CELLULAR MECHANISMS OF THYROID HORMONE HOMEOSTASIS

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Colofon

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Chapter 1

GENERAL INTRODUCTION AND AIMS OF THE THESIS

INTRODUCTION

Thyroid hormone (TH) is produced by the thyroid gland and is the common name for the prohormone T4 (3,5,3',5'-tetraiodothyronine, thyroxine) and the bioactive hormone (3,5,3'-triiodothyronine). TH is important for the normal development and metabolism of virtually all tissues, including the brain (1,2). This is illustrated by congenital hypothyroidism, which if untreated, can give rise to severe neurological and cognitive problems (3). Its effects on metabolism are demonstrated by manifestations in primary thyroid disease. In hypothyroidism symptoms as cold intolerance and bradycardia mirror a generally decreased metabolic state. In contrast, hyperthyroidism can result in heat intolerance and tachycardia, which is in line with an upregulation of metabolism. Serum TH levels are carefully regulated by the hypothalamus-pituitary-thyroid (HPT) axis (4). Thyrotropin-releasing hormone (TRH) is produced in the hypothalamus to stimulate the secretion of thyroid-stimulating hormone (TSH) in the pituitary (5). TSH stimulates the production and secretion of TH from the thyroid gland (6). These processes are dependent on the negative feedback of TH at the level of the hypothalamus and the pituitary. The genomic actions of TH are mediated by nuclear TH receptors (TRs) bound to promoter elements of TH responsive genes. Normal TH homeostasis is therefore dependent on the intracellular availability of TH, which requires adequate function of transporter proteins and deiodinating enzymes (Figure 1).

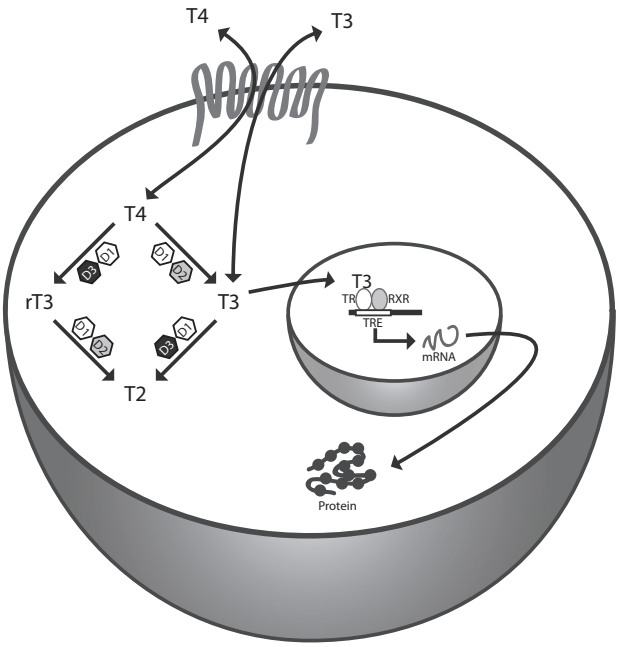

Figure 1 TH binds to nuclear receptors that will bind to promoter regions of TH target genes to regulate gene transcription. Bioavailability of the hormone is dependent on transporter proteins and deiodinating enzymes. Figure by Simone Kersseboom.

THYROID HORMONE SYNTHESIS

TH synthesis is a multi-step process which takes place in thyroid follicles. Follicles are thyrocytes that surround the colloid lumen (7,8). Iodide, the principal component of TH, is transported across the basolateral membrane of the thyrocyte by the Na/I symporter (NIS). Subsequently, iodide enters the follicular lumen from the cytoplasm by the transporter pendrin (PDS). In the colloid, I^- is oxidized to I^+ by an enzyme called thyroid peroxidase (TPO). I^+ is very reactive and rapidly iodinates 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 two DIT residues to form T4 and of a MIT and a DIT residue to form T3. Next, proteolysis of thyroglobulin in lysosomes will liberate T4 and T3 for secretion into the bloodstream. This release from the thyroid gland most likely is, at least partly, facilitated by MCT8, as it is expressed in the thyroid gland (9,10). In humans, the thyroid gland mainly secretes T4 and to a lesser extent T3. Excess MIT and DIT are deiodinated within the thyroid gland by the enzyme iodotyrosine dehalogenase (DEHAL) to allow re-utilization of the iodide for TH synthesis (11,12). Some MIT and DIT may escape from the thyroid gland to be deiodinated by DEHAL expressed in other tissues such as the liver and kidney. All iodothyronines and iodotyrosines are presented in Figure 2.

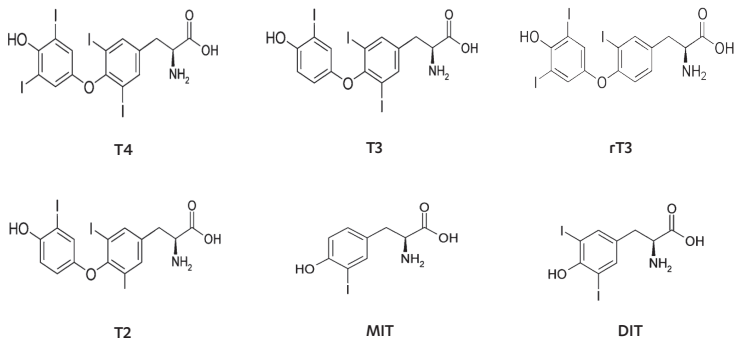

Figure 2 Chemical structures of all iodothyronines and iodotyrosines.

THYROID HORMONE ACTION

TH acts via genomic and non-genomic pathways. Genomic actions of TH are mediated by binding to nuclear T3 receptors (TRs) which regulate the transcription of target genes (2,13). Several receptor isoforms are encoded by the *THRA* (thyroid hormone receptor α) and *THRB* (thyroid hormone receptor β) genes (14), with TRα1, TRβ1, and TRβ2 as the T3-binding isoforms (15). Both TRα1 and TRβ1 are widely expressed, with TRα1 as the predominant isoform in the brain, bone, heart, and intestine, and TRβ1 as the major isoform in the liver, kidney, and thyroid (16). TRβ2 is mainly expressed in hypothalamus and pituitary and is therefore involved in the regulation of the HPT axis (17). TRs bind to TH response elements (TREs) in the promoter regions of target genes, thereby regulating gene transcription by the attraction or release of corepressors and coactivators (15,18).

Mutations in the ligand-binding domain of TRβ1/2 are associated with the resistance to TH syndrome (RTHβ). RTHβ is characterized by high serum TH levels in combination with non-suppressed TSH. The syndrome may comprise goiter, tachycardia, and raised energy expenditure (19-21). Recently, the first humans with mutations in *THRA* were identified. Their phenotype consists of relatively low serum T4 and high serum T3 levels (and thus an elevated T3/T4 ratio), neuro-cognitive impairment, growth retardation, delayed bone development, and constipation (22-25).

That TH may exert biological, non-genomic effects has only recently been recognized (26-29). In one study, T4 and rT3 could regulate actin polymerisation and D2 activity in the developing brain via non-transcriptional ways (26). Also, 3,5-T2 stimulates mitochondrial energy metabolism, and in modelling studies TH analogues could bind to the extracellular part of the integrin αvβ3, which is a cell surface protein (27,29,30). Binding of TH to αvβ3 can influence cell proliferation via mitogen activated protein kinases.

T3 is known as the active hormone, based on receptor binding studies showing that T3 binds with higher affinity (10-100x) to the TR than T4 (31-35). However, it has been shown that T4 can induce conformational changes in the TR similar to those induced by T3 (36) and the recruitment of coactivators by T4, especially to TRα1 was described (37). However, to date, it is unclear if T4 only functions as a prohormone or if it can exert direct transcriptional effects.

DEIODINASES

Intracellular TH concentrations are importantly controlled by the three deiodinating enzymes. The deiodinase type 1 (D1), type 2 (D2) and type 3 (D3) have distinct roles based on their tissue expression, substrate preference and mode of action (Table 1). D1 and D2 have an activating function,

Table 1 Characteristics of iodothyronine deiodinases. Adapted from *Bianco and Kim*, J Clin Invest. 2006.

	D1	D2	D3
Biochemical properties			
Molecular weight (kDa)	29	30.5	31.5
Preferred substrate	rT3	T4, rT3	T3, T4
Km	10^{-7}	10^{-9}	10^{-9}
Half life	Hours	± 20 min	Hours
Subcellular localization	Plasma membrane	Endoplasmic reticulum	Plasma membrane
Susceptibility to PTU	High	Low	Low
Tissues with high activity	Liver, kidney	CNS, pituitary, brown adipose tissue, placenta	CNS, placenta
Response to ↑ T3, T4			
Transcriptional	↑↑	↓	↑↑
Translational	?	↓↓↓ (ubiquitination)	?
Physiological role	Clearance rT3	Thermogenesis, development, to provide intracellular and plasma T3	Clearance T3, T4
Physiological regulation			
Induction	T3	Cold exposure, catecholamines	Tissue injury, T3
Repression	Fasting, illness	T3	Glucocorticoids

as they are capable of converting T4 to T3. D3 inactivates T4 to rT3 (3,3',5'-triiodothyronine) and T3 to 3,3'-T2 (3,3'-diiodothyronine), which are transcriptionally inactive metabolites (38,39).

Deiodinases are selenoproteins, which hold an UGA codon within the mRNA that needs to be translated into the rare amino acid selenocysteine (Sec) instead of representing a stop sign. This process requires a selenocysteine insertion sequence (SECIS) element in the 3'UTR of the mRNA. The SECIS element will form a highly complex stem-loop structure that will attract proteins like Secis Binding Protein 2 (SBP2) to assist in Sec incorporation (40). The presence of Sec is important for normal enzymatic activity of D2 (as well as D1 and D3) (41). This is illustrated by patients with mutations in SPB2, who are characterized by impaired deiodinase function and elevated serum T4, rT3 and TSH levels along with decreased T3 levels (42,43). The human D2 SECIS element is known to contain three groups of essential nucleotides and therefore introduction of a mutation in one of these groups will totally abolishes SECIS activity (44). Selenoproteins require selenium to introduce selenocysteine (45). Therefore, expression and activity of deiodinases is selenium dependent (46-48).

The exact physiological role of D1 is still unclear (38,49). As D1 is expressed in liver and kidney, its contribution to serum T3 levels by the conversion of T4 to T3 seems ideal. However, this concept is challenged by the positive regulation of D1 by T3, the normal T3 plasma concentrations in D1 deficient mice next to a normal phenotype (50,51) and the high catalytic efficiency of D1 for rT3 compared to T4. Therefore, it has been proposed that D1 could have an important function in recycling iodide from receptor-inactive metabolites for reutilization in the thyroid gland. No human mutations in *DIO1* have been identified yet. However, studies on genetic variation in D1, showed higher serum concentrations of FT4 and rT3, lower concentrations of T3 and FT3 and lower T3/rT3 or FT3/FT4 ratios, pointing toward a decreased conversion of T4 to T3 by diminished D1 protein expression or activity (52). Also, the D1-C785T polymorphism was associated with a lifetime higher risk for depression (53).

As D2 only has a function as an activating enzyme, this pathway has been hypothesized to be the major source of plasma T3, based on conversion studies in cells overexpressing D1 or D2 (38,54). However, Dio2 knockout (KO) animals showed normal plasma T3 levels (55). The role of D2 in regulating local TH homeostasis is unequivocal (39). In D2-expressing tissues, intracellular T3 levels are mainly achieved by the activity of this enzyme, which is illustrated by studies using brown adipose tissue (56). Also, the expression of TH sensitive genes in neurons is dependent on T4 to T3 conversion by D2 in the glial compartment (57), and it has been estimated that D2 generates as much as 80% of cerebral T3 levels (58,59). D2 is negatively regulated by its own substrates T4 and rT3. This process is importantly regulated at the posttranslational level by substrate-induced proteasomal degradation, a process named ubiquitination and is used to rapidly adjust cellular TH levels (39). Dio2 KO show elevated serum T4, rT3 and TSH levels along with normal serum T3 levels (55). However, despite strongly decreased T3 levels in the brains of these animals only mild neurocognitive symptoms (locomotor and memory) were observed (60). No mutations in the *DIO2* gene have been described yet. However, the commonly occurring Thr92Ala *DIO2* SNP has been associated with impaired psychological well-being in general and during T4 replacement therapy with a normalized TSH. This genetic variant was also associated with a preference for

LT3/LT4 combination therapy above LT4 monotherapy in hypothyroid patients (61). It also has been reported that D2 polymorphisms are associated with bipolar disorder and with intellectual disability (ID) in the iodine deficient areas of China (62). In contrast to genetic variation in D1, the data on the influence of genetic variation in D2 on TH parameters is conflicting (52). Altogether, D2 seems to be important for local TH homeostasis in the brain.

D3 is the major TH inactivating enzyme, as it prevents activation of T4 and terminates the action of its preferential substrate T3. As D3 is highly expressed in fetal tissues, an important role for this enzyme is implied in normal development. Indeed, *Dio3* KO mice show neonatal mortality, growth retardation and abnormal serum TH levels (63). In adult tissues D3 is expressed in brain, placenta, skin and pituitary. However, D3 can be reactivated in other tissues in critical illness or ischemic injury and this may be important to decrease energy expenditure and focus on preservation (64-66). As for the other deiodinases, no mutations have been identified in *DIO3* so far and genetic variation in D3 shows no relationship with serum TH parameters (52).

THYROID HORMONE TRANSPORT

TH action requires transport of the hormones across the plasma membrane, as the deiodinases and TRs are localized intracellularly. Based on their lipophilic structure, it was first believed that TH crossed membranes through passive diffusion. However, experimental evidence suggested the involvement of TH transporters (67). During the last decade, several transmembrane transporters have been shown to transport TH. These include Na^+/taurocholate co-transporting polypeptide (NTCP) (68,69) and the families of organic anion transporter polypeptides (OATPs) (70), L-type amino acid transporters (LATs) (71) and monocarboxylate transporters (MCTs) (72,73). Among these transporters only OATP1C1 (74-76), MCT8 (72) and MCT10 (73) are reported to have relatively high specificity towards iodothyronines.

MCT8

MCT8 (SLC16A2) is a member of the MCT family of transporters. This family is named after the substrate specificity of MCT1-4, which are known to transport monocarboxylates like lactate and pyruvate (77). MCTs have 12 putative transmembrane domains with the N-terminal and C-terminal parts localized intracellularly. The human *MCT8* gene is located on the X-chromosome and to date MCT8 is known as the most important physiological TH transporter.

Only a decade ago, MCT8 was identified as a specific and active transporter of T4, T3, rT3 and 3,3'-T2 in *Xenopus* oocytes injected with rat (r) Mct8 (72). No uptake of monocarboxylates or amino acids was observed. Later on, the uptake of iodothyronines by human (h) MCT8 was confirmed in transiently transfected cell lines and by assays in which cells that expressed MCT8 are co-transfected with deiodinases (78). hMCT8 in the presence of deiodinases induced rapid intracellular metabolism of T4, T3, rT3 and 3,3'-T2, which confirmed an increased content of intracellular iodothyronines in the presence of MCT8. The rapid TH uptake by hMCT8 reached a plateau after 10-30 minutes of incubation time, which is indicative for a balance between uptake and export. Export of iodothyronines by MCT8 was confirmed by efflux assays that showed

decreased accumulation of T4 and T3 in cells that expressed MCT8 and by studies in which hMCT8 is co-transfected with μ-crystallin (CRYM) (73). CRYM is a high-affinity cytoplasmic TH binding protein and co-transfection with this protein showed a marked decreased release of TH in efflux studies and significant amplification of TH content in uptake assays. Altogether these findings characterized MCT8 as a significant cellular TH import and efflux transporter.

MCT8 is broadly expressed, including liver, kidney, heart, placenta and brain (79-81). In the brain this transporter is expressed in various cell types and structures, such as endothelium, choroid plexus, neurons, oligodendrocytes, tanycytes, and also in the hypothalamus and pituitary (82-84).

The physiological relevance of MCT8 has been demonstrated by the identification of mutations in *MCT8* which result in a severe phenotype with psychomotor retardation: the Allan-Herndon-Dudley syndrome (AHDS) (85,86). These patients display high serum T3 and rather low circulating T4 levels, resulting in peripheral thyrotoxicosis with low body weight and muscle wasting. The neurocognitive phenotype comprises axial hypotonia, dystonia, severe ID and delayed myelination on brain MRI. Although the exact pathogenic mechanism is not understood, *Mct8* KO mice shed some light on this issue (9,10). The mice deficient in Mct8 fully replicate the endocrine abnormalities of the patients, however no overt neurological symptoms were observed, despite the strongly impaired T3 uptake into the brain (10,87,88). Subsequently, modestly decreased T4 and T3 levels were measured in the brains of *Mct8* KO mice, which was confirmed by modest decreased expression of TH sensitive genes (89). In line with these observations a diminished response of neurons towards peripherally applied T3 in hypothyroid Mct8 KO mice was shown (87). Multiple mechanisms are thought to contribute to the changed serum TH levels in AHDS patients and *Mct8* KO mice. First, TH production as well as secretion seems to be strikingly altered, as highly elevated T3 and T4 levels in the thyroid gland of Mct8 deficient animals were observed. Furthermore, it seems that the thyroid gland secretes less T4 into the bloodstream, whereas MCT8 fulfils an important export function in the thyroid (90-92). Second, increased T4 to T3 conversion due to increased deiodinase activities has been considered. In mice, D1 is highly expressed in several tissues such as the liver, kidneys and the thyroid gland. Inactivation of D1 in *Mct8* KO mice lead to normalization of the serum TH values. These findings firmly established a critical role of D1 in the generation of the abnormal serum TH levels (90,91,93). Furthermore, due to the retention of T4 inside Mct8 deficient thyrocytes, higher levels are available for thyroidal D1. Consequently, it has been hypothesized that more T4 is deiodinated to T3 already in the gland and more T3 is released in the circulation (91). Third, in particular, the kidneys may function as a "sink" for T4 as transport studies have revealed increased renal uptake of both T3 and T4 in the absence of Mct8 (94). The discrepancy in the neurological phenotype in human or mouse MCT8/mct8 deficiency can be explained by the presence of other compensatory T4 transporters in the rodent brain like Oatp1c1, Lat1 or Lat2 (88,90,91,95,96). Oatp1c1 seems to be important in mouse brain, as it is highly expressed in the blood brain barrier (BBB) and *Oatp1c1* KO mice showed a decrease in T4 and T3 brain content and downregulation of TH responsive genes (82,97). Indeed, when both Mct8 and Oatp1c1 are deficient in mice, a clear hypothyroid state of the brain is observed, including neurobehavioural symptoms (98). LAT1 and LAT2 (see below) have also been suggested to prevent a neurological

phenotype by compensating for the defect in TH transport in Mct8 deficient mice (88), as LAT1 is predominantly expressed in the murine blood brain barrier and LAT2 mainly in neurons (99-101). *Mct8/Lat2* double KO mice do not show the transient increase in T3 levels and expression of T3 target genes in the cerebral cortex as observed in neonatal *Mct8* KO mice (96). This led to the suggestion that perinatal uptake of T3 in cerebral cortex is facilitated by LAT2.

