Down-regulation of MCT8 expression in osteoblasts and rabbits with critical illness following TH treatment suggests a compensatory mechanism (33, 34). However, thyroid state is not a modulator of MCT8 *per se*, as hypothalamic MCT8 staining was unremarkable in a hyperthyroid subject (29). Moreover, hypothyroidism in athyroid *Pax8* KO animals induces expression of *D2* in brain, ensuing increased local T3 production, whereas *Mct8* expression is not affected (23). Thus, MCT8 is regulated under different conditions, although the mechanisms and appropriateness of these responses remain to be elucidated.

Mutations in *MCT8*

The importance of MCT8 for human physiology was unequivocally demonstrated by the identification of *MCT8* mutations in males with mental retardation and endocrine abnormalities (35, 36). The clinical features are most prominently manifested in the neurological abnormalities (for an extensive description of typical clinical characteristics see Ref (37)). Although there is phenotypic variability (see below), all affected males display marked cognitive disabilities with IQ values mostly below 40 (37). Speech development is typically absent and the patients communicate by non-verbal acts such as laughing, crying and making sounds.

Most patients are characterized by a severe axial hypotonia, of which poor head control is the most obvious feature. All patients display muscle hypoplasia. Hypotonia of the limbs in childhood progresses into spastic quadriplegia with advancing age. Involuntary and dystonic movements, which can be provoked upon stimuli, are commonly described. Whereas height is unremarkable, weight is below the 3rd percentile in most patients.

Heterozygous females typically do not display a neurological phenotype. Incidentally, cognitive impairments have been reported in female carriers, but a causative link with MCT8 mutations is unproven (37). To date, an obvious neurological phenotype similar to affected males has been reported in one female with a heterozygous *MCT8* mutation accompanied by unfavourable X-chromosomal inactivation (38). In the first years of life, global myelination delay may be detected by MRI (39, 40).

Because the clinical phenotype of the affected males was reminiscent of the Allan-Herndon-Dudley syndrome (AHDS), originally described in 1944, Schwartz *et al*. tested the involvement of *MCT8* in this disease (41). They demonstrated that mutations in *MCT8* are the genetic explanation for affected males in AHDS families. Recently, *MCT8* mutations have been reported in patients with Pelizaeus-Merzbacher-like disease (PMLD), which display clinical features similar to AHDS (42). Apparently, it is difficult to classify genetically distinct mental retardation syndromes on basis of clinical features. This leaves the possibility that *MCT8* is affected in other eponymous syndromes clinically resembling AHDS, of which the genetic basis is as-yet-unknown.

Characteristic for patients with *MCT8* mutations is the remarkable combination of TH serum concentrations. The most common finding are the elevated serum T3 and reduced T4, FT4 and rT3 concentrations. TSH levels are usually within the normal range, but mean TSH is doubled compared to non-affected individuals. Regarding the endocrine derangements, it is important to consider the following. Firstly, abnormalities may be subtle, depending on the severity of the disease (see below). Secondly, values must be interpreted relative to age-specific reference ranges. This is essential for serum T3 concentrations, which are higher in infants. Thirdly, the combination of the above-mentioned TH parameters is pathognomonic for MCT8 patients, rather than a single measurement, such as serum T3.

To date, *MCT8* mutations have been reported in over 50 families (for a recent overview see (43)). This suggests that *MCT8* mutations are not a rare cause of X-linked mental retardation, although the prevalence is currently unknown.

Many different types of mutations have been identified. Approximately 30% are (large) deletions or mutations introducing premature stop codons. Such mutations conceivably result in a complete loss of transporter function, but to deduce the impact of single amino acid substitutions, insertions or deletions on MCT8 function is complicated. Two models have been used to study the effects of MCT8 mutations on its transport capac-

ity: mammalian cells (transiently or stably) transfected with mutant MCT8 cDNA and fibroblasts derived from MCT8 patients.

Our lab undertook the functional analysis of MCT8 mutations by transfection studies in JEG3 cells, which express little endogenous MCT8. Cells over-expressing wild-type and mutant MCT8 cDNA alone or in combination with the deiodinases D2 or D3 were tested for T3 and T4 transport and metabolism (e.g. **chapters 1**, **2**, **5** and **10**). Most mutants were shown to be inactive, although some exhibited (significant) residual activity. The mutants with complete loss-of-function are mostly explained by a decreased plasma membrane expression as evidenced from a predominant cytosolic localization or by a diminished or absence of protein expression (44). Trafficking of mutant MCT8 to the plasma membrane appears cell-type dependent, probably reflecting differences in the cellular machinery such as regulatory proteins or the capacity to form heterodimers with wild-type MCT8 (19, 45).

In **chapters 5** and **6** we used skin fibroblasts obtained from patients as a second system to investigate the effects of *MCT8* mutations (46, 47). Although MCT8 expression was not detected by western blotting or immunocytochemistry, the transporter appears functionally important as T3 and T4 uptake were reduced by 50-60% in fibroblasts from patients as compared to controls (46, 47). The advantage of this model is that the analyses are performed in the genetic background of the individual.

Although it is too early to localize hotspots in the *MCT8* gene, several mutations have been reported twice in unrelated families (43). This may be explained if particular sequences are prone to mutation and/or if not all mutations affect transport function. The latter suggestion is supported by an MCT8 polymorphism (S107P), which is not associated with clinical symptoms or TH parameters (48, 49). Even apparently drastic mutations do not necessarily impinge on MCT8 transport function. Transport was not affected in a series of experiments in which MCT8 Cys-to-Ala mutants were studied (19). Furthermore, an M402I mutation in a male with mental retardation did not affect T3 and T4 transport in cells over-expressing the mutant MCT8 or in the patient's fibroblasts (*unpublished observations*). Thus, to address the pathogenicity of MCT8 mutations, it is required to test their effects on transport function.

Treatment is currently limited to supportive care, although the first initiatives to counteract the damaging effects of the endocrine abnormalities have been reported (see under The unknowns). Diagnosis is important for providing an etiologic explanation of the disease to the family and consequently for genetic counselling.

Relevance of *Mct8* knockout mice for patients

Different *Mct8* knockout (KO) mouse models have been created to understand the mechanisms of disease in patients with *MCT8* mutations (24, 25, 27, 50). *Mct8* KO animals have markedly decreased T4 and rT3 levels and increased T3 and TSH levels compared to

wild-type animals, thereby perfectly representing the endocrine 'fingerprint' observed in *MCT8* patients (27, 50).

Despite the significant expression of *Mct8* in liver, T3 uptake into the liver was not different in mutant *versus* control mice. This explains the increased T3 concentration in liver tissue and the elevated T3-sensitive type 1 deiodinase (D1) expression and activity. Increased D1 activity in liver of *MCT8* patients perfectly explains the decreased serum levels of rT3. Because rT3 is the preferred substrate of D1, increased D1 activity results in an increased rT3 degradation (51). Furthermore, rT3 production is probably low because of the low T4 substrate levels.

From the inappropriately high TSH levels in *MCT8* patients and in *Mct8* KO mice, it was hypothesized that the hypothalamus-pituitary-thyroid axis was compromised in *Mct8* KO mice. Therefore, the hypothalamus and pituitary were further investigated to address at which level the negative feedback loop was affected. At the hypothalamic level, expression of the T3-responsive thyrotropin-releasing hormone (TRH) was increased in *Mct8* KO animals. Only high doses of T4, but not of T3, reversed the abnormal TRH expression. This observation indicates that the hypothalamus of *Mct8* KO mice still responds to local T4 to T3 conversion. Presumably, an impaired Mct8-mediated T3 uptake is responsible for the aberrant TRH expression.

Expression of several T3-responsive genes is not affected in the pituitary of *Mct8* KO animals, indicating adequate intracellular T3 levels. This is most likely ensured by the increased pituitary D2 activity, which compensates for the decrease in serum T4, resulting in the maintenance of local T3 production. In *Mct8* KO mice rendered hypothyroid, only a high dose of T3 is able to restore TSH levels, whereas in wild-type animals similar TSH levels were achieved using a low dose of T3. This may be explained in part by the lack of effect of T3 on the hypothalamus.

Thus, the studies in *Mct8* KO mice indicate that the hypothalamus and (consequently) the pituitary are relatively insensitive to TH. Together with the expression of MCT8 in human hypothalamus and pituitary, the animal studies provide a plausible explanation for the inappropriately elevated TSH levels in patients with *MCT8* mutations.

The most unanticipated finding in *Mct8* KO mice is the absence of an overt neurological phenotype (27, 50). Recently, extensive neurological and behavioral testing revealed only subtle behavioral abnormalities in *Mct8* KO mice (25). These results are surprising, because uptake of T3 in brains of *Mct8* KO mice is largely impaired (27, 50). Consequently, T3 content in brain is also decreased.

The decreased D3 activity, which is inversely regulated by its substrate T3, probably reflects a beneficial response aimed to counteract the harmful effects of decreased T3 transport. Parallel with the decreased serum T4 levels, brains of *Mct8* KO animals contain less T4 than wild-type mice. In *Mct8* KO brains, D2 activity, which is inversely regulated by its substrate T4, is increased. Thus, local T3 production in brain may be relatively normal

due to the decrease in T4 levels and increase in D2 activity. Brain T3 levels are nevertheless markedly decreased despite high serum T3 levels and decreased T3 degradation, due to a dramatic reduction in brain T3 uptake.

T3 uptake (measured in a 15-min period) in cultured neurons from *Mct8* KO mice was diminished compared to wild-type mice (25). However, expression of the T3-sensitive gene *Hairless* with increasing concentrations of T3 added to cultured neurons did not differ between *Mct8* KO and wild-type animals (24). Also, the effect of T3 on dendritic outgrowth in cultured neurons was similar in *Mct8* KO and wild-type animals (27). This may be explained by differences in neuronal cell populations used in the different studies, where subsets of neurons may be more vulnerable to lack of functional Mct8 than others. Indeed, in contrast to other brain regions, neuronal cells in the striatum are somewhat hypothyroid, as assessed by the decrease in the T3-responsive gene *RC3* (27).

The above-mentioned studies suggest that Mct8 has an important role in the transport of T3 across the blood-brain barrier in mice. In mice, T4 transport into the brain is not dependent on Mct8 or is compensated by alternative transporters. Apparently, the level of T3 in brain of *Mct8* KO animals is sufficient to prevent gross neurological dysfunction in mice.

MCT10, OATP1C1 and other transporters

MCT10

To search novel TH transporters, it is reasonable to investigate transporter families, which have ligands related to TH. Because the basic structure of iodothyronines resides in the assembly of 2 Tyr residues, an obvious candidate to test was a T-type amino acid transporter (TAT), which mediates uptake or efflux of aromatic amino acids. In support of this hypothesis, specific interactions between T3 and aromatic amino acids, mostly Trp, have been observed in various cell types such as erythrocytes and hepatocytes (52-54). At the molecular level, TAT1 was identified as a transporter, which facilitates transport of the aromatic amino acids Phe, Tyr and Trp and L-DOPA (55, 56). Based on its homology with other MCTs, TAT1 is also named MCT10. Among the MCT family, MCT10 is most closely related to MCT8 with an amino acid sequence identity up to 49%.

MCT10 has a gene and protein structure which is similar to MCT8. It contains 6 six exons, which code for a 515-amino acid protein. It has a typical transporter structure of 12 TMDs and both protein termini are located intracellularly. Based on the above considerations, it was tested if T3 and T4 are ligands for MCT10. Therefore, cells were transfected with human MCT10 and intracellular accumulation of T3 and T4 was assayed (57). These experiments were the first showing that MCT10 facilitates TH transport with a preference for T3. This was underscored by subsequent experiments in which MCT10 was co-transfected with different deiodinases. These studies demonstrated that MCT10

enhances intracellular metabolism of T3 to a larger extent than metabolism of T4. Under identical conditions, T3 transport mediated by MCT10 exceeds that by MCT8.

T3 uptake by MCT10 is clearly dependent on the type of incubation medium used. If cells were incubated in medium containing large amounts of nutrients (DMEM/F12), MCT10-mediated T3 uptake was much less than in 'poor' medium (D-PBS). This may be partially explained by the presence of aromatic amino acids in the 'rich' medium, which impinge on net T3 uptake. Direct assessment of aromatic amino acids on T3 uptake revealed that Trp most strongly affected net accumulation of intracellular T3 in cells transfected with MCT10. These studies suggest that MCT10 is responsible for the previous observations of the interactions between T3 and Trp. Like MCT8, MCT10 is involved in bidirectional transport of TH across the plasma membrane.

Information about physiological function and expression is limited. MCT10 is ubiquitously expressed, among others in intestine, kidney, liver, muscle and placenta (54-56, 58). MCT10 appears predominantly localized in the basolateral membrane (58).

OATP1C1

OATP1C1 belongs to the OATP family, which is a large family of which many members accept a wide variety of ligands. OATP1C1 has several characteristics which distinguish this transporter from other OATPs. First, OATP1C1 displays a narrower ligand specificity towards T4, rT3, T4S, estrone-3-sulfate, estradiol-17β-glucuronide and bromosulfophthalein (12, 59). Second, it has been reported that OATP1C1 has the highest affinity for iodothyronines compared with other OATP family members (12). Recently, it has been suggested that OATP1C1 displays atypical transport kinetics, involving two different (high and low affinity) T4 binding sites (60). Third, many OATPs demonstrate a pH-dependent transport activity, except for OATP1C1. Recently, an elegant study suggested that the pH sensitivity of OATP-mediated transport depends on a His residue, which is conserved in many OATPs, but not in OATP1C1 (61). Interestingly, when the corresponding amino acid in OATP1C1 was mutated to a His residue, T4 transport mediated by OATP1C1 became pH dependent.

Expression of OATP1C1 has been noted in the Leydig cells of the testis and in brain capillaries in rat and mice, leading to the suggestion that Oatp1c1 is expressed at the blood-brain barrier (62-64). In addition, OATP1C1 staining is observed in many human brain regions (65). Species-specific expression differences were underscored by the observation that Oatp1c1 is markedly enriched in rodent cerebral microvessels, but only marginally in human microvessels (26). An interesting aspect of Oatp1c1 regulation is the inverse relationship with TH concentrations. In hypothyroid rats, Oatp1c1 expression in brain capillaries increases, whereas the opposite is observed in hyperthyroid animals (62).

The relevance of OATP1C1 for physiology is unknown. Genetic variation in the OATP1C1 gene is not associated with changes in serum TH parameters (59). However, polymorphisms in OATP1C1 were associated with subtle changes in clinical endpoints such as fatigue and depression in T4-substituted hypothyroid patients (66). These observations hint at a physiological role of OATP1C1 in brain.

Other transporters

Much less information is known about the growing number of transporters, which have been added to the list of proteins which mediate TH transport in recent years. Most of them are multispecific and the relevance *in vivo* is presently unclear. An exception is the liver-specific transporter OATP1B1, which facilitates uptake of, among other ligands, sulfated iodothyronines, estrone-3-sulfate (E1S) and bilirubin (67). The well-studied polymorphism V174A is associated with higher serum T4S, E1S and bilirubin levels in humans. *In vitro* experiments have shown that this variant is less active in transporting these ligands, providing the explanation for the associations found in humans.

TRANSPORTERS: THE UNKNOWNS

The progress made in the understanding of cellular TH transport in recent years has also increased our awareness that we are just beginning to recognize the complexity of this aspect of TH homeostasis. In the following sections, we will define gaps of knowledge, which require additional studies in the near future.

MCT8

Even though MCT8 is the TH transporter which has gained most attention during last years, much remains to be elucidated. Starting at the structural aspects of the protein, MCT8 has an intriguing N-terminal domain enriched in Pro (P) and Glu (E) repeats, defining three so-called PEST domains. PEST domains are hydrophilic stretches of 12 or more amino acids containing at least one P, E or Asp (D) and one Ser (S) or Thr (T), flanked by Lys (K), Arg (R) or His (H) residues (68). In fact, this was the reason why the gene was initially designated as XPCT, for X-linked PEST containing transporter (69). Approximately 10% of the mammalian proteins contains PEST domains and it has been demonstrated that PEST-containing proteins are subject to rapid degradation (68). However, this concept was challenged recently by whole proteome profiling of mammalian cells which demonstrated that rapidly degraded proteins are not enriched in PEST sequences (70). In **chapter 3**, we aimed to study an MCT8 variant in which the PEST domain was deleted. Unfortunately, this variant was not expressed, thus precluding direct analysis of the PEST

domain. Thus, the function of these PEST motifs in MCT8 is still elusive. Furthermore, our findings suggest that MCT8 is not subject to rapid degradation.

The human *MCT8* gene contains two translation start sites (TLSs), resulting in a 613- or 539-amino acid protein, depending on which TLS is used. The first TLS is lacking in species such as rat and mice. Most studies addressing the function of (mutated) MCT8 have used an MCT8 cDNA which only contains the second TLS (18, 20, 44, 47, 57, 71). Although the second TLS conforms better to the consensus Kozak sequence, both TLSs have an adequate sequence. This is supported by recent *in vitro* studies demonstrating that translation may also start from the first start codon (45). Proteomic analysis of membranes in mesenchymal stromal cells identified MCT8 by a sequence closely to the first TLS, which is the first evidence that the first TLS is used *in vivo* (72).