It has also been hypothesized that MCT8 can transport other biologically active substrates that could be important in human physiology. This may seem likely in view of the characteristics of the highly homologous monocarboxylate transporter 10 (MCT10), which also transports in particular aromatic amino acids in addition to iodothyronines. The hypothesis of alternative transport by MCT8 is also supported by a patient with a mutation in MCT8, with neurological symptoms, but normal TH transport capacities (102). However, so far, no alternative substrates for MCT8 have been reported. Identification of novel substrates may have important consequences for understanding the pathophysiology and possibly for treatment options of this disorder.

MCT10

MCT10 (SLC16A10) is also known as T-type amino acid transporter 1 (TAT1) and shares almost 50% of amino acid sequence identity with MCT8 (73,103,104). Our group demonstrated that in addition to the transport of the amino acids tryptophan (Trp), phenylalanine (Phe) and tyrosine (Tyr), MCT10 is also able to transport TH with a preference towards T3 (73). MCT10 is predominantly expressed at the basolateral membrane of many tissues, such as intestine, liver, kidney, muscle and placenta (103-106). To explore its physiological role, mice deficient in Mct10 were created (107). These mice showed no obvious phenotype, normal TH serum levels and some mildly decreased levels of amino acids in plasma and various tissues. No mutations have been identified yet in hMCT10 and a common polymorphism in the 3'UTR of MCT10 was not associated with TH parameters (108).

LAT1 and LAT2

System L transporters are mainly present on basolateral membranes of various tissues and preferably mediate sodium-independent exchange of neutral amino acids. They are specifically inhibited by 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) (109). LAT1 and LAT2 are highly homologous proteins consisting of the non-glycosylated 12 transmembrane domains containing light chains SLC7A5 and SLC7A8, respectively, (~40 kDa) and a common glycosylated 4F2 heavy chain (CD98/SLC3A2) containing a single transmembrane domain (~125 kDa). Compared to LAT1, which preferably transports large, neutral, amino acids with branched or aromatic side chains, LAT2 has a remarkably broad substrate specificity. Also, LAT2-mediated transport is increased at lower pH values. LAT1 is mainly expressed in brain, spleen, colon and placenta, whereas LAT2 is widely expressed at basolateral membranes of transporting epithelia, like kidney and intestine, but also in the brain, placenta and liver (110-114).

Evidence that LAT1 and LAT2 transport TH is scarce, since most studies show indirect evidence of inhibited LAT-mediated uptake of amino acids by iodothyronines (71,99,115-117). However, in 2001, our group showed direct uptake of T4 and T3 by human LAT1 in *Xenopus*

oocytes and also described the uptake of iodothyronines by mouse Lat2 (71). As mentioned above, LAT1 and LAT2 have been suggested to prevent a neurological phenotype by compensating for the defect in TH transport in Mct8 deficient mice (88).

To further explore the physiological role of these transporters, *Lat1* and *Lat2* KO mice models were studied. Homozygous *Lat1* KO mice showed to be embryonically lethal and *Lat1* heterozygous KO mice did not have any phenotype whatsoever (118). Muscle specific KO of *Lat1* showed reduced local concentrations of amino acids, but normal muscle mass. In *Lat2* KO mice urinary loss of neutral amino acids was measured with only slightly impaired movement control. Growth, development and circulating TH levels were normal (119). No mutations have been identified yet in *hLAT1* or *hLAT2*. Several polymorphisms have been identified in *LAT1* and *LAT2*; however few studies have been conducted to link polymorphisms in LATs to alterations in the capacity to transport thyroid hormone, which could lead to altered serum thyroid hormone concentrations. In one study, a polymorphism in LAT1 had no effect on the kinetics of Phe transport (108).

LAT3, LAT4 and LAT5

LAT3 (SLC43A1), LAT4 (SLC43A2) and LAT5 (SLC43A3) (~50 kDa) are transporters with a predicted membrane topology of 12 transmembrane domains with a conserved N-glycosylation site. The transport activity of LAT3 and LAT4 corresponds to that of amino acid transport system L. These transporters facilitate efflux of branched chained, neutral amino acids in a sodium independent manner at the basolateral side into the blood and are inhibited by BCH. LAT5 is an orphan transporter. LAT3 is mainly expressed in liver, pancreas, skeletal muscle and also in placenta and podocytes. LAT4 is expressed in placenta, kidney, liver and small intestine (120-124). Several features distinguish LAT1 and LAT2 from LAT3 and LAT4: (1) LAT3 and LAT4 do not need to bind to a heavy chain to traffic to the plasma membrane; (2) In particular LAT1 is an obligate exchanger, whereas LAT3 and LAT4 facilitate predominantly the efflux of substrates; (3). LAT3 and LAT4 exhibit narrow substrate selectivity and show highly complex two-component kinetics (120). LAT3 and LAT4 are, similar to LAT1 and LAT2, both expressed in the placenta and are both expected to mediate efflux of amino acids from the syncytiotrophoblast to the fetus (125). It has also been suggested that the observed up-regulation of LAT3 in liver and skeletal muscle during starvation, benefits the transport of amino acids from the liver and muscle to energy-deplete organs like the brain (126). As LAT1 and LAT2 are also able to transport TH, it can be hypothesized that LAT3, LAT4 and LAT5 may also accept iodothyronines as substrates.

As for the other transporters, various Lat3 and Lat4 deficient animal models were created to further explore its roles in physiology. A zebrafish morphant deficient in Lat3 showed collapsed glomeruli and disruption of glomerular permeability, suggesting that LAT3 may play a crucial role in the development and maintenance of podocyte structure and function (127). Recently, LAT4 was shown to facilitate the efflux of amino acids, and this transporter appeared to be important in nutrition during early development (128). In *Lat4* KO mice, they observed intrauterine and postnatal growth retardation, low amniotic amino acid concentrations and premature death after 9 days. No mutations have been identified yet in these transporters and no studies have been performed to link genetic variations in LAT3 or LAT4 to their transport capacity.

OATP1C1

OATP1C1 (SLCO1C1) belongs to the family of organic anion transporting polypeptides (OATPs) of which many members accept a wide variety of substrates (70). They are characterized as Na^+-independent transporters that consist of 12 transmembrane domains, with both the N- and C-terminal parts localized inside of the cell. OATP1C1 displays a narrow substrate specificity towards the iodothyronines T4 and rT3 and other ligands as estrone-3-sulfate, estradiol-17β-glucuronide and bromosulfophthalein (BSP) (74). OATP1C1 is expressed in the testis and many brain regions. Immuno-histochemical staining showed high expression of this transporter in the rodent BBB in contrast to low expression in the human BBB (82). This challenged the hypothesis that OATP1C1 is important for T4 transport into the human brain in contrast to the rodent brain. Indeed, as described under MCT8, Oatp1c1 has an important role in the T4 supply into the mouse brain, as *Oatp1c1* KO mice showed decreased T4 and T3 content in the brain, but otherwise with a normal phenotype and normal circulating TH levels (97). Also downregulation of T3 responsive genes and increased local D2 activity suggested a hypothyroid situation in the brain of *Oatp1c1* KO mice. As also *Mct8* KO mice did not show a neurological phenotype and only the transport of T3 into the brain was strongly diminished, Oatp1c1 was suggested to compensate for the loss of Mct8 by supplying T4 for local T3 production. For this reason *Mct8/Oatp1c1* DKO mice were generated and these mice showed strongly decreased transport of both T4 and T3 into the brain, together with a clear TH deprivation in the central nervous system (CNS) and severe locomotor abnormalities (98). Recently, a new role of OATP1C1 was suggested in the influx of amyloid-β peptide across the BBB and therefore this transporter could be relevant in the pathophysiology of neurodegenerative diseases (129). The relevance of OATP1C1 in human physiology is still unclear and no mutations have been identified so far. Polymorphisms in OATP1C1 were associated with clinical endpoints as fatigue and depression in hypothyroid patients on T4 substitution therapy (130).

TH HOMEOSTASIS IN THE BRAIN

Based on the previous information, TH homeostasis in the human brain is thought to involve at least the following steps, which is shown in the simplified Figure 3 (131). T4 is transported across the BBB by OATP1C1, MCT8 and possibly LAT1, whereas T3 is only a substrate for MCT8 and LAT1. After transport into astrocytes by OATP1C1 and other unknown transporters, D2 will convert T4 to T3. T3 will be released from the astrocytes by another unknown transporter. T3 that directly crossed the BBB and the produced T3 from the astrocytes is transported into neurons by MCT8 and LAT2. It could be possible that MCT8 is also able to supply T3 to oligodendrocytes. Finally, T3 will also be converted by D3 into the inactive metabolites 3,3'-T2 and rT3. Abnormal TH concentrations in the brain may lead to attenuated TH signalling and eventually in abnormal brain development. This is illustrated by mutations in MCT8 and in *THRA* (encoding TRα) which result in severe neurological and endocrine disorders (22-25,85,86). To which extent the other key players in TH metabolism are relevant for normal brain development is currently unknown.

Based on the importance of TH for normal brain development and the discovery that mutations in MCT8 lead to psychomotor retardation, one can hypothesize that other key players in TH

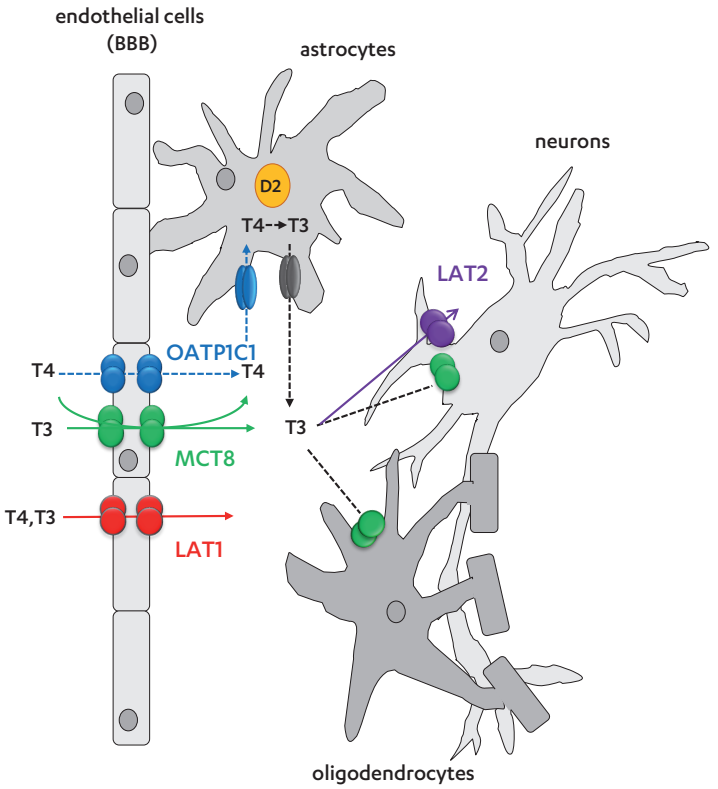

Figure 3 Simplified overview of TH transport in the central nervous system (CNS). Figure by Steffen Mayerl.

homeostasis may underlie a phenotype of ID. Therefore, a large cohort of patients with unexplained ID (IQ <50) of unknown origin (TOP-R study, the thyroid hormone origin of psychomotor retardation study) was collected, in which the characteristics were previously described (132). In this cohort several *MCT8* mutations in male patients were reported and studied. Subsequently, this cohort can be used for the identification of mutations in deiodinases, receptors and other transporters.

Essential trace elements, like copper (Cu) and selenium (Se), are nutrients that are present in very low concentrations in the human body and are dependent on sufficient dietary intake (133). Trace elements are essential for many enzymatic reactions and are therefore important for proper functioning of endocrine systems.

Sufficient levels of both TH and trace elements are important for normal development of the brain (1,134-136). Adequate levels of Cu are also important for normal development of the brain, as Cu is important in energy metabolism, anti-oxidative defence and the production of neurotransmitters (137). Also, it was shown that TH or Cu deficiencies resulted in similar defects during rodent cerebral cortical development (138). Furthermore, mice lacking selenoprotein P (SelP), which is an important Se carrier, showed very low brain Se levels together with movement disorders and occasional seizures (136).

Only few data on the relationship between TH and Se and Cu is currently available. Furthermore, in multiple studies, the usage of antiepileptic drugs (AEDs) was strongly associated with decreased T4, FT4, T3 and rT3 levels (139-141). Interestingly, AEDs have been associated with changes in serum levels of Se and Cu (132,142-146). However, the complex relationship between AEDs, THs, Se and Cu has never been directly studied. We used the TOP-R study to analyze the associations between AEDs, Se, Cu, and thyroid parameters.

GENDER RELATED DIFFERENCES IN TH SUPPRESSION DURING AGING

In humans, an age-dependent increase in serum thyroid stimulating hormone (TSH) and decrease in (free) T3 is observed, whereas (free) T4 remains unchanged (147-151) and some evidence suggests that in older individuals lowering of TH levels may be beneficial (152-154). We therefore recently, disclosed a novel link between aging and diminished TH signalling by demonstrating both decreased liver and kidney D1 activity and increased D3 activity in different models of premature aging, including *Csb*$^{m/m}$*/Xpa*$^{-/-}$ DKO mice and naturally aging mice (Visser et al. Submitted to PLoS One 2015). This may be a response to survive stressful conditions.

It is well known that there are sex-differences in aging and lifespan, with women living longer than men. It is also known that TH levels are different in men and women throughout life (147). These differences are most likely based on complex associations between TH signalling, aging and the influence of gonadal hormones (155-163). Furthermore, the females in *Csb*$^{m/m}$*/Xpa*$^{-/-}$ DKO group appeared to be in much better shape compared to the males, although the lifespan was not different. However, no studies have investigated gender differences in the attenuated TH state during aging. Therefore, we explored the possible influence of sex on TH homeostasis in different models of aging. As the benefits in treating older patients with subclinical hypothyroidism is already doubtful, it seems important to take gender differences into consideration in this question.

OUTLINE OF THE THESIS

The work in this thesis presents studies in which various key players of TH homeostasis, in health and disease, were investigated.

In the first two chapters the identification and functional analysis of mutations in the coding sequence (chapter 2) and the SECIS element (chapter 3) of D2 is described in patients with unexplained ID.

In chapter 4 we explored the role of the L-Type amino acid transporter (LAT) family members 1-5 in the transport of iodothyronines.

MCT8 is known to be a specific TH transporter. Chapter 5 challenges this claim by describing that MCT8 and MCT10 transport the TH precursors MIT and diiodotyrosine (DIT) out of the cells, which is the first evidence that the TH transporter MCT8 also accepts other substrates.

In chapter 6 we aimed to expand the current knowledge that in addition to T3, also T4, can exert transcriptional relevant effects at physiological concentrations.

In chapter 7, we have investigated gender differences in the attenuated TH state during aging.

Chapter 8 describes the associations between commonly prescribed AEDs, the trace elements Se and Cu and TH levels.

In chapter 9, the observations that are presented in this thesis are discussed in view of the current literature and possible implications of the findings are explored.

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Chapter 2

FUNCTIONAL ANALYSIS OF NOVEL GENETIC VARIATION IN THE THYROID HORMONE ACTIVATING TYPE 2 DEIODINASE

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ABSTRACT

Context

Thyroid hormones (TH) are important for normal brain development and abnormal TH regulation in the brain results in neurocognitive impairments. The type 2 deiodinase (D2) is important for local TH control in the brain by generating the active hormone T3 from its precursor T4. Dysfunction of D2 likely results in a neurocognitive phenotype. No mutations in D2 have been reported yet.

Objective

To identify D2 mutations in patients with intellectual disability (ID) and to test their functional consequences.

Design, setting and patients

Patients were selected from the multi-center TOP-R (Thyroid Origin of Psychomotor Retardation) study, which is a cohort of 946 subjects with unexplained ID. Based on characteristic serum TH values, the coding region of the *DIO2* gene was sequenced in 387 patients. Functional consequences were assessed by *in vitro* D2 assays or intact cell metabolism studies using cells transfected with wild-type or mutant D2.

Results

Sequence analysis revealed 2 heterozygous mutations: c.11T>A (p.L4H) in 3 subjects and c.305C>T (p.T102I) in 1 subject. Sequence analysis of family members revealed several carriers, but no segregation was observed with thyroid parameters or neurocognitive phenotype. Extensive tests with different *in vitro* D2 assays did not show differences between wild-type and mutant D2.

Conclusion

This study describes the identification and functional consequences of novel genetic variation in TH activating enzyme D2. Family studies and functional tests suggest that these variants do not underlie the neurocognitive impairment. Altogether, our data provides evidence of the existence of rare but apparently harmless genetic variants of D2.

INTRODUCTION

Thyroid hormones (TH) are important for homeostasis and development of virtually all tissues by regulating the expression of a variety of genes. The genomic actions of TH are mediated by nuclear TH receptors (TRs) bound to promoter elements of TH responsive genes. Binding of the active hormone T3 to TRs results in an altered interaction with co-repressor and co-activator proteins which changes the transcription of these genes. At the pre-receptor level, TH concentrations are regulated by TH transporters and deiodinases (1, 2). The type 2 (D2) and type 3 (D3) deiodinases regulate local TH bioactivity, with D2 generating T3 by outer ring deiodination of its precursor T4 and D3 inactivating T3 and T4 by inner ring deiodination.