The long MCT8 isoform contains an additional N-terminal PEST domain, but it does not change the 12 putative TMDs. Therefore, it seems unlikely that transport function is different between both isoforms. Possibly, it may have effects on expression or subcellular localization. Apparently, lack of the long isoform is not required for normal development, because a missense mutation in the first ATG (c.1A>T), making translation from this start codon impossible, was found in a non-affected male (38). However, it cannot be excluded that this mutated sequence may function as a TLS as it is known that non-AUG TLSs may also be used to start translation, although at a lower efficiency (73). Thus, the relevance of the long isoform in humans remains to be clarified.

Relating to the afore-mentioned issues, regulation of MCT8 is poorly understood. Incidental studies have reported on MCT8 expression in different (patho)physiological states (see section MCT8 under The knowns), though underlying mechanisms are presently unclear. **Chapter 3** suggests that MCT8 is ubiquinated, although it is unknown whether ubiquitinated MCT8 is degraded or has another function. Future studies may initially employ cell lines, which express endogenous MCT8, or use classical promoter analysis tools to address regulation of MCT8. Theoretically, important aspects of MCT8 regulation such as the promoter region, distinct transcriptional regulators or interacting proteins may be discovered in studies in patients with the clinical and endocrine phenotype of AHDS, but without mutations in the protein-encoding parts of the *MCT8* gene.

Two major unknowns concern observations in patients with MCT8 mutations. Firstly, the origins of the abnormally high serum T3 and low serum T4 levels are unknown. Several possibilities may explain this part of the endocrine phenotype. Assuming that MCT8 provides D3-expressing neurons in brain with T3, it is conceivable that inactivation of transport results in a decreased T3 inactivation. Consequently, serum T3 levels will increase, which stimulate D1 activity, which results in a further conversion of T4 to T3. This scenario would explain the low T4 and rT3 levels (both consumed by D1) and high T3 levels (decreased inactivation and increased T4-T3 conversion). The causative role of D3 in the pathogenesis of the abnormal serum thyroid parameters is challenged

by observations in *Mct8/Pax8* double KO mice, which lack a functional thyroid gland and are dependent on exogenous TH substitution (74). T3 treatment in these animals did not result in elevated serum T3 levels, suggesting that the increased serum T3 levels are not the consequence of decreased T3 inactivation by D3 (74).

Rather, evidence suggests that T4 (and T3) accumulates in the kidneys of *Mct8* KO mice, which may cause a further increase in renal T3 production. Moreover, a decreased thyroidal T4 and increased T3 secretion may contribute importantly to the altered serum TH levels in *Mct8* KO animals (75, 76). It is fascinating to speculate about the possible function of MCT8 in the thyroid gland. Many steps in the TH biosynthesis are well understood, but the mechanism by which TH is secreted into the bloodstream is still elusive. It should be investigated whether MCT8 in the thyroid gland plays a role in this process, and if so, to which extent a thyroid gland devoid of functional MCT8 contributes to abnormal thyroid parameters in serum.

Taken together, there are several indications to believe that a decreased D3-mediated inactivation of T3 does not play a major role in the pathogenesis of the serum TH levels. Supportive of a prominent role of D1 in the endocrine phenotype comes from observations that rT3 levels are already low at P7 in *Mct8* KO mice, whereas T4 and T3 are still normal (28). Preliminary data indicate that inactivation of *Mct8* in mice that already lack *Dio1* and *Dio2* does not further affect serum T3 and T4 levels (28). Possibly, the combination of altered deiodinase activities and accumulation of TH in tissues may explain the endocrine phenotype. Further studies are warranted to unravel the precise mechanisms of the abnormal serum T3 and T4 levels in MCT8 patients.

The other major unknown concerns the pathogenesis of the neurological phenotype in patients with *MCT8* mutations which is far from established. This is partially related to the absence of a gross neurological phenotype in *Mct8* KO mice. Several attempts have been made to explain this discrepancy. First, mouse brain may respond differently to TH deficiency compared to human brain. However, it is known for years that hypothyroidism induces structural brain abnormalities in rodents, which is why hypothyroid animals have been used as models to study the effects of TH on brain development (77). Second, MCT8 may transport alternative ligands which are important for human, but not for mouse brain development. This is mainly based on the observation that other members of the MCT family transport monocarboxylates and amino acids. However, experimental evidence for MCT8 transport of ligands in addition to iodothyronines is limited (78).

Third, the mouse may employ compensatory mechanisms such as the expression of alternative transporters, which are lacking in humans. This is suggested by observations that Oatp1c1 is present in mouse, but absent in human brain microvessels (26). It has also been suggested that LAT2 may compensate if Mct8 is deficient in mice, mainly based on different LAT2 expression levels in mice *versus* humans (25). Currently, there is little evidence to indicate which of the above mechanisms is the most likely

explanation for the human-mouse difference in phenotype resulting form inactivation of MCT8. Explanations for the neurological impairment in MCT8 patients assume that neurons suffer from an insufficient T3 supply. This scenario is based on extrapolations from other diseases in which a lack of sufficient TH plays a role in the brain phenotype, such as untreated congenital hypothyroidism or cretinism. However, this hypothesis should be experimentally validated, before any definite conclusion may be drawn. A first requirement to understand the underlying mechanisms of disease is a comprehensive knowledge of MCT8 expression in human brain in comparison with the localization of other genes important for TH regulation. The identification of target genes downstream of MCT8 is essential for our understanding of the molecular derangements if MCT8 is deficient. It is also possible that inactivation of MCT8 results in an imbalance of T3 supply to neurons which express MCT8 and neurons which express other T3 transporters. In **chapter 6**, we combined gene expression profiling in patient fibroblasts with the human brain transcriptome and specified which genes are likely disrupted in brains of MCT8 patients. In addition, the studied microarrays of human brain samples provided clues where and when MCT8 is expressed.

Apart from the role of MCT8 in providing cells with T3 to initiate a T3-dependent transcriptional program, MCT8 may also be involved in mediating so-called non-genomic actions. Possibly, MCT8 may also function in providing the brain with transcriptionally inactive iodothyronines. It has been reported that T4 and rT3 regulate actin polymerization and microfilament organization, whereas T3 does not alter these processes (79). If this requires the intracellular availability of these iodothyronines, transporters are also required to for their translocation across the plasma membrane. Given the low serum T4 and rT3 concentrations in MCT8 patients, diminished non-genomic actions in brain may contribute to the phenotype.

To date, no curative therapy is available for *MCT8* deficiency. Recently, the first initiatives for possible treatment of patients with *MCT8* mutations have been reported. In a 16-year-old MCT8 patient, referred for feeding problems and very low body weight, block-and-replace therapy as applied to patients with primary hyperthyroidism was tested (80). The combination of the anti-thyroid drug PTU and L-thyroxine replacement resulted in a normalization of T4, T3 and TSH levels. During this therapy, body weight significantly increased. Unfortunately, neurological improvements were not observed and perhaps also not expected. If exposure of the developing brain to abnormally low serum T4 and high T3 levels plays an important role in the pathogenesis of AHDS, early normalization of these TH levels may well be an effective therapeutic option.

Theoretically, compounds which mimic the action of TH, but rely on other transporters than MCT8 for cellular entry, would counteract the neurological damage. This prompted the investigation of the effects of 3,5-diiodothyropropionic acid (DITPA) in comparison with T4 in hypothyroid *Mct8* KO mice (81). Normal doses of T4 did not correct the el-

evated TSH levels and D2 activity in brain of *Mct8* null mice. Treatment with supraphysiological T4 doses normalized these parameters, but also increased D1 activity in the liver, indicating peripheral hyperthyroidism. Promising effects were observed with high doses of DITPA, which produced similar beneficial effects on the brain parameters, but avoided thyrotoxicity in the liver. Importantly, in *Mct8* KO mice without experimental hypothyroidism, beneficial effects of DITPA treatment on brain remained without affecting thyroid state in the liver.

Obviously, further detailed studies need to be done to rule out possible negative effects of DITPA. Furthermore, it should be investigated to which extent DITPA replaces TH in normal brain development, for example by comparing the effects of DITPA *versus* T4 on brain development in *Pax8* KO mice. However, given the large differences in neurological phenotype of humans *versus* mice and the absence of an appropriate model to investigate possible treatment regimens, it seems reasonable to explore such treatment possibilities in MCT8 patients (82). Alternatively, naturally occuring TH analogues such as Triac (3,5,3′-triiodothyroacetic acid) and Tetrac (3,5,3′,5′-tetraiodothyroacetic acid) should also be investigated. Ultimately, early initiated gene therapy might largely prevent the neurological phenotype. Although these technologies are still being developed and are surrounded with a variety of challenges, several successes have been achieved (83-85). Of particular interest is the recent report demonstrating beneficial effects of gene therapy in adrenoleukodystrophy, a severe progressive brain disease caused by mutations in the X-chromosome located *ABCD1* transporter gene. In two boys with adrenoleukodystrophy gene therapy successfully stopped or reversed the progressive disease course (86).

Obviously, the earlier treatment is started the higher the potential to prevent or reverse abnormalities. This requires early detection of *MCT8* mutations. Most patients with *MCT8* mutations have been identified from several months onwards. However, serum TH abnormalities may already be detectable at birth. Retrospective analysis of the T4-based neonatal screening for congenital hypothyroidism revealed that most patients had low T4 levels in the blood spot (47). However, based on the current screening strategy in The Netherlands, no further action was undertaken as TSH levels and T4/TBG ratios were not abnormal. Therefore, it should be investigated if high T3 and low rT3 levels are already present in neonates with *MCT8* mutations, and if screening protocols could be adapted to identify MCT8 patients. Taken together, the first steps towards a treatment for MCT8 patients have been made. Future research should be dedicated to explore novel therapeutic options and early detection of disease.

In vivo **relevance of MCT10 and OATP1C1**

Although the expression patterns of MCT10 and OATP1C1 are compatible with an important role of these transporters in TH physiology, the functional relevance *in vivo* of

MCT10 and OATP1C1 remains to be established. Because MCT10 and OATP1C1 transport other ligands in addition to TH, fundamental questions for thyroid research are whether these transporters are primarily involved in regulating TH homeostasis or whether transport of other ligands is their principal function. Mice deficient in *Mct10* and *Oatp1c1* are the most straightforward models to answer these questions. Similar to the story of MCT8, it is important to realize that species-specific gene expression and compensatory mechanisms may mask certain phenotypes.

In cultured cells, endogenous expression of transporters may largely differ from the cells they represent *in vivo*. For example, *Mct8* and *Oatp1c1* were highly down-regulated in cultured microvascular endothelial cells compared with freshly isolated cells (87). Once the importance of MCT10 and OATP1C1 for TH physiology has been defined, further studies are needed to characterize the interaction between TH and other ligands as well as the expression patterns and regulatory mechanisms. Ultimately, novel syndromes caused by pathogenic mutations in these transporters may reveal their relevance for human physiology.

Are there novel TH transporters to be expected?

The identification of numerous TH transporters during last decade raises the question if all TH transporters have been identified. Several basic and clinical studies suggest otherwise, implicating that other TH transporters are left in store to be discovered in the future.

From studies investigating TH transport into hepatocytes evidence emerged for the existence of a high-affinity, Na^+-dependent transporter (2). It should be realized, however, that most of these experiments to investigate the Na^+-dependence of transport were carried out using the Na-K-ATPase blocker ouabain. Inhibition of transport by ouabain is not necessarily explained by direct effects of Na^+ on the transporter, but may also be caused by indirect effects of Na^+, for example, on cellular energy state or intracellular TH binding proteins. Furthermore, ouabain is a ligand for OATPs and may thus compete with TH transport (88).

Notwithstanding these remarks, the possibility of a high-affinity, Na^+-dependent liver TH transporter remains an appealing explanation for these observations. In support of this view, detailed kinetic studies in a patient with abnormal serum TH concentrations suggested a defect in iodothyronine uptake by the liver (89). Thus, the molecular identity of the putative high-affinity, Na^+-dependent TH transporter in the liver remains to be clarified.

In view of the complexity of the brain, it would be surprising if MCT8 and OATP1C1 are the only TH transporters in brain. Different brain areas and different cell types may express various transporters for optimal specificity in TH regulation. An obvious candidate for further research is OATP3A1, which exhibits a relative specificity for T4 transport (90).

Interestingly, there are two functionally active OATP3A1 isoforms with highly specific expression patterns in rat and human brain.

MCT8 and MCT10 are localized in the basolateral membrane of tissue cells and both facilitate uptake and efflux of TH. Other TH transporters may exist, located in the basolateral or apical membrane of polarized cells, which facilitate only uptake or efflux. This is particular relevant in the current working hypothesis of local TH control in brain, where T4 is transported into astrocytes by an as-yet-unknown transporter and subsequently converted to T3 by D2. It is assumed that an unidentified T3 exporter exists, which ensures the release of T3 from the astrocytes, which becomes available for adjacent neurons.

So far, most studies have been dedicated to characterize TH uptake, and little information is available about TH efflux processes (10, 18, 57, 91-94). Studies of TH efflux are technically complicated as it requires prior loading of cells through specific TH uptake transporters. Such limitations may be overcome by the use of polarized cells cultured in double-chamber dishes.

Several strategies may be used to identify novel TH transporters. In **chapter 7** we tested family members of a transporter (SLC10A1) which facilitate TH transport. Thus, sequence and ligand homology can be used to prioritize transporters. In addition, modified TH molecules may be covalently linked with proteins, among others with transporters. The usefulness of this approach remains to be established since the affinity-label BrAcT3, which modifies the TR, does not modify MCT8, MCT10 or OATP1C1 (**chapter 4**). Unbiased strategies such as gene expression profiles or proteomics may be applied to cells whose transport characteristics alter if experimental conditions are changed. Furthermore, the identity of transporters which are affected in subjects with highly abnormal TH serum levels might be deduced from such patients.

Taken together, there are firm reasons to believe that novel TH transporters are waiting to be discovered.

Intracellular transport

The intracellular availability of TH is not only determined by transporters and deiodinases, but also by intracellular binding proteins. An important protein is μ-crystallin (CRYM) which has a high affinity for the iodothyronines T3, T4 and rT3 in the presence and for T2 in the absence of NADPH (95, 96). **Chapter 7** identifies CRYM also as a binding protein of sulfated iodothyronines (9). In CRYM KO animals, serum T3 and T4 concentrations are decreased and T3 rapidly leaves tissues (97). Nevertheless, peripheral T3 action appears not to be changed and, thus, it remains to be established which role CRYM plays in physiology. Furthermore, it is unknown whether CRYM is involved in trafficking TH to subcellular compartments or that other proteins fulfil this function. Future research may

answer the basic question how and when TH is transported within the cell to various cellular compartments.

TRANSPORTERS: THE GUESSES

As extensively illustrated by the characterization of plasma membrane transporters in this thesis, the intrinsic properties of iodothyronine molecules prevent the possibility of diffusion through the lipid bilayer. However, there seems no *a priori* reason to restrict this type of TH transport to the plasma membrane. What about cellular compartments which are also surrounded by lipid bilayers such as mitochondria and the nucleus?

Several transporters from the SLC family have been identified as essential for transporting molecules into mitochondria. A well-known example is the ornithine transporter SLC25A15, which results in serious symptoms if functional lacking in humans (98). As T3 largely influences transcription of mitochondrial DNA, transport of the hormone across the mitochondrial membranes is obligatory (99). To our knowledge, TH transport across mitochondrial membranes has not been studied yet, and molecular identification of one or more mitochondrial TH transporters is awaited in the future.

It has been established that transport of macromolecules (typically > 40 kDa) from the cytoplasm to the nucleoplasm is mediated by nuclear-pore complexes (NPC). Many different NPCs, which are each an assembly of multiple proteins, have been identified and mutations therein have been linked to diseases (100). Because NPCs facilitate translocation of transcription factors across the nuclear envelope, T3 bound to its receptor may enter the nucleus and as such mediates its effects on transcription. This scenario is consistent with the observation of rapid cytoplasmic-nuclear transport of TH receptors (101, 102). This nuclear uptake of TR-bound T3 may also result in an uphill cytoplasmic-nuclear gradient of free T3, a phenomenon that has already been proposed by Oppenheimer *et al* in 1985 (103). In view of the size of the pores in the nuclear membrane, it is doubtful if transporters are required to transport the iodothyronines apart from the TRs into the nucleus.

Undoubtedly, novel syndromes will be recognized, the genetic basis of which resides in TH transporters mutations. The development and application of novel genomic technologies promise that understanding of genetic mechanisms of diseases will rapidly progress in the next coming years.

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Summary

Thyroid hormone is crucial for normal development and function of virtually all tissues. The thyroid gland secretes predominantly the prohormone T4 and to a lesser extent the bioactive hormone T3. The net effects of thyroid hormone are exerted at the cellular level. Cellular thyroid hormone homeostasis is regulated at different levels: a) thyroid hormone transporters mediate transport of the hormone across the plasma membrane; b) different deiodinating enzymes activate T4 to T3 or 'inactivate' these hormones and c) thyroid hormone receptors affect gene expression of T3-target genes upon binding or release of T3.

Primary diseases of the thyroid gland may affect thyroid hormone production. In hypothyroidism, decreased thyroid hormone production results in clinical symptoms such as weight gain and cold-intolerance. Opposite clinical features are observed in hyperthyroidism.