Given the pre-eminent role of TH for brain development, mutations in different key players of TH regulation result in neurocognitive impairments. This is illustrated by mutations in the TH transporter monocarboxylate transporter 8 (MCT8), which results in a severe neurological and endocrine disorder, including intellectual disability (ID), motor abnormalities, and altered TH parameters (2, 3). Mutations in *THRA* (encoding TRα) also result in mild to severe cognitive impairments (4-7).

D2, encoded by the *DIO2* gene, is the only enzyme known to convert T4 to T3 in the brain and it has been estimated that D2 generates as much as 80% of cerebral T3 levels (8, 9). The importance of D2 for cognitive function in humans is supported by the observation that the commonly occurring Thr92Ala *DIO2* SNP has been associated with impaired psychological well-being (10) in general and during T4 replacement therapy with a normalized TSH. This genetic variant was also associated with a preference for LT3/LT4 combination therapy above LT4 monotherapy in hypothyroid patients (10). It has been observed that hypothyroid patients on LT4 replacement therapy can have reduced quality of life, despite normal serum TH levels (11). Although speculative, this may be explained in part by the influence of genetic variation in D2 on local conversion of T4 to T3 in the brain. It also has been reported that D2 polymorphisms are associated with bipolar disorder and with ID in the iodine deficient areas of China (12). Based on these observations, an important role has been ascribed to D2 in the regulation of TH bioactivity in the brain. However, no pathogenic D2 mutations have been identified so far in humans.

We hypothesized that mutations in D2 may result in impaired neurological development. Therefore, we collected a large cohort of 946 subjects with unexplained ID, in whom we profiled TH parameters (13). In this cohort several novel MCT8 mutations were identified as a proof of principle (14). Subsequently, we screened subjects with characteristic TH parameters for mutations in the *DIO2* coding sequence. We identified 2 heterozygous non-synonymous mutations and tested their functional consequences on D2 activity.

PATIENTS AND METHODS

Patients

Patients were selected from the TOP-R (Thyroid Origin of Psychomotor Retardation) study, which is a cohort consisting of 946 subjects with unexplained ID (IQ <50) in whom extensive profiling of TH parameters has been performed (13). The study was approved by the medical ethics committee of the Erasmus University Medical Center.

Serum analysis

Serum T4, T3, FT4 and TSH were measured by chemiluminescence assays (Vitros ECI, Ortho-Clinical Diagnostics Inc., Rochester, NY). rT3 was measured using a commercial RIA (Immunodiagnostic Systems, Scottsdale, AZ).

Screening and genetic analysis

Because the biochemical phenotype in humans with *DIO2* mutations is unknown, we used non-stringent criteria to select subjects for sequence analysis to maximize the chances of identifying such mutations. We based the selection criteria on the role of D2 in converting T4 to T3 and rT3 to T2, on the serum TH abnormalities in patients with *SBP2* mutations, as well as those in D2 knock-out mice (15-17). For inactivating mutations in *DIO2*, the following criteria were used: a) a T3/rT3 ratio <20th percentile and a FT4xlnTSH product >80th percentile, or b) a T3/T4 ratio <20th percentile and for possible activating mutations: c) a T3/T4 ratio >90th percentile and a TSH <10th percentile in the cohort. Applying these arbitrary, non-stringent criteria, a total of 387 patients from our cohort were selected.

The coding region of *DIO2* (NM_013989.3) was analyzed using the primers described in Supplementary Table 1. Oligonucleotides were synthesized by Invitrogen (Bleiswijk, The Netherlands). PCR and sequence reactions were carried out by BaseClear (Leiden, The Netherlands). For PCR reactions of family members, we used the Taq polymerase, dNTPs and buffers from Qiagen (Venlo, The Netherlands). The PolyPhen tool was used to predict pathogenicity of non-synonymous mutations (18).

DNA constructs and mutagenesis

A human D2 construct in pUHD10-3 was used as described previously (19). The identified mutations were introduced in this construct using the QuickChange Site-Directed Mutagenesis protocol (Stratagene, Amsterdam, The Netherlands). The presence of the introduced mutations was confirmed by DNA sequencing.

D2 activity in cell lysates

COS1 cells were cultured at 37 C° and 5% CO_2 in 75 cm^2 flasks with DMEM/F12 (Life Technologies, Bleiswijk, The Netherlands) supplemented with 9% heat-inactivated fetal bovine serum (Sigma Aldrich, Zwijndrecht, The Netherlands), penicillin-streptomycin, and 100 nM Na_2SeO_3. At confluence, cultured cells were split and seeded in six-well plates. At 70% confluence, cells were transiently transfected with 500 ng empty vector, wild-type (WT) or mutant D2 cDNA using X-treme Gene 9 Transfection Reagent (Roche, Mannheim, Germany).

Two days after transfection, cells were harvested and D2 activity was measured in lysates using HPLC, as described previously (20). Protein levels were determined using the method of Bradford (21). Transfection efficiency was similar in all conditions as assessed in preliminary experiments carried out by co-transfection with human GH cDNA and measurement of growth hormone concentrations in the medium. Unlabeled iodothyronines were obtained from Sigma Aldrich. [^{125}I]T4 and [^{125}I]rT3 were prepared as previously described (22).

For D2 half-life studies, transfected cells were treated for 60 min before harvesting with 100 µM cycloheximide (CHX; Sigma Aldrich) or vehicle (DMSO). To study substrate-induced D2 degradation, the above protocol was modified by treatment for 60 min before harvesting with 10 µM MG132 (Cayman Chemical, Tallinn, Estonia), 100 nM rT3, or vehicle (DMSO or NaOH).

D2 metabolism in intact cells

COS1 cells were cultured in 24-well plates using 500 µl DMEM/F12 supplemented with 9% FCS and with 100 nM Na_2SeO_3. At 70% confluence, cells were transiently transfected with 200 ng WT or mutant D2 cDNA plus 80 ng of the transactivator pUHD15 (23). Two days after transfection, cells were incubated for 24 h with 1 nM [^{125}I]T4, and T4 metabolism was analyzed using HPLC as described previously (20).

Statistical analysis

All results are presented as the means ± SEM of 2-4 independent experiments performed in duplicate. Statistical significance between WT and mutant D2 activity in cell lysates and intact cells was determined using Student's t test for unpaired observations. For the D2 half-life studies, we used Student's t test for unpaired observations to calculate significant differences in the decrease or increase in D2 activity between WT and mutant D2.

The associations with thyroid parameters of the T92A SNP were assessed using SPSS 22 for Windows (SPSS, Inc, Chicago, IL). Deviation from Hardy-Weinberg equilibrium was analyzed using a χ^2 test. Differences between groups were compared using 1-way AN[C]OVA to adjust differences between genotype groups for age, sex and use of antiepileptic drugs. Logarithmic transformations were performed if appropriate. All analyses were performed with exclusion of patients who used thyromimetic or thyrostatic medication and patients with overt hypothyroidism (TSH >4.3 mU/L and FT4 <11 pmol/L) or hyperthyroidism (TSH <0.4 mU/L and FT4 >25 pmol/L).

RESULTS

DIO2 sequence analysis

Sequence analysis of the coding region of *DIO2* revealed 2 heterozygous mutations in 4 patients (Figure 1). In 3 patients (#9, #26 and #622) an identical missense mutation was observed, resulting in a Leu to His substitution at amino acid position 4 (c.11T>A; p.L4H). Patient #370 showed a missense mutation resulting in a Thr to Ile substitution at position 102 (c.305C>T; p.T102I). The serum thyroid parameters of the patients with *DIO2* mutations, which persisted during follow-up, are depicted in Table 1.

T92A SNP

The D2 common SNP (c.274A>G; p.T92A; rs225014) was detected in 215 (60.5%) of the 355 remaining patients after exclusion of patients with overt thyroid disease or on LT4 and/or thyrostatic drugs. Distribution of genotypes (39.5% AA, 43.9% AG, 16.6% GG) did not deviate from Hardy-Weinberg equilibrium. In this selection of patients with ID, the D2 T92A SNP was

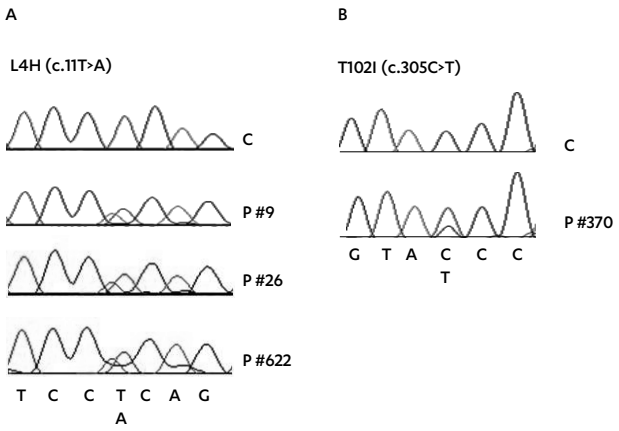


Figure 1 Partial sequence profiles of the *DIO2* gene. **A** c.11T>A (p.L4H) mutation in patients (P) #9, #26 and #622 and control (C). **B** c.305C>T (p.T102I) mutation in patient #370 and control.

associated with slightly lower levels of TSH (2.08 mU/L AA, 1.95 mU/L AG, 1.67 mU/L GG, p=0.021) but not with FT4 levels or the T3/T4 ratio.

L4H mutation

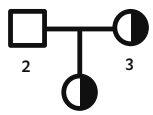
After the identification of the L4H missense mutation in 3 patients, we aimed to screen the family members of the patients. Only relatives of patients #9 (mother) and #26 (father and mother) were available for this screening. Sequence analysis in family members of patient #26 revealed the same mutation in the mother (#26-3), but not in the father (#26-2) of the patient (Figure 2A). Clinically, the mother (#26-3) does not have a neurocognitive phenotype. Also, no clear co-segregation was observed with thyroid parameters or ratios thereof (Figure 2A). Sequence analysis of the father of patient #9 did not show the mutation (Figure 2B). The increase in publicly available databases with next generation sequence profiles of healthy individuals allowed us to identify this variant in the 1000 Genomes Catalog (ESP_14_80677805) (24) and in the NHLBI Exome Variant Server (25) with a minor allele frequency of 0.056.

The PolyPhen tool predicted that this mutation could be possibly damaging and alignment of *DIO2* sequences from several species revealed that amino acid Leu4 is highly conserved (Figure 2C) (26). To functionally test the L4H mutation, we measured D2 activity in cells transfected with WT D2 or the L4H mutant. No significant differences in deiodinase activities were observed in both cell lysates (Figure 2D) and intact cells (Figure 2E). Kinetic studies did not show differences in apparent Km and Vmax values between WT D2 and the L4H mutant (data not shown).

T102I mutation

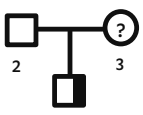
After the identification of the T102I mutation in patient #370, subsequent sequence analysis revealed the T102I mutation in two of the patient's relatives as well. No co-segregation was observed in this family of the mutation with serum TH levels or ratios thereof (Figure 3A). The T102I

A



		1(P#26)		Normal range
TSH	-	0.69	0.77	0.4-4.3 mU/L
T4	77.8	106	95.1	58-128 mmol/L
FT4	25.8	21.1	21.0	11-25 pmol/L
T3	1.48	1.75	1.70	1.4-2.5 mmol/L

B



	1 (P#9)		Normal range
TSH	1.56	1.90	0.4-4.3 mU/L
T4	75.1	55	58-128 mmol/L
FT4	14.1	7.5	11-25 pmol/L
T3	2.03	1.53	1.4-2.5 mmol/L

C

H

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HsapD2      : MGI L SVDLLIT
PtroD2      : MGI L SVDLLIT
MmulD2      : MGI L SVDLLIT
DnovD2      : MGI L SVDLLIT
LafrD2      : MGI L SIDLLIT
MlucD2      : MGI L SVDLLIT
PvamD2      : MGI L SVDLLIT
BtauD2      : MGI L SVDLLIT
TtruD2      : MGI L SVNLLIT
EcabD2      : MGI L SVDLLIT
SscrD2      : MGI L SVDLLIT
CfamD2      : MGI L SVDLLIT
ChofD2      : MGI L SVNLLIT
OgarD2      : MGI L SVDLLIT
CporD2      : MGI L SIDLLIT
OpriD2      : MGI L SVDLLIT
DordD2      : MGI L SVDLLIT
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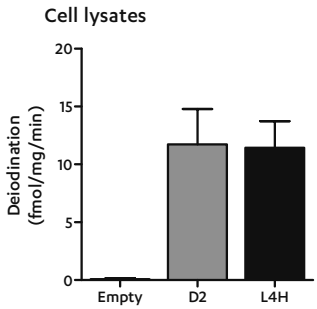
D



E

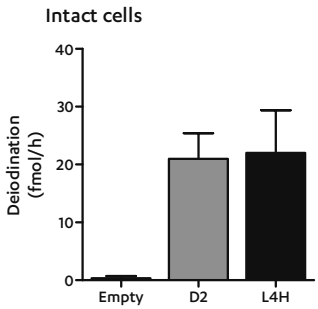


Figure 2 A,B Pedigree and TH values of L4H index patients and family members. **C** Alignment of the N-terminal amino acid sequence of D2 in different species. The amino acid change of the L4H mutation is indicated above the sequence. **D** D2 activity, corrected for protein concentration (fmol/mg/min), in cell lysates of COS1 cells transfected with empty vector, WT or L4H D2 cDNA. **E** Metabolism of [^{125}I]T4 in intact transfected COS1 cells after incubation for 24 hours (fmol/h). Results are the means ± SEM of three experiments. Significance represents WT D2 versus L4H mutant.

missense mutation has also been identified in the 1000 Genomes Catalog (ESP_14_80669549) and in the NHLBI Exome Variant Server with a minor allele frequency of 0.008.

The T102I mutation was predicted to be a benign variant by the PolyPhen tool. Indeed, testing the functional impact of this mutation, we could not demonstrate differences in deiodinase activity

between WT D2 and the T102I in cell lysates or intact cell metabolism studies (Figure 3B,C). Also kinetic studies did not show differences in apparent Km and Vmax values between WT D2 and the T102I mutant (data not shown). Although T102 is not conserved in D2 sequences from different species (Suppl. Figure 3), this mutation is localized within an 18-amino acid loop (92-109) which among the deiodinases is unique for D2. This loop is critical for recognition by the ubiquitination complex, thereby targeting the protein for (substrate-induced) proteasomal degradation (27).

To test the impact of this mutation on the process of ubiquitination, we studied the half-life of WT D2 and the T102I mutant, and their substrate-induced degradation. After incubation with the protein synthesis inhibitor cycloheximide (CHX), we observed the expected decrease in D2 activity (Fig 3D). However, enzyme activity decreased to a similar extent for WT D2 and T102I mutant (Figure 3D). These studies were extended using the proteasome inhibitor MG132 to inhibit ubiquitination or rT3 which enhances ubiquitination of D2. As expected, incubation with MG132 increased D2 activity by preventing proteasomal degradation. However, the increase in activity was similar between WT and mutant D2 (Figure 3E). Also, incubation with rT3 did not reveal a significant difference in substrate-induced enzyme inactivation between WT D2 and the T102I mutant (Figure 3F).

DISCUSSION

During recent years, it has become increasingly clear that TH levels at the cellular level are importantly controlled by D2 and D3 (28). Manipulation of either D2 or D3 results in impressive changes in gene expression and, consequently, in differentiation and proliferation of various tissues including the brain (28-33). As a consequence, mutations in D2 may give rise to neurocognitive impairments in humans. Until now, no pathogenic D2 mutations in humans have been reported.

In the present study, we report on the identification and functional consequences of 2 novel heterozygous mutations in D2 and the occurrence of the common T92A SNP. The prevalence of the T92A SNP is this selection of patients is comparable with previous studies (10, 34-39). This observation suggests that this SNP is not related to ID. We found an association between this SNP and slightly lower TSH levels. Some, but not all previous studies also reported a slightly lower TSH associated (10, 34, 36, 37, 39-41). However, in line with other studies, no association was observed with FT4 levels or the T3/T4 ratio. Apparently, this SNP is not related to obvious changes in serum TH levels. Functional studies have not shown differences in D2 activity for this SNP (39).

In this study we report on the L4H and T102I mutations that were discovered in the TOP-R cohort, which consists of subjects with severe ID. However, the mutations were also present in unaffected family members. After we identified these mutations, the same genetic variants became available in different databases with next generation sequences of healthy individuals at a low frequency. There is no clear explanation why the prevalence of these variants is higher in our cohort compared to the databases. The occurrence of these polymorphisms in healthy family members and genomic databases suggests that the D2 mutations identified in our cohort do not underlie the neurocognitive impairment in these patients and that mutations in D2 are not a frequent cause of severe ID. This is in contrast to several MCT8 mutations, which were identified in the TOP-R study and are causally linked to ID (15). The co-existence of mutations in D3 were ruled out (data not shown).