Changes in any of the factors important in peripheral thyroid hormone regulation (transporters, deiodinases and receptors) alter thyroid hormone signalling. Under physiological conditions, these different levels of control are positioned to modify cellular content and action of thyroid hormone. Abnormalities in thyroid hormone signalling the consequence of altered cellular circumstances. In addition, primary defects in the local thyroid hormone homeostasis may cause disease. This thesis presents studies in which thyroid hormone regulation under normal and pathological conditions has been investigated. In particular, key players involved in thyroid hormone regulation in brain have been studied.

Chapter 1 provides a general background of thyroid hormone physiology. Particular attention is paid to cellular transport, metabolism and action of thyroid hormone under normal and pathological conditions. General aims and outline of the thesis are presented.

The first part of this thesis details characteristics of the thyroid hormone transporter MCT8, which has a crucial function in brain development. In **chapter 2**, several basic properties of MCT8 are explored. In contrast to other MCTs, MCT8 expression and function is not dependent on ancillary proteins. MCT8 appears to exist as a homodimer. Furthermore, individually mutated Cys residues do not affect T3 transport by MCT8. **Chapter 3** demonstrates that MCT8 is ubiquitinated. Apparently, ubiquitination of MCT8 does not result in rapid degradation. In **chapter 4** previous statements about affinity labelling of MCT8 by BrAcT3 are challenged. MCT8 nor the homologous MCT10 are modified by BrAcT3, but the intracellular protein disulfide isomerase. Inhibition studies with thyroid hormone analogues showed different effects on T3 and T4 transport by MCT8 and MCT10, suggesting different ligand binding sites in these transporters.

In **chapter 5**, we report several novel mutations in *MCT8* and analyzed their effects in skin fibroblasts from patients. T3 and T4 transport is largely decreased in fibroblasts from patients compared with controls. Furthermore, we demonstrate that in fibroblasts

with an F501del mutation in MCT8, T3 uptake is mildly affected. This parallels the much milder clinical phenotype in patients with this mutation and demonstrates the existence of genotype-phenotype differences in patients with mutations in *MCT8*. We pursued the use as patients' fibroblasts as a model for the disease in **chapter 6**. Transcriptional profiling of patients' versus control fibroblasts reveals numerous differentially expressed genes, many of which are implicated in brain development. In addition, we investigated MCT8 expression in human brain by exploring different data sets consisting of gene expression profiles of human brain samples. Comparative transcriptome analysis specifies the genes which are likely disrupted in brains of MCT8 patients. In fact, this chapter describes how genome-wide expression data from patient-derived non-neuronal tissue related to the human brain transcriptome may be successfully employed to improve our understanding of neurological diseases.

In **chapter 7**, we investigate which members of the solute carrier family SLC10 transport thyroid hormone (sulfates). We demonstrate that among the 7 members of the SLC10, only human SLC10A1 mediates transport of T4, and the sulfated thyroid hormones T3S and T4S. Cells co-expressing the liver-specific SLC10A1 and the deiodinase D1 demonstrate a dramatic increase in T3S and T4S metabolism. These findings suggest that SLC10A1 may fulfill a critical step in providing liver D1 with iodothyronine sulfates for rapid degradation. **Chapter 8** reports on thyroid hormone transport processes in human umbilical vein endothelial cells, which is a commonly used model for transport processes across the endothelium. Kinetic, inhibition, affinity labeling and expression studies suggest that T3 and T4 uptake is mediated by the same transport system, whose characteristics do not correspond to known thyroid hormone transporters.

Since thyroid hormone is crucial for normal brain development, it was hypothesized that mutations in key players of thyroid hormone metabolism, analogous to *MCT8* mutations, result in mental retardation. We initiated a nation-wide multi-center study to include subjects with unexplained mental retardation (TOP-R study for Thyroid Origin of Psychomotor Retardation study). **Chapter 9** describes the characteristics of the TOP-R study. We extensively profiled thyroid hormone parameters in 946 participants. Large differences in thyroid hormone measurements were attributed to the use of anti-epileptic drugs. In addition, the prevalence of primary thyroid diseases was assessed. This study may serve as a background for interpreting thyroid hormone indices in patients with mental retardation. To our knowledge, the TOP-R study represents the largest cohort of institutionalized subjects with mental retardation of whom serum thyroid parameters have been profiled. This strategy enabled us to screen for mutations in key genes regulating thyroid hormone signalling in brain. **Chapter 10** describes the identification of several *MCT8* mutations by selecting candidates based on low T4 and high T3 serum levels. *In vitro* transport assays demonstrate the pathogenicity of the identified nonsynonymous mutations. The mild clinical phenotype corresponds with the marked residual

transport capacity of the mutants. Our study indicates that mutations in *MCT8* are a frequent cause of X-linked mental retardation. The first results of the genetic screening for mutations in the *DIO2* and *DIO3* genes, encoding the type 2 and type 3 deiodinases, respectively, are presented in **chapter 11**. In both genes several novel DNA variants were identified. The mutations in *DIO2* are of particular interest, because several mutations result in amino acid substitutions. In addition, stem-loop distorting mutations are found in the SECIS element of *DIO2*. The functional impact of these variants is subject of future studies.

Although the clinical features associated with changes in thyroid state are well-known, the molecular basis is largely lacking. In **chapter 12**, we report the gene expression profiles of skeletal muscle from patients in hypothyroid versus euthyroid state. Numerous genes are changed upon different thyroid state and many novel genes are identified as responsive to thyroid hormone. Furthermore, the primary transcripts of the muscle-specific noncoding microRNA pair miR-206/miR-133b are largely decreased in hypothyroid versus euthyroid state, hinting at a new layer of cellular regulation by thyroid hormone.

Changes in thyroid hormone regulation as a consequence of aging are analyzed in **chapter 13**. In different progeroid mouse models and naturally old mice, thyroid hormone concentrations in serum and liver, kidney and brain have been assayed. The activities of the deiodinases D1 and D3 are measured in different tissues. In general, thyroid hormone metabolism has been decreased. Of interest, an increased D3 activity in liver appears to contribute to the lowered thyroid hormone concentrations. This chapter suggests a direct link between DNA damage, aging and lowered TH signaling and may help to understand the molecular basis of known clinical phenomena.

In **chapter 14** the observations presented in the thesis are discussed. Furthermore, the possible implications of the findings are explored. The gaps in the current understanding of cellular thyroid hormone regulation in health and disease are discussed and future directions of research are presented.

Samenvatting

Schildklierhormoon is cruciaal voor een normale ontwikkeling en functie van vrijwel alle weefsels. De aanmaak van schildklierhormoon vindt plaats in de schildklier, een vlindervormig orgaan gelegen in de hals. De schildklier scheidt voornamelijk het prohormoon T4 en in mindere mate het bio-actieve hormoon T3 af. Primaire aandoeningen van de schildklier kunnen schildklierhormoonproductie beïnvloeden. Een verminderde productie van schildklierhormoon (hypothyreoïdie) resulteert in klinische symptomen zoals gewichtstoename en koude-intolerantie. Daarentegen treden gewichtsverlies en warme-intolerantie juist op bij hyperthyreoïdie, een verhoogde schildklierhormoonproductie.

De effecten van schildklierhormoon zijn uiteindelijk het gevolg van haar werking op in de cel. De beschikbaarheid en werking van schildklierhormoon in de cel wordt gereguleerd op verschillende niveaus: a) schildklierhormoontransporters fungeren als 'poortjes' in de celwand (plasmamembraan) en faciliteren op die manier transport van het hormoon door het plasmamembraan; b) verschillende enzymen (dejodases) 'activeren' schildklierhormoon door T4 naar T3 om te zetten en 'inactiveren' schildklierhormoon door T4 naar rT3 en T3 naar T2 af te breken en c) receptoren voor schildklierhormoon in de celkern (schildklierhormoonreceptoren) moduleren genexpressie van T3-gevoelige genen na binding aan de receptor. Genexpressie betekent dat een gen (DNA) wordt gekopieerd naar messenger RNA (mRNA). Dit mRNA wordt vervolgens vertaald in aminozuren. Deze keten van achtereenvolgende aminozuren vormt samen het eiwit. Eiwitten zijn grote moleculen die vele cellulaire functies kunnen hebben (bijvoorbeeld communicatie, enzymatische reacties, transport etc.) en zijn dus essentieel voor het normaal functioneren van een organisme. Transporters en dejodases beïnvloeden de schildklierhormoonconcentraties in de cel. De schildklierhormoonreceptoren zijn direct betrokken bij de werking van schildklierhormoon.

Veranderingen in activiteit en werking van transporters, dejodases of kernreceptoren resulteren in veranderingen van het uiteindelijke effect van schildklierhormoon. In normale omstandigheden zijn deze verschillende controleniveaus op elkaar ingespeeld om heel nauwkeurig de hoeveelheid en werking van schildklierhormoon in de cel aan te passen. Veranderingen in één van de schakels in deze keten kunnen het gevolg zijn van gewijzigde omstandigheden en onderdeel zijn van aanpassingsmechanismen. Wanneer bijvoorbeeld de T4 concentratie daalt, zal de activiteit van het type 2 dejodase (dat T4 naar het actieve T3 omzet) toenemen om netto een voldoende T3 niveau in de cel te handhaven. Defecten in de lokale schildklierhormoonhomeostase (transporters, dejodases of receptoren) kunnen resulteren in verschillende ziekteprocessen. In dit proefschrift worden experimenten beschreven waarmee verschillende niveaus van schildklierhormoonhomeostase onder normale en abnormale omstandigheden zijn bestudeerd.

Hoofdstuk 1 geeft een algemene achtergrond van de schildklierhormoonfysiologie. In het bijzonder wordt er aandacht besteed aan het cellulaire transport door transporters, metabolisme van schildklierhormoon via dejodases en de uiteindelijke werking van schildklierhormoon via de kernreceptoren onder normale en pathologische omstandigheden. De ontdekking dat transportereiwitten in de celwand nodig zijn om schildklierhormoon te transporteren is van recente datum. Aangezien schildklierhormoon goed oplost in vet, en de celwand bestaat uit vetten, werd altijd aangenomen dat schildklierhormoon vanzelf de celwand passeerde. Het is de laatste jaren steeds meer duidelijk geworden dat hiervoor specifieke transporters in de celwand noodzakelijk zijn. Het wezenlijke belang van deze transporters werd duidelijk toen er mutaties (afwijkingen) in het *MCT8* gen gevonden werden bij mensen met ernstige verstandelijke en motorisch beperkingen en zeer afwijkende schildklierhormoonconcentraties in het bloed. Het eerste hoofdstuk besluit met het introduceren van de hoofdlijnen en doelstellingen van het proefschrift.

In het eerste deel van dit proefschrift zijn verscheidene fundamentele eigenschappen van de schildklierhormoontransporter MCT8 bestudeerd. In **hoofdstuk 2** worden verschillende basale kenmerken van MCT8 onderzocht. In tegenstelling tot andere leden van de MCT familie lijkt functie en expressie van het MCT8 eiwit niet afhankelijk van zogenaamde 'helpereiwitten'. MCT8 wordt gekenmerkt door een zogenaamde homodimerische structuur: twee MCT8 eiwitten gaan met elkaar een fysieke verbinding aan en vormen mogelijk op die manier de functionele transporterstructuur. Verder heeft het muteren van individuele Cys aminozuren geen invloed op T3 transport door MCT8. **Hoofdstuk 3** laat zien dat MCT8 kan worden gemodificeerd door binding van ubiquitinemoleculen. Doorgaans worden geubiquitineerde eiwitten snel afgebroken. Bovendien bevat MCT8 een zogenaamd PEST-domein. PEST-domeinen worden in verband gebracht met een snelle afbraak van eiwitten. Echter, ondanks dat MCT8 geubiquitineerd worden en een PEST-domein bevat, vindt er geen snelle afbraak plaats. In tegenstelling tot eerdere beweringen, leert **hoofdstuk 4** dat het affiniteitlabel BrAcT3 niet bindt aan MCT8 noch aan diens homologe transporter MCT10, maar aan een andere intracellulair gelegen eiwit. Met schildklierhormoonanaloga (moleculen die sterk op schildklierhormoon lijken) laten we verschillende effecten zien op T3 en T4 transport door MCT8 en MCT10. Dit wijst erop dat T3 en T4 op verschillende gedeelten in deze homologe transporters aangrijpen.

In **hoofdstuk 5** wordt een aantal nieuwe mutaties in *MCT8* geïdentificeerd. De effecten van deze mutaties zijn geanalyseerd in cellen van patiënten (huidfibroblasten). T3 en T4 transport is sterk verminderd in fibroblasten van patiënten vergeleken met fibroblasten van gezonde controles, hetgeen uiteindelijk resulteert in een verminderde expressie van T3-gevoelige genen. Bovendien tonen we aan dat in fibroblasten met de F501del mutatie, T3 opname veel minder is verstoord dan in fibroblasten van andere patiënten.

Het mildere klinische fenotype bij patiënten met deze mutatie wordt gereflecteerd in de restcapaciteit voor T3 opname. Dit onderstreept dat niet alle mutaties gelijke effecten hebben op het transport van schildklierhormoon. Dit is de moleculair basis van genotype-fenotype verschillen bij patiënten met mutaties in MCT8. Vervolgens hebben we in **hoofdstuk 6** de fibroblasten van patiënten verder gekarakteriseerd door expressie van alle genen (ongeveer 20.000 – 25.000 bij de mens) te bestuderen. Genexpressieprofielen van fibroblasten van patiënten en controles laten grote verschillen zien. Ongeveer 2000 genen komen lager of juist hoger tot expressie in cellen van patiënten, waarvan er vele betrokken bij ontwikkeling van de hersenen. Daarnaast onderzochten we MCT8 expressie in hersenen door genexpressieprofielen van normale hersenen te analyseren. Genen die sterk met MCT8 gecorreleerd zijn in hersenen (dus genen die een hoge expressie hebben waar MCT8 hoog is en omgekeerd) zijn sterk overgerepresenteerd in de genen die een veranderde expressie hadden in patiënten fibroblasten. Deze vergelijkende analyse van genexpressie specificeert dus de genen die waarschijnlijk verstoord zijn in de hersenen van MCT8 patiënten. Dit hoofdstuk legt de basis om meer te begrijpen waarom precies de verstandelijke handicap het gevolg is van een mutatie in MCT8. Deze methode kan bredere toepassing krijgen omdat dit hoofdstuk beschrijft hoe expressieprofielen afkomstig van niet-neuronaal weefsel (huidfibroblasten) van patiënten vergeleken met expressieprofielen van gezond hersenweefsel gebruikt kan worden om ons begrip van neurologische aandoeningen te verbeteren.

In **hoofdstuk 7** onderzoeken we welke leden van de SLC10 transporter familie (gesulfateerd) schildklierhormoon transporteren. Gesulfateerd schildklierhormoon is biologisch inactief en wordt snel afgebroken door het type 1 dejodase (D1) in de lever. We laten zien dat van de 7 leden van de SLC10 familie, alleen leverspecifieke SLC10A1 T4 en gesulfateerd schildklierhormoon (T3S en T4S) transporteert. Cellen die zowel SLC10A1 als D1 bevatten, laten een sterke toename in afbraak van T3S en T4S zien. Deze bevindingen suggereren dat SLC10A1 een cruciale stap is in het aanleveren gesulfateerd schildklierhormoon aan D1 in de lever, zodat dit snel kan worden afgebroken. De eerste stap om schildklierhormoon vanuit het bloed de weefsels (bijvoorbeeld de lever) te laten gaan is het passeren van de bloedvatwand. **Hoofdstuk 8** bestudeert schildklierhormoontransport in cellen afkomstig van de menselijke navelstrengader endotheelcellen. Deze cellen worden veel gebruikt als model voor transportprocessen van het endotheel, de bekleding van de bloedvatwand. Studies waarin kinetische, remming, affiniteitslabeling en expressie experimenten zijn gedaan suggereren dat T3 en T4 opname gemedieerd wordt door hetzelfde transport systeem, waarvan de kenmerken niet overeenkomen met bekende schildklierhormoontransporters.

Schildklierhormoon is een essentieel hormoon voor de normale ontwikkeling van de hersenen. Daarvoor is een optimale samenwerking nodig tussen de eiwitten die de schildklierhormoonhomeostase in de cel reguleren. Indien dit proces abnormaal