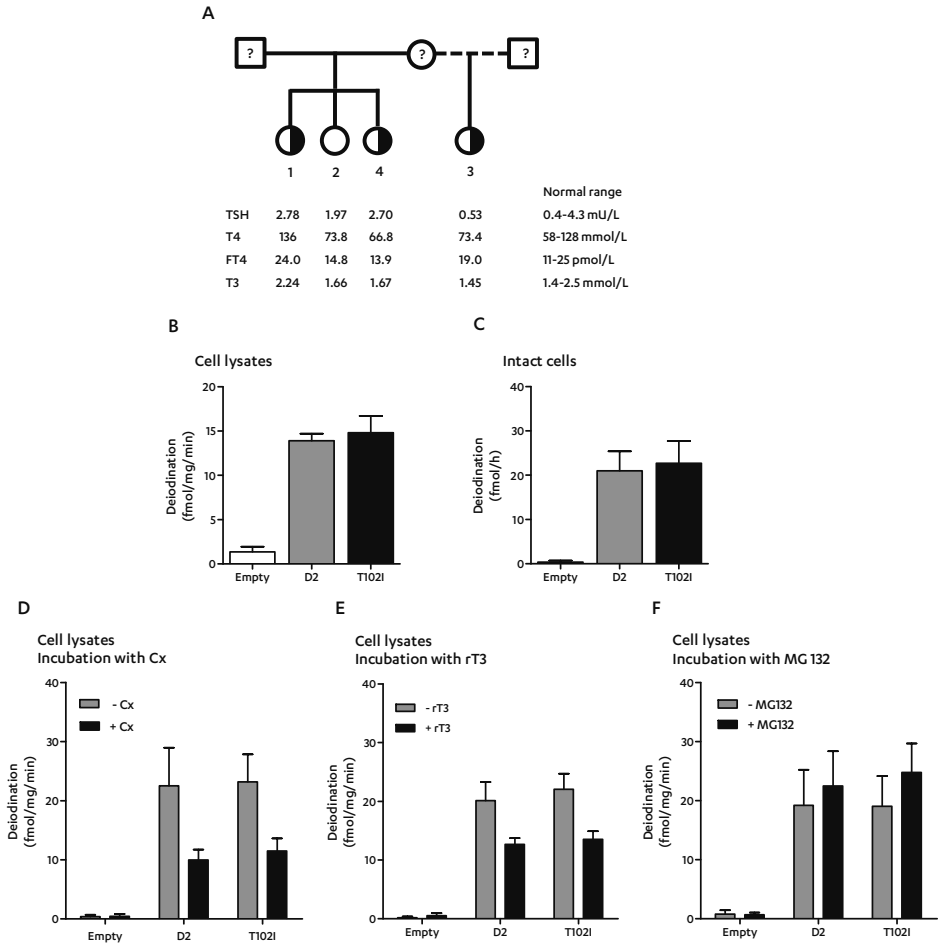


Figure 3 A Partial pedigree and TH values of T102I index patient and family members. **B** D2 activity, corrected for protein concentration (fmol/mg/min), in cell lysates of COS1 cells transfected with empty vector, WT or T102I D2 cDNA. **C** Metabolism of [^{125}I]T4 in intact transfected COS1 cells after incubation for 24 hours (fmol/h). **D,E,F** Cells transfected with empty vector, WT or T102I D2 cDNA were treated for 1 h with 100 μM cycloheximide (D), 10 nM rT3 (E) or 10 μM MG132 (F) or vehicle. D2 activity is corrected for protein concentration and is shown in fmol/mg/min. Results are the means ± SEM of 2-3 experiments. Significance represents WT D2 versus T102I mutant.

The functional consequences of these novel genetic variants were studied using cell lysates containing recombinant WT or mutant D2 in the presence of DTT as synthetic cofactor, or in intact cells expressing D2 under natural conditions. Neither the L4H nor the T102I variant differed in activity compared to WT D2. Elegant studies have shown that D2 is inactivated by ubiquitination which is accelerated in the presence of substrate (42-44). The T102I variant is localized in the middle of an 18-amino acid loop which is critical for recognition of the ubiquitination complex (27). Therefore, we studied the possible interference of the T102I mutation with D2 ubiquitination. However, no differences were observed when the

ubiquitination process was modulated with the proteasomal inhibitor MG132 or with rT3. Thus, the functional studies suggest that these genetic variants do not have functional consequences.

Although the mutations appear not to be related to a specific phenotype, it cannot be excluded that these variants exert effects under different conditions. For example, based on the importance of D2 in influencing local T3 levels, it can be speculated that patients harboring these variants, who are dependent on treatment with exogenous T4 (e.g. in primary hypothyroidism), respond differently than non-carriers. If the newly identified variants interfere with D2 function they may play a role in the level of efficacy of LT4 treatment (10).

Altogether, our data provide evidence of the existence of rare but apparently harmless genetic variants of D2 that were also published in genomic databases. This field of next generation sequencing is rapidly expanding, leaving us with numerous variants in different genes without having knowledge about possible pathogenic effects. Here we described the screening of almost 400 subjects with severe ID, selected based on their thyroid function tests that could fit with a defect in D2. The identified variants in D2 were extensively functionally analyzed and no harmful effect was found. Pathogenic mutations in D2 therefore do not seem to be a frequent cause of ID, which is important information for endocrinologists and clinical geneticists. Therefore, the phenotype of patients with pathogenic D2 mutations remains elusive.

ACKNOWLEDGEMENTS

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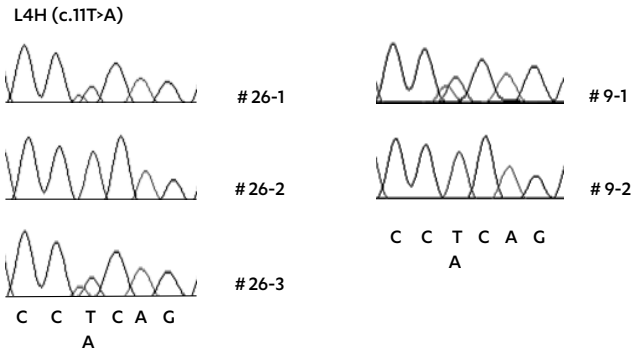
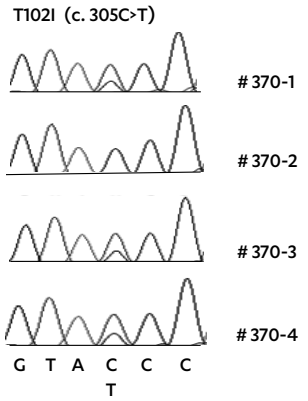
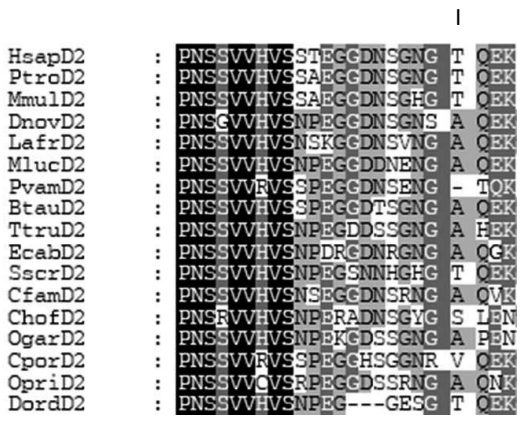


Supplemental Figure 1 Partial sequence profiles of the L4H index patient and family members.



Supplemental Figure 2 Partial sequence profiles of the T102I index patient and family members.



Supplemental Figure 3 Alignment based on the amino acids of the first part of the D2 protein is shown for different species. The amino acid changes of the T102I mutation is indicated above the sequence.

Supplemental Table 1 Synthetic oligonucleotides for PCR (P) and sequencing (S) of DIO2.

DIO2	Forward primer (5'-3')	Reverse primer (5'-3')
Exon 1 (P&S)	CCACCCCCTTTATCACCAC	ATGCTCCCAATGGCCTCT
Exon 2 (P)	TGTGAATTCAAGTGGCAATG	CCAATAGGGCTCTGTTGAAA
Exon 2 (S)	TCCTCAGTGGCTGACTTCCT	GCACATCGATCTTCCTGGTT

Chapter 3

FUNCTIONAL ANALYSIS OF NOVEL GENETIC VARIATION IN THE SECIS ELEMENT OF THYROID HORMONE ACTIVATING TYPE 2 DEIODINASE

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To be submitted

ABSTRACT

Context

Thyroid hormone (TH) is important for normal brain development and the type 2 deiodinase (D2) is important for local TH control in the brain by activating T4 to T3. D2 activity is dependent on the incorporation of selenocysteine (Sec) by the SECIS element. We therefore hypothesized that mutations in the SECIS element could result in a neurocognitive phenotype.

Objective

To identify mutations in the SECIS element of *DIO2* in patients with intellectual disability (ID) and to test their functional consequences.

Design, setting and patients

In the TOP-R cohort of 946 subjects with unexplained ID, the SECIS element of *DIO2* was sequenced in 387 patients, based on characteristic serum TH values. SECIS element read-through in wild-type (WT) or mutant D2 was quantified by a luciferase reporter system in transfected cells. Functional consequences were assessed by cell lysate or intact cell metabolism studies.

Results

Sequence analysis revealed 2 heterozygous mutations: c.5703C>T and c.5730A>T. Sequence analysis of family members revealed several carriers. Extensive tests with different *in vitro* D2 assays did not show differences between WT and 5703C>T SECIS mutant. However, the presence of the 5730A>T mutation decreased SECIS element read-through by 75% without affecting D2 activity in cell lysates or intact cells.

Conclusion

We identified 2 heterozygous mutations in this cohort of patients with unexplained ID. The 5703C>T mutation did not show any functional consequences. Our data provide evidence that nucleotide 5730 seems to be important for normal SECIS element read-through. However, we could not demonstrate any deleterious effect on D2 activity. At present, the phenotype of ID appeared not to be related to the presence of this mutation. Whole genome sequencing will exclude other causes of neurological impairment.

INTRODUCTION

Thyroid hormone (TH) is crucial for normal development and function of virtually all tissues, including the brain (1). This is illustrated by mutations in the TH transporter MCT8, which results in a severe neurological and endocrine disorder, including intellectual disability (ID), motor abnormalities, and altered TH parameters (2,3).

D2 is the only enzyme known in the brain that converts T4 to the active form T3 (4,5) and the expression of TH sensitive genes in neurones is dependent on T4 to T3 conversion by the type 2 deiodinase in the glial compartment (6). Also the commonly occurring Thr92Ala *DIO2* SNP has been associated with impaired psychological well-being, which supports the importance of D2 for cognitive function in humans (7). No pathogenic mutations in the coding sequence of D2, encoded by the *DIO2* gene, have been reported in humans until now. We hypothesized that mutations in D2 may result in impaired neurological development.

Recently, we reported on a study in which we aimed to identify mutations in the type 2 deiodinase (D2) (8). In this study, patients were selected from the TOP-R (Thyroid Origin of Psychomotor Retardation) study, which is a cohort consisting of 946 subjects with unexplained intellectual disability (ID). In this cohort of patients with ID, we identified 2 heterozygous non-synonymous mutations in the coding sequence of D2. However, functional studies suggested that these mutations do not affect D2 enzyme activity and are in fact rare but harmless variants.

In the present study we focused on the identification of mutations in the SECIS element of D2 using an identical approach. Deiodinases are selenoproteins, which hold an UGA codon in the middle of the mRNA that needs to be translated into the rare amino acid selenocysteine (Sec) instead of representing a stop signal. This process requires a selenocysteine insertion sequence (SECIS) element in the 3'UTR of the mRNA. The SECIS element has a stem-loop structure that forms a complex with Secis Binding Protein 2 (SBP2) which in turn interacts with a specific elongation factor leading to the incorporation of Sec (9). The presence of Sec is important for normal enzymatic activity of D2 (10). The human D2 SECIS element is known to contain three groups of essential nucleotides, and, therefore introduction of a mutation in one of these groups will totally abolish SECIS activity (11). Obviously, selenium (Se) is essential for the synthesis and activity of all selenoproteins, including D2 (12) (13-15). The crucial role of Sec in deiodinases was shown in families with SPB2 mutations, who have a multi-system disorder including impaired deiodinase function and abnormal TH levels in serum (16,17).

Although the identified variations in the coding sequence of *DIO2* appeared to be harmless, we still hypothesized that mutations in the SECIS element could be pathogenic. Subsequently, we used the same approach to sequence the SECIS elements of 387 patients with unexplained ID. Here we report on the identification of 2 heterozygous mutations in the SECIS element of D2 and the testing of the functional consequences.

PATIENTS AND METHODS

Patients

Patients were selected from the TOP-R (Thyroid Origin of Psychomotor Retardation) study, which is a cohort consisting of 946 subjects with unexplained ID (IQ <50) in whom extensive

profiling of TH parameters has been performed (18). The study was approved by the medical ethics committee of the Erasmus University Medical Center.

Serum analysis

Serum T4, T3, FT4 and TSH were measured by chemiluminescence assays (Vitros ECI, Ortho-Clinical Diagnostics Inc., Rochester, NY). rT3 was measured using a commercial RIA (Immunodiagnostic Systems, Scottsdale, AZ).

Screening and genetic analysis

Because the biochemical phenotype in humans with *DIO2* mutations is unknown, we used non-stringent criteria to select subjects for sequence analysis to maximize the chance of identifying such mutations. The selection criteria were previously described (8). A total of 387 patients from our cohort were selected for *DIO2* SECIS sequencing.

The SECIS element of *DIO2* (NM_013989.3) was analyzed using the primers described in Supplementary Table 1. Oligonucleotides were synthesized by Invitrogen (Bleiswijk, The Netherlands [NL]). PCR and sequence reactions were carried out by BaseClear (Leiden, NL). For PCR reactions of family members, we used the Taq polymerase, dNTPs and buffers from Qiagen (Venlo, NL). The SeciSearch and Mfold programs were used to analyze the effects of mutations on the structure of the SECIS element (19,20).

DNA constructs and mutagenesis

The cloning of the luciferase SECIS reporter construct in pcDNA3.1 was previously described (21). This expression vector contains a WT or mutant SECIS element, that is placed directly after the cDNA coding for a Sec mutant of luciferase. This construct is used to quantify the recoding efficiency of the D2 SECIS element. The identified patients' mutations (5703C>T and 5730 A>T) were introduced in this construct using the QuickChange Site-Directed Mutagenesis protocol, according to the manufacturer (Stratagene, Amsterdam, NL). The artificial 5730A>C and 5679 G>A mutations were introduced using the same protocol. The 5679 G>A is known to inactivate the SECIS element and is used in our experiments as a positive control (11).

To determine the effect of SECIS mutations on D2 activity, we used a construct that contained the human D2 coding sequence and part of the 3'UTR (~2.5 kb) including the SECIS element cloned in pUHD10-3, as described previously (11). The identified patients' mutations 5703C>T and 5730 A>T and the artificial mutations 5679G>A and 5730A>C were introduced in this construct by mutagenesis as described before.

The described constructs that we used in our experiments are cloned in different expression vectors and also the presence of 3'UTR is not similar. That is why we first used two *Afe1* restriction sites to digest part of the 3'UTR (~2 kb) from the D2- pUHD10-3 construct to match the luciferase reporter construct. Next we used *HindIII* and *Xho1* restriction sites to subclone the short construct into pcDNA3. Again we performed mutagenesis to introduce the 5730A>T mutation.

SECIS element recoding efficiency assay

COS1 cells were cultured in 96-well plates using 100 µl DMEM/F12 supplemented with 9% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin and with 100 nM Na_2SeO_3. At 70% confluence cells were transiently transfected for 48 h with 25 ng of the luciferase D2 SECIS element reporter construct (WT or mutant) and 25 ng of a control renilla luciferase reporter. After washing, luciferase and renilla signals were analyzed in lysed cells using the Dual-Glo Luciferase Assay System (Promega, Leiden, NL). Firefly luciferase activity was normalized to renilla luciferase activity to adjust for transfection efficiency.

D2 activity in cell lysates

COS1 cells were cultured at 37 C° and 5% CO_2 in 75 cm^2 flasks with DMEM/F12 (Life Technologies, Bleiswijk, NL) supplemented with 9% FCS (Sigma Aldrich, Zwijndrecht, NL), 1% penicillin-streptomycin and with or without 100 nM Na_2SeO_3. At confluence, cultured cells were split and seeded in six-well plates. At 70% confluence, cells were transiently transfected with 500 ng empty vector, WT or mutant D2 cDNA using X-treme Gene 9 Transfection Reagent (Roche Diagnostics, Almere, NL).

Two days after transfection, cells were harvested and D2 activity was measured in lysates using HPLC, as described previously (22). Protein levels were determined using the method of Bradford (23). Transfection efficiency was similar in all conditions as assessed in preliminary experiments carried out by co-transfection with human GH cDNA and measurement of growth hormone concentrations in the medium. Unlabeled iodothyronines were obtained from Sigma Aldrich. [^{125}I]T4 and [^{125}I]rT3 were prepared as previously described (24).

D2 metabolism in intact cells

COS1 cells were cultured in 24-well plates using 500 µl DMEM/F12 supplemented with 9% FCS and with 100 nM Na_2SeO_3. At 70% confluence, cells were transiently transfected with 200 ng WT or mutant D2 cDNA plus 80 ng of the trans-activator pUHD15 (25). Two days after transfection, cells were incubated for 24 h with 1 nM [^{125}I]T4, and T4 metabolism was analyzed using HPLC as described previously (22).

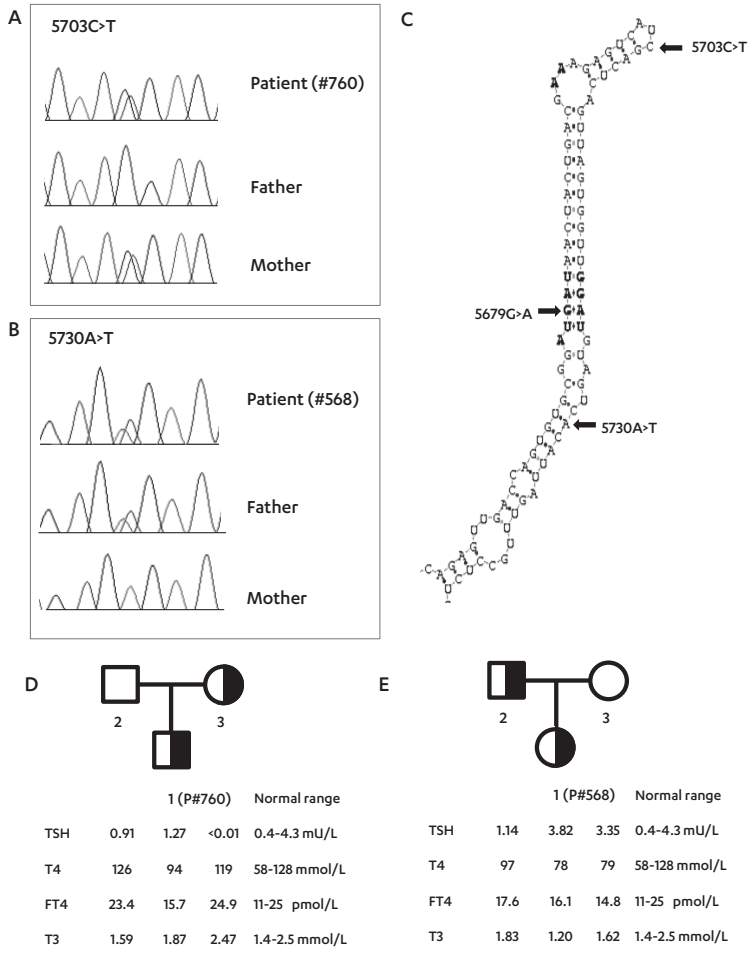
Statistical analysis

All results are presented as the means ± SEM of 2-4 independent experiments performed in duplicate. Statistical analysis was carried out in GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Statistical significance of WT D2 compared to the various mutants in the luciferase, cell lysate and intact cell assays, was determined using one-way ANOVA followed by a Bonferroni post. $^*P$ <0.05; $^{**}P$ <0.01; $^{***}P$ <0.001.

RESULTS

Sequence analysis of the SECIS element of *DIO2* revealed heterozygous mutations in patient #760 (c.5703C>T) and in patient #568 (c.5730A>T) (Figure 1A-B). After identification of these mutations in two patients, subsequent sequence analysis revealed the presence of these mutations also in

relatives of both patients (Figure 1A-B). None of the relatives had ID. The patients with mutations in the SECIS element did not exhibit identical biochemical phenotypes and this was persistent during follow up (Figure 1D-E). Also no clear co-segregation of serum TH levels, or ratios thereof, was observed in the subjects carrying a mutation (Figure 1D-E). The SECIS element mutations have not been described in the 1000 Genomes Catalog (26) or in the NHLBI Exome Variant Server (27). Although the mutations are not localized in the 3 clusters of highly conserved nucleotides (depicted in bold) that are known to be most important for SECIS element functionality (Figure 1B), c.5703C>T and c.5730A>T putatively change the stem-loop structure of the SECIS element (19). The 5679G>A is an artificial mutation that is localized within the AUGA motif of the SECIS element and this mutation has been shown to totally abolish SECIS element activity (Figure 1C) (11).



		1 (P#760)		Normal range
TSH	0.91	1.27	<0.01	0.4-4.3 mU/L
T4	126	94	119	58-128 mmol/L
FT4	23.4	15.7	24.9	11-25 pmol/L
T3	1.59	1.87	2.47	1.4-2.5 mmol/L

	1 (P#568)			Normal range
TSH	1.14	3.82	3.35	0.4-4.3 mU/L
T4	97	78	79	58-128 mmol/L
FT4	17.6	16.1	14.8	11-25 pmol/L
T3	1.83	1.20	1.62	1.4-2.5 mmol/L

Figure 1 A-E Partial sequence profiles of the 5703C>T **(A)** and 5730A>T **(B)** index patients and family members. Loop structure of hD2 SECIS element with the localization of the patients mutations and the artificial 5679G>A mutation **(C)**. Pedigree and TH values of the 5703C>T **(D)** and 5730A>T **(E)** index patients and family members.

SECIS element recoding efficiency assay

To quantify the SECIS element read-through in WT and SECIS mutants, we used an expression vector containing a WT or mutant SECIS element placed after a luciferase gene. In this construct a TGC, coding for Cys, has been replaced by TGA, coding for Sec. Decreased SECIS function will result in less efficient incorporation of Sec and decreased luciferase activity will be measured. The SECIS element read-through in the 5703C>T patients mutation was comparable to the WT (Figure 2A). However, the luciferase-renilla ratio for the second mutation decreased by ~75% (Figure 2A). As expected, the 5679G>A artificial mutation showed no read-through and can be used as a positive control (Figure 2A).

Next we mutated the 5730A>T mutation back to WT, resulting in 100% activity, confirming the specificity of this mutation (Figure 2A). As a final approach we mutated nucleotide 5730 from A to C and showed an even more pronounced decrease in the luciferase activity, compared to the patients' mutation (Figure 2A). Therefore the nucleotide at position 5730 in the SECIS element appears to be important for SECIS element read-through.

D2 activity in cell lysates and intact cells

Next, we studied the effects of the SECIS mutants on D2 activity. In cell lysates, we observed no significant difference in D2 activity between WT and the SECIS mutants (Figure 2B). As anticipated, the 5679G>A mutant showed almost no activity in all experiments. As D2 activity depends on Se availability, we also tested the function of the SECIS variants under Se deficient culture conditions. In Se deficient cultures, the total D2 activity in cell lysates decreased around 50%, however, there was no significant difference between WT and mutants (Figure 2C). Next, we used the same approach in intact cell metabolism studies. In normal and Se deficient culture conditions, we could not demonstrate a difference in function between WT and SECIS mutants (Figure 2D-E). Also kinetic studies did not show differences in apparent Km and Vmax values between WT D2 and the SECIS mutants (data not shown). As the artificial 5730A>C mutant showed an even more impaired SECIS function, we assessed WT and 5730A>C mutant D2 activity. In cell lysates, a non-significant trend was observed towards a decreased D2 activity compared to the WT (Figure 2F). However, this could not be confirmed in the intact cell metabolism assay (Figure 2G).

Inter-assay variability by different constructs

As we observed inconsistent results in the SECIS element read-through assay and the measurement of D2 activity in the 5730A>T mutant, we aimed to minimize the differences between these constructs. In the luciferase construct, the SECIS element is placed directly after the coding sequence, whereas in the D2-pUHD10-3 vector, part (~2.5 kb) of the *DIO2* 3'UTR is present. We reasoned that the large 3'UTR could modulate deiodinase activity, as has been shown before (28).

After deletion of almost all 3'UTR (~2 kb) in the D2-pUHD10-3 constructs, we observed higher total D2 activities in cell lysates, compared to constructs containing the longer 3'UTR. However no differences could be measured in D2 activities between WT and 5730 A>T mutant (Figure 3A). Also in intact cell metabolism studies WT and mutant D2 activities were similar (Figure 3B). After sub-cloning the D2 insert into pcDNA3 to maximally match the luciferase

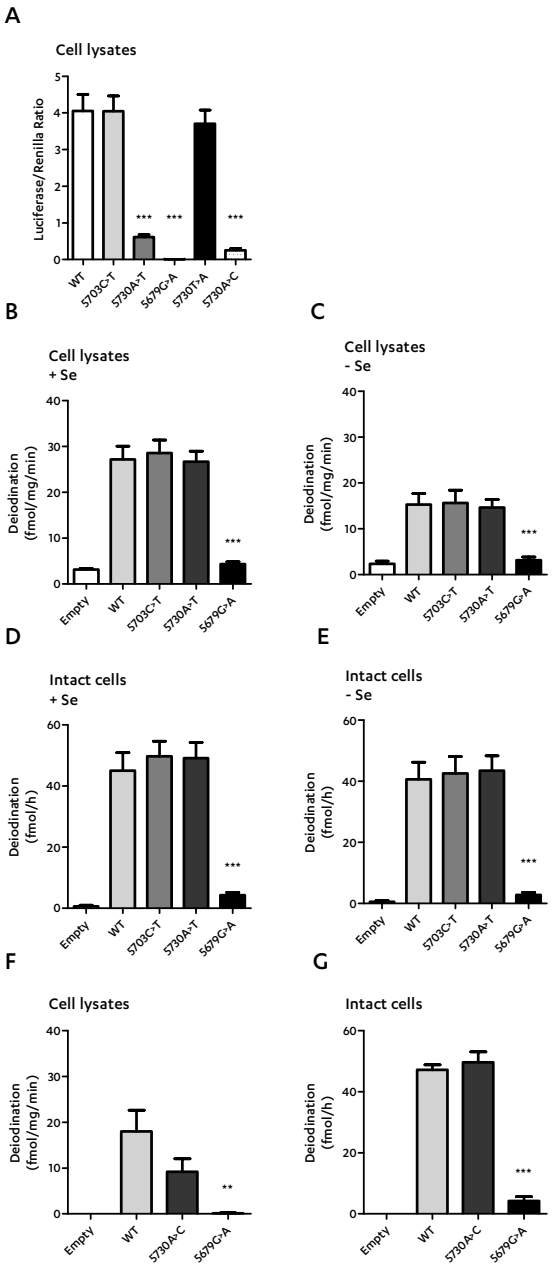


Figure 2 A-G Luciferase-renilla ratios in cell lysates of COS1 cells transfected with WT or mutant cDNA **(A)**. D2 activity, corrected for protein concentration (fmol/mg/min), in cell lysates of COS1 cells transfected with empty vector, WT or mutant cDNA with or without Se **(B,C)**. Metabolism of [^{125}I]T4 in intact COS1 cells transfected with empty vector, WT or mutant cDNA with or without Se after incubation for 24 hours (fmol/h) **(D,E)**. D2 activity, corrected for protein concentration (fmol/mg/min), in cell lysates of COS1 cells transfected with empty vector, WT or 5730 A>C **(F)**. Metabolism of [^{125}I]T4 in intact transfected COS1 cells with empty vector, WT or 5730 A>C after incubation for 24 hours (fmol/h) **(G).** Significance represents WT D2 versus SECIS mutants.

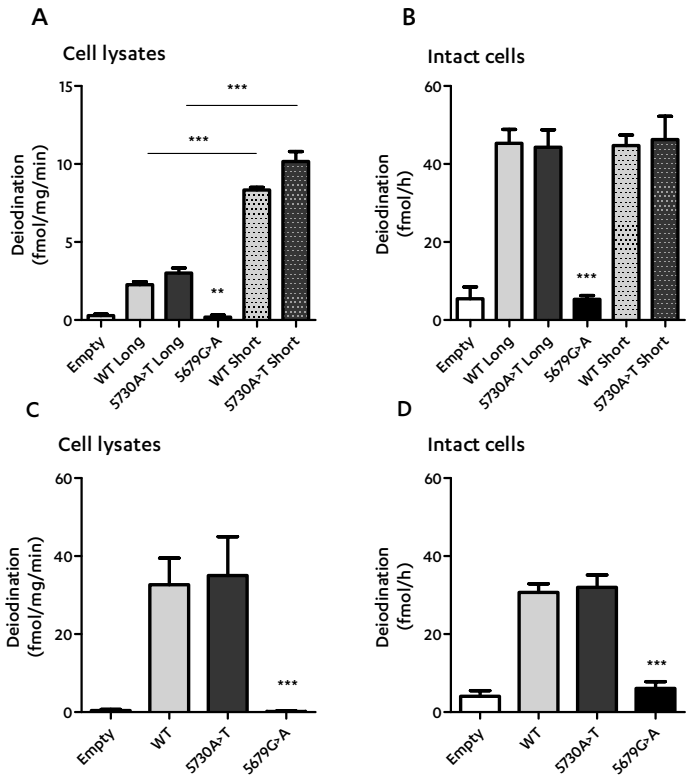


Figure 3 A-D D2 activity, corrected for protein concentration (fmol/mg/min), in cell lysates of COS1 cells transfected with empty vector, WT or mutant cDNA in long versus short constructs **(A)**. Metabolism of [^{125}I]T4 in intact COS1 cells transfected with empty vector, WT or mutant cDNA in long versus short constructs after incubation for 24 hours (fmol/h) **(B)**. D2 activity, corrected for protein concentration (fmol/mg/min), in cell lysates of COS1 cells transfected with empty vector, WT or mutant cDNA in pcDNA3 **(C)**. Metabolism of [^{125}I]T4 in intact transfected COS1 cells with empty vector, WT or mutant cDNA in pcDNA3 after incubation for 4 hours (fmol/h) **(D).** Significance represents WT D2 versus SECIS mutants or WT long versus WT short and mutant long versus mutant short in cell lysates.

construct, we still did not observe differences between WT and SECIS mutants in both cell lysates and intact cell metabolism assays (Figure 3C-D).

DISCUSSION

As D2 is important for the activation of T4 to T3 in the brain (1) and T3 is necessary for normal neurological development (5), we hypothesized that mutations in D2 can give rise to neurological impairment. Recently, we reported on the existence of rare, but harmless variants in the coding sequence of D2 in a cohort of patients with ID (TOP-R) (8).

In D2, the SECIS element is crucial to introduce Sec and the presence of this amino acid is important for normal enzyme activity (10,11). As we hypothesized that mutations in the SECIS element of D2 could be pathogenic, we used an identical approach to sequence the SECIS

elements in this cohort of patients with ID. We identified two heterozygous mutations in the SECIS element of D2 and here we report on the functional consequences of these mutations.

The mutations were also present in unaffected family members and did not segregate with a clinical or biochemical phenotype. As the mutations were not localized within the groups of highly conserved nucleotides of the SECIS element, it was not surprising that the 5703C>T mutant did not interfere with normal SECIS element and D2 functionality (Figure 2). Interestingly, we noticed a 75% decrease in SECIS element read-through in the 5730A>T mutant (Figure 2A). However, D2 activity was not affected as assessed by *in vitro* D2 activity assays in cell lysates and intact cells under both Se replete and Se deplete conditions (Figure 2B-E). The 5730A>C artificial mutation showed an even more pronounced decrease in SECIS element read-through (Figure 2A). However, only in lysates, in which the synthetic cofactor DTT is used, we observed an insignificant decrease in D2 activity (Figure 2F,G). This may suggest that position 5730 has an important function for the SECIS element under certain conditions.

We may speculate that the intact cell metabolism assays represent the more physiological situation as these cells express D2 under natural conditions. However, the supply of the substrate to the intracellular compartment is dependent on transporters. It is therefore possible that transport is the rate-limiting step in this system, leaving it impossible to measure small differences in D2 activity when mutations are present.

The decreased SECIS function of the 5730A>T mutant in the luciferase assay was not confirmed by the D2 activity assays. In both systems D2 was over-expressed by transient transfection. However, the constructs that we used in initial luciferase and deiodinase experiments had different 3'-UTR lengths. Human WT D2 has a 3'UTR of ~5 kb , the D2-pUHD10-3 construct of ~2 kb and the luciferase construct did not contain any 3'UTR other than the SECIS element. As it has been shown that spacing between the UGA codon and the SECIS element interferes with efficiency of Sec incorporation (28), it is possible that the SECIS element is more vulnerable for mutations when no 3'UTR is present. To overcome this problem, we removed almost complete 3'UTR in the D2-pUHD10-3 construct. However, this did not result in a different D2 activity between WT and 5730A>T mutant in cell lysates and intact cells (Figure 2).

Altogether, we identified 2 heterozygous mutations in this cohort of patients with unexplained ID. The 5703C>T mutation did not show any functional consequences and most likely this mutation can be described as a harmless, rare variant. Our data provided evidence that nucleotide 5730 seems to be important for normal SECIS element read-through. However, we could not demonstrate any deleterious effect on D2 activity. It is interesting that the cDNA for the human D2 coding region contains two in-frame UGA codons (29). Mutation of this second UGA codon to cysteine (Cys) or an unambiguous stop codon, did not affect deiodination properties of the enzyme and therefore the function of this second codon remains unsolved. In our study, we showed that the identified mutations in the SECIS element also did not affect enzymatic properties. However, we cannot conclude anything on the effects on other, non-enzymatic functions of the protein or the influence on incorporation of the second UGA.

At present, the phenotype of ID appeared not to be related to the presence of this mutation. Possibly, an additional mutation in one of the key players in SECIS element processing may

result in decreased D2 activity in the presence of a more vulnerable SECIS element. Therefore, we will perform genome sequencing on DNA of the index patient and her relatives to search for mutations in critical genes involved in SECIS element processing and to identify possible other genetic causes of neurological impairment.

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Supplemental Table 1 Synthetic oligonucleotides for PCR (P) and sequencing (S) of DIO2.

DIO2	Forward primer (5'-3')	Reverse primer (5'-3')
SECIS (P&S)	CCAGTTTTGTTTAGTTTTGCATCA	CACATAGCACTCAGCACCAA

Chapter 4

TRANSPORT OF IODOTHYRONINES BY HUMAN L-TYPE AMINO ACID TRANSPORTERS

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ABSTRACT

Thyroid hormone (TH) transporters facilitate cellular TH influx and efflux, which is paramount for normal physiology. The L-type amino acid transporters LAT1 and LAT2 are known to facilitate TH transport. However, the role of LAT3, LAT4 and LAT5 is still unclear. Therefore, the aim of this study was to further characterize TH transport by LAT1 and LAT2 and to explore possible TH transport by LAT3, LAT4 and LAT5. FLAG-LAT1-5 constructs were transiently expressed in COS1 cells. LAT1 and LAT2 were co-transfected with the CD98 heavy chain. Cellular transport was measured using 10 nM ^{125}I-labeled T4, T3, rT3, 3,3'-T2 and 10 μM MIT as substrates. Intracellular metabolism of these substrates was determined in cells co-transfected either of the LATs with type 1 (D1) or type 3 (D3) deiodinase. LAT1 facilitated cellular uptake of all substrates and LAT2 showed net uptake of T3, 3,3'-T2 and MIT. Expression of LAT3 or LAT4 did not affect transport of T4 and T3 but resulted in decreased cellular accumulation of 3,3'-T2 and MIT. LAT5 did not facilitate transport of any substrate. Co-transfection with LAT3 or LAT4 strongly diminished cellular accumulation of 3,3'-T2 and MIT by LAT1 and LAT2. These data were confirmed by metabolism studies. LAT1 and LAT2 show distinct preferences for uptake of the different iodocompounds, whereas LAT3 and LAT4 specifically facilitate 3,3'-T2 and MIT efflux. Together, our findings suggest that different sets of transporters with specific influx or efflux capacities may cooperate to regulate cellular thyroid state.

INTRODUCTION

Thyroid hormone (TH) is the common name for the prohormone T4 and the active hormone T3 which are important for development and metabolism of virtually all tissues. T3 exerts its action by binding to nuclear TH receptors (TRs) which regulate the transcription of target genes (1,2). The biological activity of TH is therefore dependent on the intracellular T3 concentration, which is importantly controlled by deiodinases and plasma membrane TH transporters. The deiodinases can either convert T4 to T3 (D1, D2) or degrade T4 to rT3 (D1, D3), rT3 to 3,3'-T2 (D1, D2) and T3 to 3,3'-T2 (D1, D3) (3).

It has been well established that TH transporters mediate the uptake and efflux of iodothyronines across the plasma membrane (4,5). So far, only organic anion transporting polypeptide 1C1 (OATP1C1) (6), monocarboxylate transporter 8 (MCT8) (7) and MCT10 (8) have been identified as relatively specific TH transporters. The importance of TH transporters has been demonstrated by the identification of mutations in MCT8 which result in a severe phenotype with psychomotor retardation and disturbed serum TH levels (9). Mct8 deficient mice display similar TH levels as patients, but lack a neurological phenotype (10,11). In this context, members of the L-type amino acid transporter (LAT) family have gained attention (12,13).