verloopt, leidt dit tot een verminderde ontwikkeling van de hersenen. Dit blijkt onder meer uit de mutaties in de MCT8 die leiden tot ernstige verstandelijke en motorische beperkingen als een gevolg van abnormale cellulaire schildklierhormoonstatus in de hersenen. Leiden afwijkingen in één van de (andere) schakels (transporters, dejodases en receptoren) in de cellulaire schildklierhormoonhomeostase tot verstandelijke handicaps (mentale retardatie)? Om deze hypothese te onderzoeken hebben we een landelijke multi-center studie opgezet. Het doel was om mensen met onverklaarde mentale retardatie te onderzoeken op afwijkingen in het schildklierhormoonmetabolisme. Deze studie wordt de TOP-R (TOP-R voor Thyroid Origin of Psychomotor Retardation; Schildklier Oorzaken van Psychomotore Retardatie) studie genoemd. **Hoofdstuk 9** beschrijft de kenmerken van de TOP-R studie. In 946 deelnemers zijn verschillende schildklierhormoonparameters gemeten. Er zijn verschillen met schildklierhormoonconcentraties in gezonde controles. Het gebruik van antiepileptica interfereert sterk met de schildklierhormoonbepalingen. Deze waarnemingen zijn van belang om testen voor de schildklierfunctie in mensen met een verstandelijke handicap goed te kunnen interpreteren. De TOP-R-studie is het grootste cohort van mensen met een verstandelijke handicap van wie schildklierhormonen zijn gemeten. De TOP-R-studie stelde ons in staat om mensen met abnormale schildklierhormoonconcentraties nader te onderzoeken. In mensen met de meest afwijkende schildklierhormoonconcentraties hebben we belangrijkste genen die betrokken zijn bij schildklierhormoonhomeostase geanalyseerd op mutaties. **Hoofdstuk 10** beschrijft hoe het *MCT8* gen is onderzocht in mensen met afwijkende schildklierhormoonwaarden die verdacht zijn voor *MCT8* mutaties, i.e. hoge T3 en lage T4 waarden. Deze aanpak heeft geleid tot de identificatie van verschillende nieuwe *MCT8* mutaties. Vervolgens zijn de functionele consequenties van deze mutaties onderzocht met *in vitro* transport assays. De pathogeniciteit van de mutaties die leiden tot een aminozuurverandering in MCT8 is hiermee vast komen te staan. De resterende transportcapaciteit van de mutanten correspondeert met het relatief milde klinische fenotype van deze MCT8 patiënten. Onze studie geeft aan dat mutaties in *MCT8* een niet zeldzame oorzaak van X-gebonden mentale retardatie zijn. De eerste resultaten van de genetische screening voor mutaties in de *DIO2* en *DIO3* genen, die coderen voor D2 en D3 respectievelijk, worden gepresenteerd in **hoofdstuk 11**. In beide genen is een aantal nieuwe DNA-varianten geïdentificeerd. De mutaties in *DIO2* zijn van bijzonder belang, omdat een aantal mutaties resulteert in aminozuursubstituties. Bovendien zijn er interessante mutaties gevonden die de structuur van een essentieel gedeelte in *DIO2* (het zogenaamde SECIS element) verstoren. De functionele gevolgen van deze varianten moeten blijken uit toekomstige studies.

De klinische symptomen die het gevolg zijn van veranderingen in schildklierhormoonstatus zijn algemeen bekend. Echter, de moleculaire basis hiervan ontbreekt grotendeels. In **hoofdstuk 12** is skeletspier bestudeerd van mensen die eerst normale en

vervolgens verlaagde schildklierhormoonconcentraties hadden. In deze spierbiopten is de expressie van alle genen bestudeerd. Een groot aantal genen (ongeveer 600) bleek te reageren op veranderingen in schildklierstatus. Tot nu toe was het van de meeste hiervan onbekend dat ze gevoelig zijn voor schildklierhormoon. Bovendien vonden we dat de primaire transcripten van heel kleine RNA moleculen, die niet voor eiwit coderen reageerden op schildklierhormoonstatus. De spierspecifieke niet-coderend microRNAs (miRs) miR-206 en miR-133b reageren sterk en consistent op een verandering in schildklierhormoonconcentraties. Deze bevindingen wijzen erop dat schildklierhormoon een nieuw niveau van cellulaire regulatie aanstuurt.

Schildklierhormoon is niet alleen belangrijk voor ontwikkeling, maar ook voor de functie van nagenoeg alle weefsels tijdens het volwassen leven. Gedurende het verouderingsproces neemt de schildklierfunctie geleidelijk af, maar het is onduidelijk of dit al dan niet gunstige gevolgen heeft. Daarom zijn in **hoofdstuk 13** de veranderingen in schildklierhormoonhomeostase tijdens het ouder worden bestudeerd. We hebben hiervoor verschillende muismodellen voor (vroegtijdige) veroudering gebruikt. Progeria muizen hebben een defect in het herstellen van DNA schade. Dit resulteert in een accumulatie van niet herstelde DNA schade en wordt gekenmerkt door vroegtijdige veroudering. In verschillende muismodellen (progeria muizen en natuurlijk verouderende muizen) hebben we schildklierhormoonconcentraties in bloed en weefsels (lever, nieren en hersenen) bepaald. Bovendien hebben we de activiteit van de dejodases D1 en D3 gemeten. In de bestudeerde modellen is er een verminderd metabolisme van schildklierhormoon in een aantal weefsels. De lever en nier worden gekenmerkt door een duidelijk verminderde werking van schildklierhormoon, terwijl dit niet wordt gezien in de hersenen. Deze bevindingen kunnen geïnterpreteerd worden als een aanpassingsmechanisme. Het metabolisme in weefsels die belangrijk zijn voor het metabolisme wordt preferentieel verminderd, terwijl het metabolisme in de hersenen gespaard lijkt te worden. Blijkbaar is een verlaging van het metabolisme in de meer vitale organen, zoals hersenen, niet gunstig. Een interessante waarneming was de sterke inductie van D3, het enzym dat schildklierhormoon afbreekt. D3 is met name van belang tijdens de foetale ontwikkeling. Onze bevindingen suggereren dat D3 gereactiveerd kan worden tijdens veroudering. Dit hoofdstuk impliceert een direct verband tussen DNA-schade, veroudering en verminderde werking van schildklierhormoon en helpt om de moleculaire basis van bekende klinische verschijnselen te begrijpen.

In **hoofdstuk 14** worden de resultaten van dit proefschrift samengevat en in een breder perspectief geplaatst. De mogelijke implicaties van de bevindingen worden verkend. De hiaten in de huidige kennis van cellulaire schildklierhormoonregulatie in gezondheid en ziekte worden aangegeven. Dit hoofdstuk besluit met suggesties voor verder onderzoek om de vragen die de studies in dit proefschrift oproepen verder te exploreren.

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ASVZ Zuidwest, Baalderborg, Bartimeüs, Bronlaak-Heimdal, Cello, De Brink, De Bruggen, De Kleine Johannes, De Zijlen, Dichterbij, Esdege-Reigersdaal, It Fugellan, 's Heeren Loo, Ipse, Leekerweide, Ons Tweede Thuis, ORO, Prinsenstichting, Reinaerde, Sint Anna, Stichting Siza Dorp Groep, Stichting Tragel, Swetterhage, Vanboeijen en Stichting Zuidwester). De grote interesse en belangeloze werkijver van alle betrokken AVG's en huisartsen heeft grote indruk gemaakt. Luc Imschoot, Paul Vrijmoeth, Peter Linssen, Elles Gremmen, Marijke Nieberg, Arjen Louisse, Erik Boot, Hans Wessels, Ietje van Gelderen, Jacques van der Kleij, Quinta Bergman, Marion Gruijters, Tonny Coppus, Gert de Leijer, Mathijs Kersten, Hans Steegmans, Lizette Deen, Jacinta Bruin, Theo Bakker, Marijke Tonino, Joop van de Berg, Luc Bastiaanse, Maarten Boer, Nicole van Paaschen, Margriet Meershoek, Rob Schreuel, Elsbeth Booij, Margriet van Duinen, Suzanne Duffels, Constant Hoedemaekers, Sylvia Huisman, Susanne Neuvel, Angelique Dijkgraaf, Willem Kleinveld, Frank Visser, Frans Scholte, Herman de Waal, Koos de Geest, Barber Tinselboer, Lukas de Groote, Wiebe Braam, Esther Askes, Carmen Bakker, Hans Verheij, Marien Nijenhuis, Sonja Soudant, Willy Pesch, Mascha Schulpen, Esther Bakker, Luc Goffin, Arthur de Jong, Gerda de Kuijper, Dagmar Douma, Corine Vermoen, Jelle de Koning, Marjolein Willems, Riet Niezen en Josje Kingma. Zonder jullie allemaal was er geen TOP-R studie van de grond gekomen.
Bij de praktische uitvoering van de studie zijn tientallen doktersassistenten en ''prikzusters'' en laboranten betrokken geweest die de coördinatie van het afnemen van bloed op zich genomen hebben: in ieder geval Marion, Loes, Manuela, Nicolette, Judith, Madelon, Hedy, Karin (3x), Nel, Ingrid, Mien, Lenie, Margareth, Angela, José, Marianne, Yvonne, Willy, Hetty, Cinta, Ina, Henny, Mariëtte (2x), Ingrid, Marijke, Rian, Angelina,

Maddy, Peter, Jeannine, Antia, Annelies, Frouke, Marian (2x), Ilna, Esther, Will, Nel, Lian, Gien, Anneke en mogelijk nog anderen die anoniem blijven en des te belangelozer zich hebben ingespannen. In een woord: geweldig! De bereidheid van alle participerende laboratoria en klinisch chemici (H.A. Assink, R. Baumgarten, G.J.J. Beukeveld, M. M. Buijs, J.L.P. van Duijnhoven, F.P.L. van der Dijs, H. Fleuren, D. Hardeman, R. de Keijzer, P.J.M.J Kok, R.J. Kraaijenhagen, R. Kusters, A.W.H.M. Kuypers, L.J. Mostert, A. Naus, J. van de Ouweland, M. Paanakker, C.J. Pronk-Admiraal, G.L.A. Reijnierse, R. Sanders, J.H.M. Souverijn, J. van Suijlen, A.L. Szakály en S.T. Ijpma en uit eigen huis natuurlijk Yolanda de Rijke) om bloed af te nemen en tijdelijk op te slaan is een belangrijke schakel geweest. Zo'n omvangrijke studie was niet mogelijk geweest zonder deze fantastische samenwerking.