System L transporters are mostly present on the basolateral membrane of various tissues and mediate sodium-independent exchange of neutral amino acids and are specifically inhibited by 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) (14). LAT1 and LAT2 are heterodimeric proteins (~40 kDa) composed of a common heavy chain (CD98, SLC3A2) and different light chains (SLC7A5,8), whereas LAT3, LAT4 and LAT5 are monomeric proteins (~50 kDa) from an entirely different family (SLC43A1,2,3). LAT1 and LAT2 have been suggested to prevent a neurological phenotype by compensating for the defect in TH transport in Mct8 deficient mice (12), as LAT1 is predominantly expressed in the murine blood brain barrier and LAT2 mainly in neurons (15-17). Mct8/Lat2 double KO mice do not show the transient increase in T3 levels and expression of T3 target genes in the cerebral cortex as observed in neonatal Mct8 KO mice (13). This led to the suggestion that perinatal uptake of T3 in cerebral cortex is facilitated by LAT2.

Evidence that LAT1 and LAT2 transport TH is scarce, since most studies show indirect evidence of inhibited LAT-mediated uptake of amino acids by iodothyronines (15,18-21). The first direct evidence of TH transport by LAT1 was presented by Ritchie *et al.* (22). Also, our group showed direct uptake of T4 and T3 by human LAT1 in oocytes and described the uptake of iodothyronines by mouse LAT2 (18). The possibility that LATs may participate in the transport of TH in the brain may have important consequences for understanding the neurological phenotype of patients with MCT8 mutations. Therefore, we have studied the characteristics of iodothyronine transport by LAT1 and LAT2 in detail. Furthermore, we explored the possibility of iodothyronine transport by the recently identified LAT3, LAT4 and LAT5.

METHODS

Constructs

Cloning vectors containing full length cDNA inserts of human (h) LAT1, LAT2, LAT3, LAT4 and LAT5 were obtained from Thermo Fisher Scientific (Landsmeer, The Netherlands (NL)) and subcloned into the expression vector pcDNA3 using oligonucleotides (Integrated DNA Technologies, Leuven, Belgium) carrying the sequence for the FLAG tag and suitable restriction sites for the individual inserts. All constructs were verified by sequencing (BaseClear, Leiden, NL). The cloning of hMCT8.pcDNA3 (7), pSG5.CRYM (7) rD1.pcDNA3 (23) and hD3.pCIneo(23) constructs have been previously described. Mouse CD98 was amplified from kidney cDNA and cloned into the expression vector pcDNA3 (Invitrogen, Bleiswijk, NL) using HindIII and XbaI cloning sites.

Cell culture and transfection

COS1 cells were cultured at 37 °C and 5% CO_2 in 75 cm^2 flasks with DMEM/F12 (Life Technologies, Bleiswijk, NL) supplemented with 9% heat-inactivated fetal bovine serum (Sigma Aldrich, Zwijndrecht, NL), 1% penicillin-streptomycin, and 100 nM Na_2SeO_3. At confluence, cultured cells were split and seeded in 24-well dishes for the uptake and metabolism studies and in 6-well dishes to prepare lysates for immunoblots. At 70% confluence, cells were transiently transfected using X-tremeGENE 9 Transfection Reagent (Roche Diagnostics, Almere, NL) according to the manufacturer's protocol.

For uptake studies, COS1 cells were transiently transfected in duplicate with 5-50 ng of FLAG-LAT1, LAT2, LAT3, LAT4 or LAT5.pcDNA3 or MCT8.pcDNA3 together with 50 ng of CD98.pcDNA3 in case of LAT1 or LAT2 and with or without CRYM.pSG5. In some experiments 50 ng of FLAG-LAT1 or LAT2.pcDNA3 plus 50 ng CD98.pcDNA3 were co-transfected with 5-50 ng FLAG-LAT3 or LAT4. pcDNA3. For metabolism experiments, cells were additionally transfected with 50 ng D1.pcDNA3 or D3.pCIneo. To study the influence of transfection of LAT3, LAT4 or LAT5 on the endogenous expression of LAT1, COS1 cells were transfected with 5-50 ng LAT3, LAT4 or LAT5.pcDNA3. Empty pcDNA3 vector (EV) was always added to bring the total amount of plasmid to 250 ng per well. For immunoblots, cells were transfected with 200 ng of each FLAG-LAT1, LAT2, LAT3, LAT4 or LAT5.pcDNA3, adjusted with EV to a total amount of 1000 ng plasmid.

Uptake experiments

Two days after transfection, cells were washed with assay buffer (Dulbecco's phosphate buffered saline (DPBS) + Ca^{2+}/Mg^{2+} + 0.1% D-glucose) and incubated for 5-60 minutes at 37 °C with 10 nM (50,000 cpm) [^{125}I]T4, [^{125}I]T3, [^{125}I]rT3, [^{125}I]3,3'-T2 or 10 μM (50,000 cpm) [^{125}I]3'-iodotyrosine (MIT) or [^{3}H]leucine (Leu) in 0.5 ml assay buffer with or without 0.1% BSA. All radioactive iodocompounds were prepared as previously described (24). Nonradioactive iodothyronines were obtained from Henning (Berlin, Germany), and MIT and Leu from Sigma Aldrich. To evaluate the pH dependence of 3,3'-T2, rT3 and MIT uptake, the pH of the assay buffer was adjusted to 7.3, 6.3 or 5.3 with 1 M HCl or KOH. After incubation, cells were washed with assay buffer and lysed with 0.1 M NaOH. Radioactivity in the lysate was measured in a γ-counter (^{125}I) or a β-counter (^{3}H).

Metabolism experiments

Two days after transfection, cells were washed with assay buffer and subsequently incubated for 2 or 4 hours at 37 °C with 1 nM (200,000 cpm) [^{125}I]T4, [^{125}I]T3, [^{125}I]rT3 or [^{125}I]3,3'-T2 in 250 µl assay buffer +0.1% BSA. After incubation, 100 µl of incubation medium was added to 125 µl ice-cold ethanol (pH 2.3) and incubated for 30 minutes on ice. After centrifugation, 125 µl supernatant was mixed with 100 µl 0.1% TFA in water. Metabolites were separated by UPLC as described previously for the HPLC method (23).

Immunoblotting

Two days after transfection, COS1 cells were rinsed with DPBS, lysed in 100 µl ice-cold lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris/HCl pH 8.0, 1 mM EDTA) and centrifuged for 5 minutes at 800×g. Post-nuclear supernatants (2.5–25 µg) were separated on 12% precise Tris-HEPES SDS-gel (Fisher Scientific), and transferred to a nitrocellulose membrane (GE Healthcare, Eindhoven, NL). The membrane was blocked for 1 hour in DPBS/0.1% Tween/5% milk and incubated overnight at 4 °C in DPBS/0.1% Tween/5% milk with 1:1000 mouse anti-FLAG M2 antibody (F1804 Sigma Aldrich) or 1:10,000 mouse anti-human GAPDH antibody (MAB374 Chemicon International, Amsterdam, NL). The membranes were washed 3 times for 5 minutes with DPBS/0.1%Tween, and incubated for 1 hour at room temperature in DPBS/0.1% Tween with 1:3000 goat anti-mouse IgG HRP conjugate (#172-1011 Bio-Rad Life Science, Veenendaal, NL). Finally, the blots were washed 3 times for 5 minutes with DPBS/0.1% Tween and incubated for 1 minute in enhanced chemiluminescent substrate for detection of HRP (Thermo Scientific) before scanning by the Alliance 4.0 using Uvitec software (Cambridge, United Kingdom).

qRT-PCR

Total RNA was isolated from COS-1 cells using the High Pure RNA Isolation kit (Roche Diagnostics). RNA (1 µg) was reverse-transcribed using the Transcriptor High Fidelity cDNA Synthesis kit (Roche Diagnostics). Quantitative real-time PCR was performed using the qPCR Core kit for SYBR® Green I No dUTP (Eurogentec, Maastricht, NL). Results were normalized using GAPDH as the housekeeping gene. The PCR primers used are listed in Supplemental Table 1.

Statistical analysis

All results are presented as the means ± SEM of 2-6 independent experiments performed in duplicate. Statistical analysis was carried out in GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). The statistical significance of differences in substrate uptake after transfection with LAT1, LAT2, LAT3, LAT4 or LAT5 vs. EV or after co-transfection with LAT1 or LAT2 plus LAT3 or LAT4 vs. LAT1 or LAT2 alone, and of differences in endogenous LAT1 mRNA levels in cells transfected with LAT3, LAT4 or LAT5 vs EV was determined using the Student's t-test for unpaired observations.

For the plasmid titration experiments of 3,3'-T2 and MIT uptake and time courses of 3,3'-T2, rT3 and MIT uptake, we used two-way ANOVA followed by a Bonferroni post test to calculate

significant differences between transfection with LAT1 or LAT2 alone *vs.* co-transfection with LAT1 or LAT2 plus LAT3 or LAT4. Statistical significance in the metabolism and pH experiments was determined using one-way ANOVA followed by a Bonferroni post test. $P < 0.05$ was considered statistically significant.

RESULTS

To study the transport characteristics of TH by LAT1, LAT2, LAT3, LAT4 and LAT5, we transiently transfected COS1 cells with these transporters and used MCT8 as a control. LAT1 and LAT2 were combined with the CD98 heavy chain. All constructs were designed with a FLAG tag to visualize expression of the proteins. First, we confirmed expression of the transporter proteins by immunoblot analysis using an antibody against the FLAG tag that is present in the different constructs. Cells co-transfected with CD98 and LAT1 or LAT2 showed bands of ~40 and ~45 kDa, respectively (Supplemental Figure 1). For cells transfected with FLAG-tagged LAT3, LAT4 or LAT5 we observed the most pronounced bands at ~50 kDa (Supplemental Figure 1). All bands were in agreement with the predicted molecular weights of the different LATs. Additional bands with a higher molecular mass in cells transfected with LAT3, LAT4 or LAT5 are likely explained by glycosylation (25). To confirm functional expression of transporters, we measured Leu uptake in cells transfected with LAT1, LAT2, LAT3, LAT4 or LAT5 (Figure 1A). As anticipated, in cells expressing LAT1 or LAT2 we observed increased Leu uptake, whereas in cells expressing LAT3 or LAT4 Leu uptake was decreased compared with control EV-transfected cells. Also LAT5-expressing cells showed slightly diminished Leu accumulation. These results support the functional expression of LAT1-5 in our cell system (Figure 1A).

We next measured the uptake of T4, T3, rT3, 3,3'-T2 and MIT by COS1 cells transfected with LAT1, LAT2, LAT3, LAT4 or LAT5. Preliminary data showed that substrate uptake was linear with time for at least 30 minutes, we chose this incubation time for our initial experiments. Cells transfected with LAT1 showed significantly higher uptake of all substrates than cells transfected with EV (Figure 1B-F) Net uptake of rT3 and 3,3'-T2 was even higher for LAT1 than for MCT8. In cells transfected with LAT2 there was significant uptake of T3, 3,3'-T2 and MIT, and MIT uptake even exceeded that mediated by LAT1 (Figure 1D,E). Interestingly, transfection with LAT3 or LAT4 induced a significant decrease in cellular 3,3'-T2 and MIT accumulation similar to our findings with Leu, suggesting that LAT3 and LAT4 predominantly facilitate the efflux of these substrates (Figure 1E,F). For LAT5, we could not detect any differences in uptake of the iodothyronines and MIT (Figure 1B-F). These experiments were also performed in JEG3 cells, with similar results (data not shown).

An alternative explanation for the decreased uptake of 3,3'-T2 and MIT in cells transfected with LAT3 or LAT4 is a diminished expression of endogenous LAT1 or LAT2. Therefore, we explored how sensitive substrate uptake responds to a decrease in LAT1-4 expression. Uptake of 3,3'-T2 by LAT1 and LAT2 only decreased by ~15% when the amount of plasmid was reduced from 50 to 25 ng, and only by ~50% when plasmid was further reduced to 5 ng (Figure 2A). MIT uptake by LAT1 was hardly affected when the amount of plasmid was reduced from 50 to 10 ng (Figure 2B). Cellular accumulation of MIT was similarly reduced after transfection of cells with 25 or 50

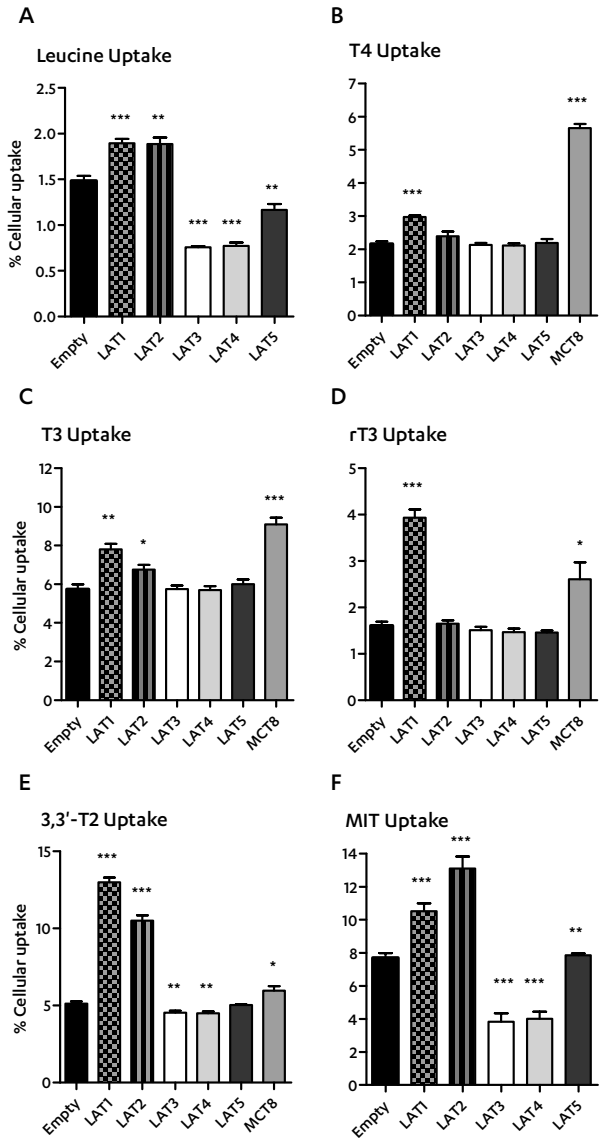

Figure 1 A-F Cellular uptake of 10 nM T4, T3, rT3, 3,3'-T2 or 10 μM of MIT or Leu by COS1 cells transfected with EV, FLAG-tagged LAT1, LAT2, LAT3, LAT4, LAT5 or MCT8 after 30 minutes or 10 minutes (Leu) of incubation. Uptake is presented as a percentage of added substrate. Results are the means ± SEM of 4-6 experiments. Significance represents EV versus different transporters. **P* <0 .05; ***P* <0.01; ****P* <0.001.

ng LAT3 or LAT4 plasmid. Even when only 5 ng of LAT3 or LAT4 plasmid was transfected, cellular MIT accumulation was still significantly decreased (Figure 2B). In view of these results and to maximize the effects on cellular iodothyronine and MIT accumulation without compromising endogenous transport, we used 50 ng of transporter plasmid in all further experiments.

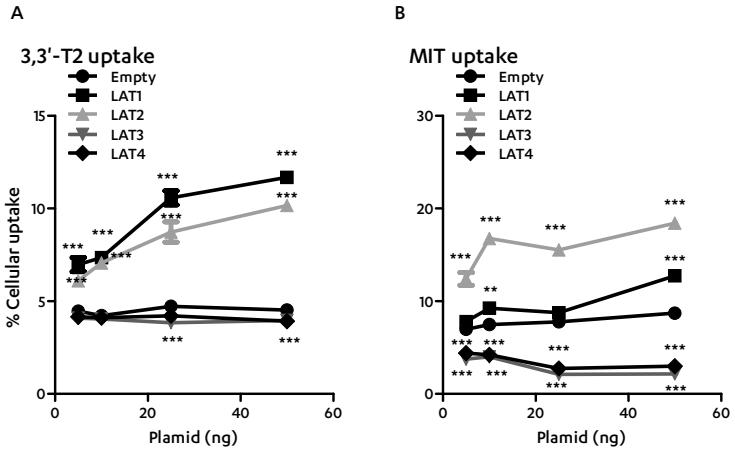

Figure 2 A-B Cellular uptake of 10 nM 3,3'-T2 and 10 μM of MIT by COS1 cells transfected with 5-10-25 or 50 ng of FLAG-tagged LAT1, LAT2, LAT3 or LAT4 after 30 minutes of incubation. Uptake is presented as a percentage of added substrate. Significance represents EV versus different transporters. **P* <0 .05; ***P* <0.01; ****P* <0.001.

The cellular accumulation of T4 induced by LAT1 and T3 by LAT1 and LAT2 was only modest, which could be explained if these transporters mediate not only influx but also efflux of TH. To test this, we compared uptake of T4 and T3 in cells co-transfected with CRYM, a high-affinity cytoplasmic TH-binding protein (8), or EV as control. Co-transfection with CRYM resulted in an expected increase in accumulation of T4 and T3 in cells expressing MCT8, which facilitates both uptake and efflux of TH (8), but not in cells expressing LAT1 or LAT2 (Figure 3). Co-transfection of CRYM with LAT3 or LAT4 did not result in any uptake of T4 or T3 (Figure 3). These results indicate that LAT1 and LAT2 facilitate influx but not efflux of T3 and T4, and further confirm that T4 and T3 are not substrates for LAT3 and LAT4.

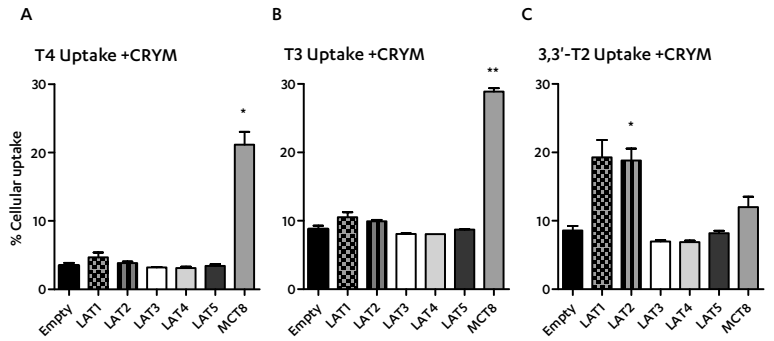

Figure 3 A-C Cellular uptake of 10 nM T4, T3 and 3,3'-T2 by COS1 cells transfected with CRYM in combination with EV, FLAG-tagged LAT1, LAT2, LAT3, LAT4, LAT5 or MCT8 after 30 minutes of incubation. Uptake is presented as a percentage of added substrate. Results are the means ± SEM of 2-3 experiments. Significance represents EV versus different transporters. **P* <0 .05; ***P* <0.01; ****P* <0.001.

To get further insight in the transport characteristics of LAT1, LAT2, LAT3 and LAT4, we studied the time course of 3,3'-T2, rT3 and MIT uptake. The uptake of 3,3'-T2 by LAT1 and LAT2 increased significantly over time until a steady state was almost reached after 30 minutes (Figure 4A-D).

To further investigate the efflux of 3,3'-T2 by LAT3 and LAT4, we analyzed transport of 3,3'-T2 by cells co-transfected with LAT1 or LAT2 together with LAT3 or LAT4. We observed a significant decrease in cellular 3,3'-T2 accumulation in the co-transfected cells compared to cells expressing LAT1 or LAT2 alone (Figure 4A-D). This suggests that 3,3'-T2 taken up by LAT1 or LAT2 is exported by LAT3 or LAT4, when these transporters are co-expressed.

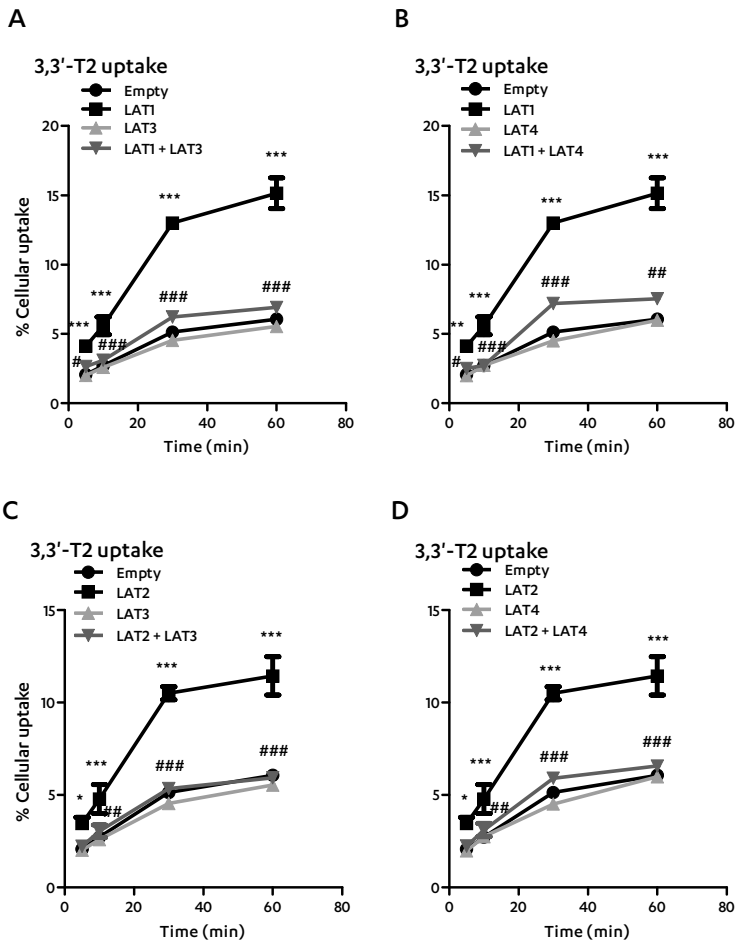

Figure 4 A,B Cellular uptake of 10 nM 3,3'-T2 by COS1 cells transfected with EV, LAT1, LAT3 or LAT4 alone or co-transfected with LAT1 plus LAT3 or LAT4 after 5-60 minutes of incubation **C,D** Cellular uptake of 10 nM 3,3'-T2 by COS1 cells transfected with single EV, LAT2, LAT3 or LAT4 alone or co-transfection of LAT2 together with LAT3 or LAT4 after 5, 10, 30 and 60 minutes of incubation. Uptake is presented as a percentage of added substrate. Results are the means ± SEM of 3 experiments. Significance represents EV versus LAT1 or LAT2 (*) and single LAT1 or LAT2 alone versus LAT1 or LAT2 plus LAT3 or LAT4 (#). *P <0 .05; **P <0.01; ***P <0.001.

Transfection with LAT1, but not with LAT2, LAT3 or LAT4 (data not shown), induced a significant increase in rT3 uptake at all time points, beyond 5 minutes of incubation time (Figure 5A,B). Co-transfection of LAT1 with LAT3 or LAT4 decreased rT3 uptake at all-time points (Figure 5A,B), indicating rT3 efflux by LAT3 and LAT4.

MIT uptake peaked after 10 minutes in control cells transfected with EV, followed by a marked decline (Figure 6A-D). At all time points, both LAT1 and LAT2 induced a marked increase, whereas LAT3 and LAT4 produced an even greater decrease in cellular MIT accumulation compared to cells transfected with EV (Figure 6A-D). Cellular accumulation of MIT was equally decreased in cells transfected with LAT1 or LAT2 in combination with LAT3 or LAT4 as in cells transfected with LAT3 or LAT4 alone, supporting the hypothesis that MIT is actively exported by the latter transporters (Figure 6A-D).

Transport of iodothyronines by LAT1, LAT2, LAT3 and LAT4 will change their intracellular levels and consequently their metabolism by deiodinating enzymes. To study this, we co-transfected cells with LAT1-4, or MCT8 as a positive control, together with D1 or D3 and analyzed metabolism of T4, T3, rT3 and 3,3'-T2. We measured metabolites in the medium which contains >80% of added radioactivity and reflects total cellular iodothyronine metabolism (23). LAT1 and LAT2 induced insignificant increases in the metabolism of T4, T3 and 3,3'-T2 by D3 (Figure 7A-C). LAT3 and LAT4 had little, insignificant effect on the metabolism of T4 and T3 by D3, but induced an almost 25%, albeit insignificant decrease in 3,3'-T2 metabolism by D3. This suggests that LAT3 and LAT4 export 3,3'-T2 before this substrate can be metabolized by D3. Again, we co-transfected LAT1 or LAT2 with LAT3 or LAT4 to test their effects on the intracellular availability of 3,3'-T2 for metabolism by D3. As anticipated, metabolism of rT3 by D1 was only increased in cells expressing LAT1, but not LAT2 (Figure 7D-E). Both LAT3 and LAT4 expression decreased 3,3'-T2

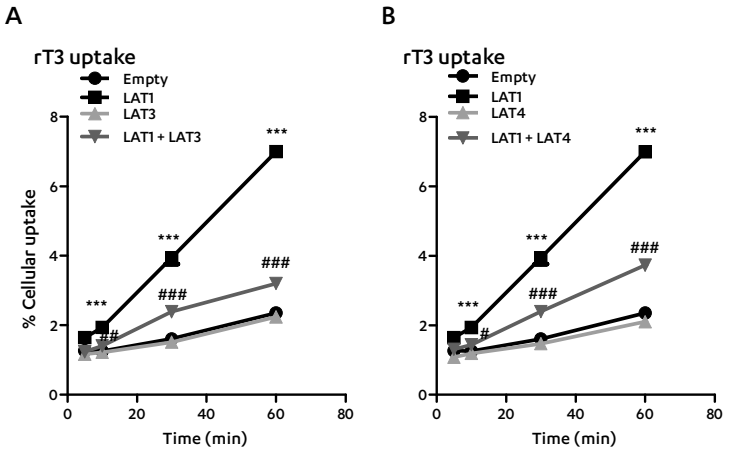

Figure 5 A,B Cellular uptake of 10 nM rT3 by COS1 cells transfected with EV, LAT1, LAT3 or LAT4 alone or co-transfected with LAT1 plus LAT3 or LAT4 after 5-60 minutes of incubation. Results are the means ± SEM of 3 experiments. Significance represents EV versus LAT1 or LAT2 (*) and LAT1 or LAT2 alone versus LAT1 or LAT2 plus LAT3 or LAT4 (#). **P* <0 .05; ***P* <0.01; ****P* <0.001.

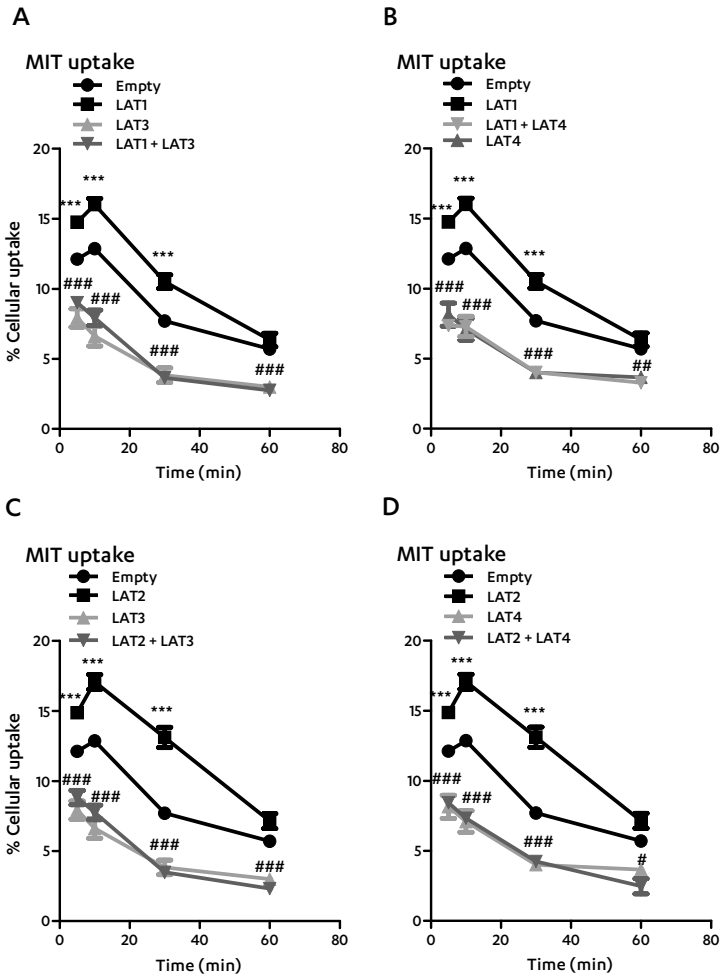

Figure 6 A,B Cellular uptake of 10 μM MIT by COS1 cells transfected with single EV, LAT1, LAT3 or LAT4 alone or co-transfected with LAT1 plus LAT3 or LAT4 after 5-60 minutes of incubation. **C,D** Cellular uptake of 10 μM MIT by COS1 cells transfected with EV, LAT2, LAT3 or LAT4 alone or co-transfected with LAT2 plus LAT3 or LAT4 after 5-60 minutes of incubation. Uptake is presented as a percentage of added substrate. Results are the means ± SEM of 3 experiments. Significance represents EV versus LAT1 or LAT2 (*) and LAT1 or LAT2 alone versus LAT1 or LAT2 plus LAT3 or LAT4 (#). **P* <0 .05; ***P* <0.01; ****P* <0.001.

metabolism (Figure 7D) and modestly decreased rT3 metabolism by D1 (Figure 7E), which was abolished by co-transfection with LAT1 or LAT2 (Figure 7D,E).

As mentioned above, the decrease in cellular accumulation of MIT, 3,3'-T2 or rT3 by LAT1 or LAT2 after co-transfection with LAT3 or LAT4 may be explained by the direct efflux of these iodocompounds by LAT3 or LAT4 and/or by a decreased expression of LAT1 or LAT2 protein. Therefore, we co-transfected cells with LAT1 or LAT2 and increasing amounts of LAT3 or LAT4. Then, we analyzed 3,3'-T2 and MIT uptake in parallel with LAT1 and LAT2 protein expression by

Figure 7 A,B,C Metabolism of 1 nM T4, T3 and 3,3'-T2 during 2-4 hours of incubation with COS1 cells co-transfected with D3 plus EV, LAT1, LAT2, LAT3, LAT4 or MCT8, or with D3 plus LAT1 or LAT2 plus LAT3 or LAT4. **D,E** Metabolism of 1 nM 3,3'-T2 and rT3 after a 2-4 hours of incubation with COS1 cells co-transfected with D1 plus EV, LAT1, LAT2, LAT3, LAT4 or MCT8, or with D1 plus LAT1 or LAT2 plus LAT3 or LAT4. Metabolism is shown as percentage metabolites in the incubation medium. Metabolites are expressed as percentage of total radioactivity in the medium. Results are the means ± SEM of 3 experiments. Significance represents D1 or D3 alone versus D1 or D3 plus transporter (*), or D1 or D3 plus LAT1 or LAT2 versus D1 or D3 plus LAT1 or LAT2 plus LAT3 or LAT4 (#). $^{*}P$ <0 .05; $^{**}P$ <0.01; $^{***}P$ <0.001.

immunoblotting. Cellular accumulation of 3,3'-T2 and MIT was already significantly decreased when 50 ng of LAT1 or LAT2 was co-transfected with as low as 5 ng LAT3 or LAT4, which did not affect LAT1 and LAT2 protein expression (Supplemental Figures 2A-D, 3A,B). This indicates that the decreased accumulation of MIT and 3,3'-T2 and decreased metabolism of 3,3'-T2 in cells (co-)transfected with LAT3 or LAT4 are due, at least in part, to direct export of these compounds by LAT3 and LAT4. This may also hold to some extent for rT3 although we did not detect decreased rT3 uptake by cells transfected with LAT3 or LAT4 alone (Figure 1C). As a final approach, we measured endogenous levels of LAT1 and LAT2 in COS1 cells after transfection with increasing concentrations of LAT3, LAT4 and LAT5. First, COS1 only expressed LAT1 and not LAT2 (data not shown). Next, we measured LAT1 levels after co-transfection with 5, 10, 25 or 50 ng of LAT3 and LAT4 and showed that 5-50 ng of LAT3 or LAT4 did not decrease LAT1mRNA levels (Supplemental Figure 2E,F).

The uptake of amino acids by LAT2 depends on the pH of the incubation medium, but this has not been tested for LAT1 (11). Therefore, we first studied the effects of pH on the uptake of the preferred iodocompounds by LAT1 or LAT2. Adjusting the pH from 7.3 to 5.3 significantly increased uptake of rT3 by LAT1 (Figure 8A), whereas uptake of rT3 by LAT2 was very low at all pH values tested (Figure 8B). Uptake of 3,3'-T2 by LAT1 and LAT2 was optimal at pH 6.3 although not significantly different from pH 5.3 or 7.3 (Figure 8C-D). Uptake of MIT by LAT1 was significantly higher at pH 5.3 than at pH 7.3 (Figure 8E), whereas uptake of MIT by LAT2 was already significantly increased at pH 6.3 (Figure 8F). To exclude the possibility that the increased substrate uptake at lower pH was caused by a decreased substrate binding to BSA, the experiments were also performed in the absence of BSA, yielding very similar results for all substrates (data not shown). Finally, we studied the pH dependence of T4 and T3 by LAT1 or LAT2. The uptake of T4 and T3 by LAT1 increased when the pH was adjusted from 7.3 to 5.3. The modest transport of T3 by LAT2 was not pH dependent (data not shown).

DISCUSSION

In this study, we characterized iodothyronine transport by LAT1 and LAT2. Furthermore, we explored the possibility of iodothyronine transport by the recently identified LAT3, LAT4 and LAT5. LAT1 and LAT2 are highly homologous heterodimeric proteins consisting of the non-glycosylated 12 transmembrane domains containing light chains SLC7A5 and SLC7A8, respectively, and a common glycosylated 4F2 heavy chain (CD98/SLC3A2) containing a single transmembrane domain. Compared to LAT1, which preferably transports large, neutral, amino acids with branched or aromatic side chains, LAT2 has a remarkably broad substrate specificity. Also, LAT2-mediated transport is increased at lower pH values. LAT1 is mainly expressed in brain, spleen, colon and placenta, whereas LAT2 is widely expressed mostly at basolateral membranes of various tissues, like kidney and intestine, but also in the brain, placenta and liver (26-30).