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Collega arts-assistenten en specialisten in het Sint Franciscus Gasthuis: hoewel we nog maar net kennen, voel ik me prima op m'n gemak. Dank voor de prettige opvang.

Vrienden, ook al hebben jullie volstrekt niets bijgedragen aan dit proefschrift, betekenen jullie ontzettend veel daarbuiten. Vriendschap laat zich niet uitdrukken in maat en getal. Daarom wil ik het goede gebruik volgen om vrienden te bedanken. Paul en Walter, vrienden van het eerste uur! Dank voor alles door de jaren heen: over de groten zaken des levens, mountainbiken en pizza met blikjes bier. Erik-Jan, ik hoop dat we nog tijden herinneringen blijven delen. Marc-Jan, wat ben ik blij dat we elkaar hebben leren kennen. Een gevoel van melancholie komt altijd boven als we mijmeren over ''onze" stad. Alle bekende proeflokalen passeren dan de revue en de Griek van tegenwoordig is mager surrogaat. Cornelis, het samenzijn is altijd goed. Al is het tegenwoordig met heel wat meer vrouwen om ons heen dan vroeger. Peter-Willem, Robert en Maarten: wanneer gaan we weer biertjes drinken? Martin, niet vaak, maar altijd goed. Het Parijs-weekend samen met Koen en Gerard zal niet licht meer uit m'n hoofd verdwijnen.

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En dan natuurlijk het allermooiste: ''de meisjes''. Lieve Liza, lieve Eva, het leven valt niet te denken zonder jullie. Gelukkig dat jullie geen enkele boodschap eraan hebben dat ik zo nodig moest promoveren. Met jullie voetballen, boekjes lezen en over de grond rollen: het is me liever dan wat ook! En hier een nieuw boekje om uit de kast te trekken en als kleurboek te gebruiken.
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Ik hou van je.

Curriculum vitae

Willem Eduard Visser was born on September 12th, 1980 in Dordrecht. After completing secondary school at the Van Lodenstein College in Amersfoort, he moved to Antwerp to study Medicine. After succesful completing the first and second year, he continued studying Medicine in Rotterdam. In May 2005 he obtained his medical degree and started as a resident at the Dept of Internal Medicine at the Ikazia Hospital, Rotterdam. In April 2006 he entered the fascinating scientific world of thyroidology at the laboratory of the thyroid expert prof. dr. ir. Theo Visser. The results of four subsequent exciting years are presented in this thesis. For his studies about several aspects of thyroid hormone signalling, he received two travel grant awards in 2008: at the Transporters meeting (Murten, Switzerland) and from the American Thyroid Association (Chicago, USA). Furthermore, he obtained a travel grant from the Endocrine Society in 2009. In addition, he was honoured as a presidential poster competition winner (ENDO 09, Washington, USA). In 2010 he received the Endocrine Society outstanding abstract award (ENDO 2010, San Diego, USA). In May 2010 he started his training residencies in Internal Medicine at the Sint Franciscus Gasthuis, Rotterdam under supervision of A.P. Rietveld. He married Evelien Schakelaar and they have two beautiful daughters Liza and Eva.

PhD Portfolio

Name PhD Student	Edward Visser
Erasmus MC Department	Internal Medicine – Thyroid lab
Research school	MolMed
PhD period	April 2006 – May 2010
Promotor	prof. dr. ir. T.J. Visser

1. PhD training

General academic skills	**Year**	**Workload**
Annual Course on Molecular Medicine, Erasmus MC, Rotterdam, The Netherlands	2007	2 days
Basic Fellows Track, ATA, Chicago, USA	2008	2 days
Endocrine Trainee Day, Endocrine Society, Washington, USA	2009	1 day
Research skills		
Bioinformatic Analysis, Tools and Services	2008	1 day
(Inter)national conferences		
77th Annual Meeting of the American Thyroid Association, Phoenix, USA	2006	4 days
6th Meeting of European Federation of Internal Medicine, Lisbon, Portugal	2007	3 days
32nd Annual Meeting of the European Thyroid Association, Leipzig, Germany	2007	4 days
78th Annual Meeting of the American Thyroid Association, New York, USA	2007	4 days
90th Meeting of the Endocrine Society, San Francisco, USA	2008	4 days
Transporters 2008, Murten, Switzerland	2008	3 days
33rd Annual Meeting of the European Thyroid Association, Thessaloniki, Greece	2008	4 days
79th Annual Meeting of the American Thyroid Association, Chicago, USA	2008	4 days
91st Meeting of the Endocrine Society, Washington, USA	2009	4 days
34th Annual Meeting of the European Thyroid Association, Lisbon, Portugal	2009	4 days
92nd Meeting of the Endocrine Society, San Diego, USA	2010	4 days

Presentations

Large X-chromosomal deletion affecting the MCT8 gene in a severely retarded boy with elevated serum T3. *American Thyroid Association.*	2006	Poster
Large X-chromosomal deletion affecting the MCT8 gene in a severely retarded boy with elevated serum T3. *Internal Medicine Science Days*.	2007	Poster
A prostatectomy related osteomyelitis caused by Pseudomonas *Aeruginosa*. *EFIM*.	2007	Oral
Differences in T3 uptake and metabolism in fibroblasts of MCT8 patients reflect phenotypic variability. *European Thyroid Association.*	2007	Poster
Differences in T3 transport in fibroblasts from MCT8 patients reflect phenotypic variability. *American Thyroid Association.*	2007	Poster
Differences in T3 transport in fibroblasts from MCT8 patients reflect phenotypic variability. *Internal Medicine Science Days*.	2008	Poster
Differences in T3 transport in fibroblasts from MCT8 patients reflect phenotypic variability. *MolMed Day.*	2008	Poster
Structure and function of the human thyroid hormone transporters MCT8 and MCT10. *ENDO 08.*	2008	Poster
Novel physiological implications of co-transport of (sulfated) iodothyronines and taurocholate by liver SLC10A1. *European Thyroid Association.*	2008	Oral
Importance of differential regulation of deiodinase activities in the protective metabolic response in rapidly aging mice. *American Thyroid Association.*	2008	Oral
Novel physiological implications of co-transport of (sulfated) iodothyronines and taurocholate by liver SLC10A1. *Transporters.*	2008	Oral
Importance of differential regulation of deiodinase activities in the protective metabolic response in rapidly aging mice. *Internal Medicine Science Days*.	2008	Poster
Physiologic levels of thyroid hormone regulate a myriad of transcripts in human skeletal muscle. *ENDO 09.*	2009	Poster
Largely different gene expression profiles in cells from MCT8 patients. *European Thyroid Association.*	2009	Oral
Comparative transcriptome analysis of patients' fibroblasts and normal brain specifies the deranged molecular pathways in brains of mct8 patients. *Internal medicine science days*.	2010	Oral
Large scale profiling of thyroid hormone parameters in a large mental retardation cohort: the TOP-R (Thyroid Origin of Psychomotor Retardation) study. *ENDO 10.*	2010	Poster
Transcriptional profiling in fibroblasts of patients with mutations in *MCT8* and comparative analysis with the human brain transcriptome. *ENDO 10.*	2010	Poster
Novel *MCT8* mutations in a cohort of patients with unexplained mental retardation. *ENDO 10.*	2010	Poster
Thyroid hormone signaling is suppressed in progeroid and normal aging. *ENDO 10.*	2010	Poster

Teaching activities	**Year**	**Workload Lecturing**
Vaardigheidsonderwijs second year medical students	2007	5 days
Vaardigheidsonderwijs second year medical students	2008	5 days
Vaardigheidsonderwijs second year medical students	2009	5 days
Vaardigheidsonderwijs first year medical students	2009	5 days
Supervising internships and (bachelor) thesis		
Wing San Wong, Hogeschool Rotterdam Biology and Medical Laboratory Research *Identificatie en karakterisering van (mutaties in) schildklierhormoontransporters*	2008	4 months
Norah Oudesluis, Erasmus MC, Medicine *Thyroid hormone transport in liver cells*	2008	4 months
Hatice Barutçi, Hogeschool Rotterdam, Biology and Medical Laboratory Research *Analysis of the intracellular distribution of thyroid hormone mediated by the different transporters/Preliminary study of thyroid hormone analogs for treatment of MCT8* patients *(Bachelor's thesis)*	2009	7 months
Alies van Mullem, Erasmus MC, Medicine *Thyroid hormone transport in the liver*	2009	5 months