LAT3 (SLC43A1), LAT4 (SLC43A2) and LAT5 (SLC43A3) are transporters with a predicted membrane topology of 12 transmembrane domains with a conserved N-glycosylation site. The transport activity of LAT3 and LAT4 corresponds to that of amino acid transport system L. These transporters facilitate efflux of branched chained, neutral amino acids in a sodium independent manner at the basolateral

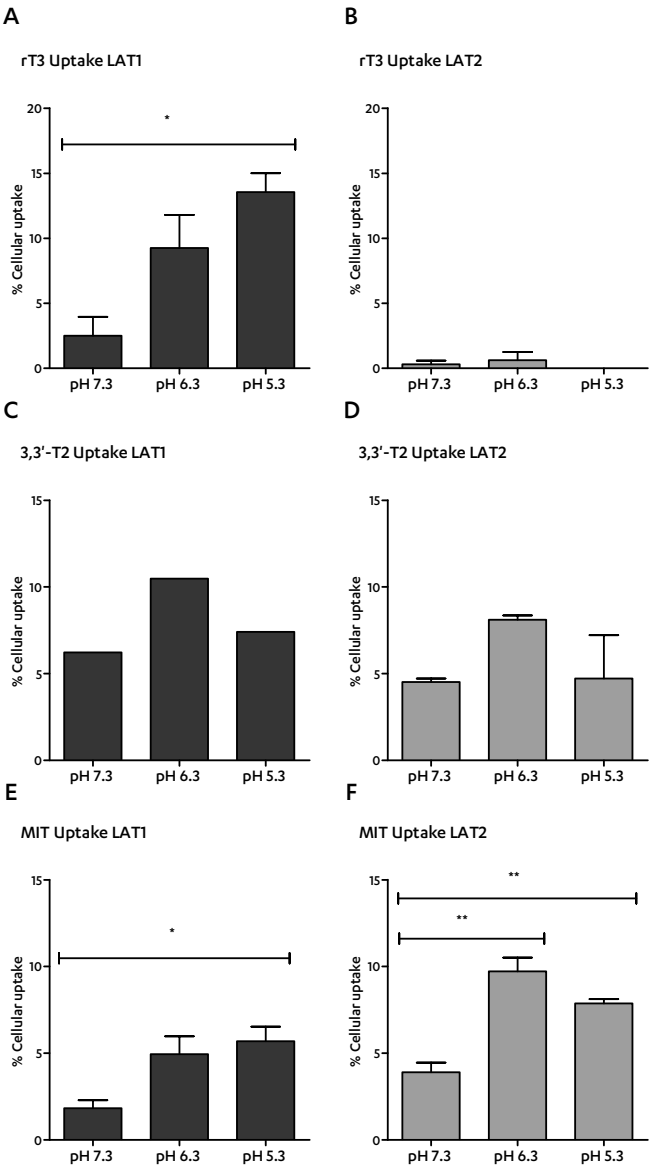

Figure 8 A-E Effects of pH 5.3-7.3 on the uptake of 1 nM rT3, 3,3'-T2 or MIT by COS1 cells transfected with LAT1 or LAT2 after 30 minutes of incubation. Uptake is presented as a percentage of added substrate. Results are the means ± SEM of 3 experiments. Significance represents pH 5.3 or 6.3 versus pH 7.3. *P <0 .05; **P <0.01; ***P <0.001.

side into the blood and are inhibited by BCH. LAT5 is an orphan transporter. LAT3 is mainly expressed in liver, pancreas, skeletal muscle and also in placenta and podocytes. LAT4 is expressed in placenta, kidney, liver and small intestine (25,31-35). Table 1 presents an overview of the tissue distribution of LAT1, LAT2, LAT3, LAT4 and LAT5 (26,28,29,31-33,35-39).

Table 1 Tissue distribution of LAT transporters

	Brain	Spleen	Intestine	Placenta	Liver	Kidney	Pancreas	Skeletal muscle	Heart	Testis	Refs.
LAT1	Human, rat	Human, rat	Rat	Human, rat				Human, rat		Human, rat	26,29, 36-39
LAT2	Human, rat	Human	Human, rat	Human, rat	Human, rat	Human, rat		Human, rat	Human	Rat	28,29,36
LAT3				Human	Human	Human	Human	Human	Human		31,35
LAT4		Mouse		Human, mouse		Mouse		Mouse	Mouse		32
LAT5				Human	Human	Human			Human		33

Several features distinguish LAT1 and LAT2 from LAT3 and LAT4: (1) LAT3 and LAT4 do not need to bind to a heavy chain to traffic to the plasma membrane; (2) LAT1 and LAT2 are exchangers, whereas LAT3 and LAT4 are known to efflux substrates; (3) LAT3 and LAT4 exhibit narrow substrate selectivity and show highly complex two-component kinetics (25).

In our studies, LAT1 facilitated intracellular accumulation of T4 and T3 (Figure 1) and this transporter can therefore be important for normal uptake of TH in various tissues. LAT1 is highly expressed in the syncytiotrophoblast mainly at the maternal, apical side and also less pronounced at the fetal, basolateral side (26,40). This suggests that LAT1 could also regulate TH transport from mother to child. It is noteworthy that LAT1 transports the receptor inactive metabolites rT3, 3,3'-T2 and MIT, whereas it is not expressed in the liver, kidney or thyroid, where these compounds are mainly metabolized by D1 and DEHAL1 (41-43). However, iodothyronines may also exert non-genomic biological effects (41,44-46). It has been shown that T4 and rT3 regulate actin polymerization and D2 activity in the developing brain via non-transcriptional ways (44). Also, 3,3'-T2 can influence mitochondrial energy metabolism (46,47). rT3 levels in the human fetus are relatively high due to high placental and fetal tissue D3 activities (48). Notably, Lat1 KO mice are embryonic lethal (39). The expression of LAT1 in the syncytiotrophoblast may therefore indicate a role for LAT1 in transport of iodothyronines from the fetus back to the mother. However, since LAT1 is predominantly expressed at the maternal side of the syncytiotrophoblast (40), the exact role of LAT1 in placental TH transport, if any, remains to be elucidated.

In neonatal Mct8 KO mice, local hyperthyroidism and expression of TH target genes in cerebral cortex are prevented by additional deletion of Lat2 (13). It has been suggested that LAT2 facilitates T3 transport in neurons in the prenatal and perinatal period. Our data indicate that LAT2 is a poor T4 and T3 transporter (Figure 1). These observations may be reconciled if mouse and human LAT2 differ in substrate preference. Alternatively, if LAT2 expression is sufficiently high, it may still contribute to neuronal T3 uptake despite its low intrinsic T3 transport capacity. Given the net accumulation of T4 and T3 by LAT1 and its expression in the mouse brain, LAT1 could be a compensatory TH transporter in Mct8 deficient mice (15).

In our studies, we found significant uptake of 3,3'-T2 and MIT by LAT2 (Figure 1D,E). The accumulation of MIT by LAT2 is even more pronounced compared to LAT1. LAT2 is expressed in

almost all tissues (28), including the thyroid gland (49). LAT2 may have a role in the transport of 3,3'-T2 and MIT in the liver and thyroid, where these substrates are metabolized and the iodide produced is re-utilized for the production of TH.

Our findings indicate that LAT1 facilitates cellular accumulation of all tested substrates and LAT2 showed net uptake of T3, 3,3'-T2 and MIT. This size selectivity for the iodothyronines is consistent with known preferences of LAT1 for larger amino acids and LAT2 for smaller amino acids (26-30).

The uptake of MIT in control cells, shows a marked decline over time, which could be explained by extensive intracellular metabolism of MIT. However, UPLC analysis indicated that MIT was completely stable during these incubations (data not shown). It is more likely that MIT uptake by COS1 cells is driven by the exchange with intracellular amino acids, since LAT1 and LAT2 are obligatory exchangers (26-30). Cells were cultured in amino acid-replete medium but during the uptake assay, the cells were incubated in amino acid-deplete PBS, which probably results in a decline in intracellular amino acids and, thus, a decrease in cellular MIT uptake with time of incubation. The physiological role of the possible exchange of TH and amino acids needs to be elucidated.

LAT3 and LAT4 are known system L transporters (25). Similar to LAT1 and LAT2, both LAT3 and LAT4 are expressed in the placenta, and they are both expected to mediate efflux of amino acids from the syncytiotrophoblast to the fetus (35). It has also been suggested that the observed up-regulation of LAT3 in liver and skeletal muscle during starvation, benefits the transport of amino acids from the liver and muscle to energy depleted organs such as the brain (50). A zebrafish morphant lacking Lat3 function showed collapsed glomeruli and disruption of glomerular permeability, suggesting that LAT3 may play a crucial role in the development and maintenance of podocyte structure and function (51). Recently, the group of Verrey *et al*. showed that LAT4 facilitates the uptake and efflux amino acids and that LAT4 is important in nutrition during early development (52). In *Lat4* KO mice, they observed intrauterine and postnatal growth retardation, low amniotic amino acid concentrations and premature death after 9 days. In our studies we showed efflux of 3,3'-T2 and MIT by LAT3 and LAT4 (Figure 1). We confirmed these data by using metabolism assays (Figure 7), and were also able to observe a decrease in net accumulation of 3,3'-T2 and MIT by co-transfecting LAT1 or LAT2 together with LAT3 or LAT4 (Figure 4,6).

The decreased intracellular accumulation of 3,3'-T2 and MIT in the presence of LAT3 or LAT4, was not caused by lower levels of endogenous LAT levels, as transfection with 5-50 ng of LAT3 or LAT4 did not alter LAT1 mRNA levels (Supplemental Figure 2E,F) and 5 ng of plasmid already resulted in decreased accumulation of 3,3'-T2 and MIT (Figure 2). Furthermore, in co-transfection studies, we showed that the accumulation of 3,3'-T2 and MIT by LAT1 and LAT2 could be abolished in the presence of LAT3 and LAT4. We used different approaches to show that this effect was not caused by lower endogenous or exogenous LAT1 or LAT2 expression. First, 5 ng of LAT3 or LAT4 already significantly decreased the intracellular accumulation of 3,3'-T2 and MIT by LAT1 and LAT2, without affecting protein levels (Supplemental Figures 2,3). Second, we showed that the 50 ng of LAT3 or LAT4 did not affect LAT1 mRNA levels (Supplemental Figure 2E,F). Third, the plasmid titration curve showed that the uptake of 3,3'-T2 and MIT only decreased by half when 10-fold lower concentrations of plasmid were used (Figure 2). Altogether, these data strongly

suggest that LAT3 and LAT4 directly contribute to the efflux of 3,3'-T2 and MIT. The physiological role of iodothyronine export by LAT3 and LAT4 *in vivo* remains to be elucidated, for example by measuring TH levels in the Lat3 KO zebrafish model and the lat4 knockout mice (51,52).

Uptake of the different substrates by LAT1 and LAT2 is dependent on the pH in the incubation medium as we found a significant increase in uptake by decreasing the pH from 7.3 to 6.3 or 5.3 (Figure 8). Although in the range studied, pH may affect the charge at the phenolic hydroxyl group of T4 and rT3, the increased MIT uptake by LAT1 and LAT2 at lower pH agrees with previous data showing higher uptake of substrates by LAT2 at lower pH. The physiological role of increased transport by LAT1 and LAT2 at lower pH remains to be elucidated.

In conclusion, our data highlight novel functions for LAT1, LAT2, LAT3 and LAT4 in the transport of iodothyronines. LAT1 and LAT2 facilitate mainly uptake of TH, whereas LAT3 and LAT4 can be regarded as T2 and MIT exporters. Further studies are necessary to explore the physiological role of these transporters *in vivo*.

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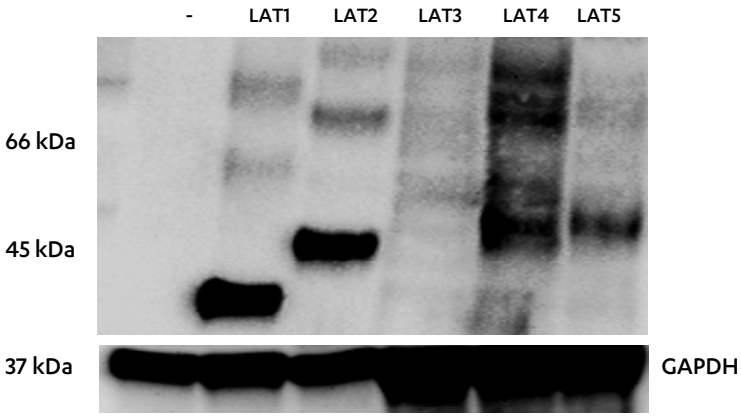
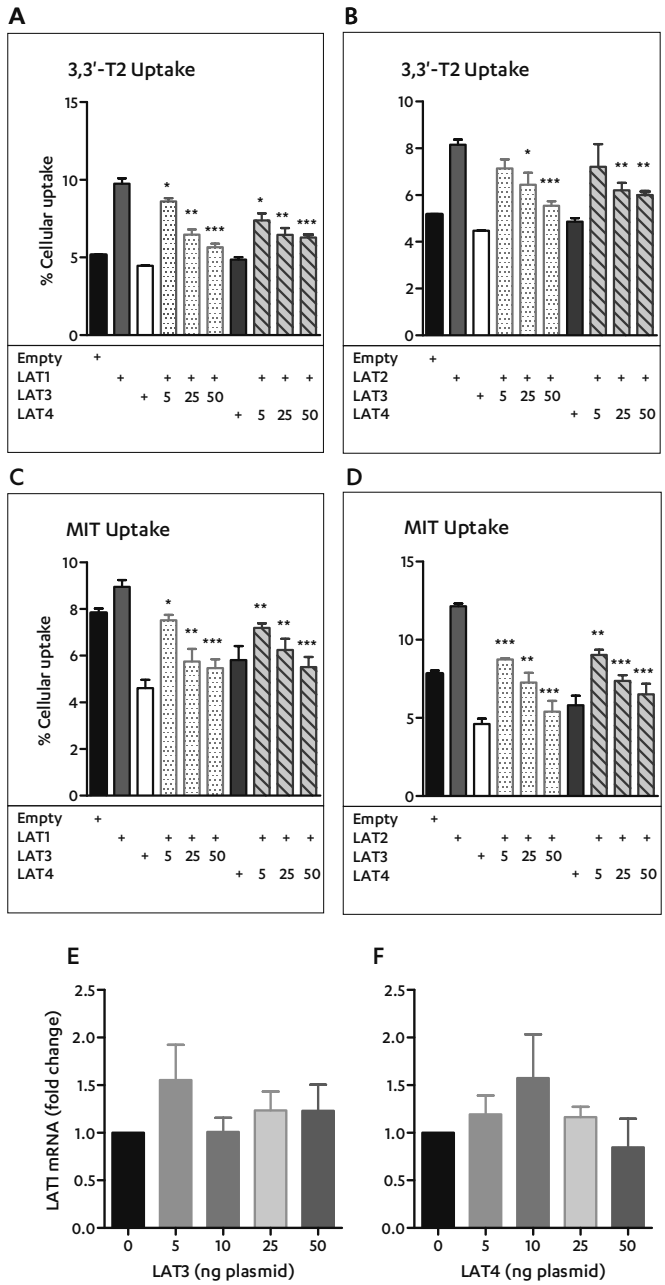
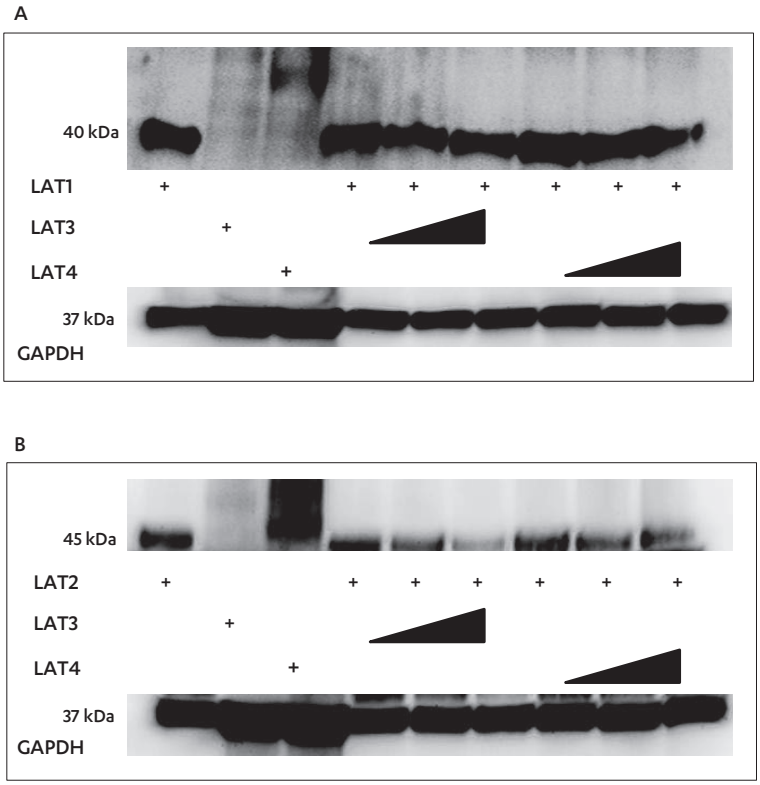


Supplemental Figure 1 Western Blots of postnuclear supernatants of transfected COS1 cells with LAT1, LAT2, LAT3, LAT4 or LAT5. 12% SDS-gels for LAT1 or LAT2 were loaded with 2.5 µg protein and for LAT3, LAT4 or LAT5 with 25 µg protein. LAT1 and LAT2 show a specific monomer band ~ 40 kDa. For LAT3, LAT4 and LAT5 the most pronounced bands are at ~ 50 kDa. All bands are in agreement with the predicted molecular weights of the different LATs. Extra bands with a higher molecular mass are present in cells transfected with LAT3, LAT4 or LAT5, which is likely explained by glycosylation. GAPDH was used as a housekeeping protein. Immunoblots were carried out at least two times.



Supplemental Figure 2 A-D Uptake of 10 μM 3,3'-T2 or MIT by COS1 cells transfected with 50 ng LAT1 or LAT2 plus 5-50 ng LAT3 or LAT4 after 30 minutes of incubation. Uptake is presented as a percentage of added substrate. Results are the means ± SEM of 3 experiments. **E,F** Endogenous LAT1 mRNA levels in COS-1 cells after transfection with 5-50 ng LAT3, LAT4 or LAT5. GAPDH was used as internal control. Results are the means ± SEM of 2 experiments performed in triplicate. Significance represents LAT1 or LAT2 alone versus LAT1 or LAT2 plus LAT3 or LAT4, or EV versus LAT3, LAT4 or LAT5. **P* <0.05; ***P* <0.01; ****P* <0.001.



Supplemental Figure 3 A,B Western blots of post-nuclear supernatants of COS1 cells co-transfected with 50 ng LAT1 or LAT2 plus 5-50 ng LAT3 or LAT4. GAPDH was used as a housekeeping protein. Immunoblots were carried out at least two times.

Supplemental Table 1 Primers used for qRT-PCR

GAPDH_Fw	AATGACCCCTTTATTGAC
GAPDH_Rv	TCCACGACGTACTCAGCGC
LAT1_Fw	GAAGGGTGATGTGTCCAATCT
LAT1_Rv	GCAAAGAGGCCGCTGTATAA

Chapter 5

EXPORT OF IODOTYROSINES BY THE HUMAN THYROID HORMONE TRANSPORTERS MCT8 AND MCT10

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ABSTRACT

MCT8 and MCT10 are highly homologous and highly effective thyroid hormone (TH) transporters. Despite this high homology, MCT8 seems to be specific for iodothyronines, whereas MCT10 also transports aromatic amino acids. The iodotyrosines MIT and DIT are intermediates in TH biosynthesis, showing structural resemblance with iodothyronines. Therefore, we investigated if in addition to the iodothyronines T4, T3, rT3 and 3,3'-T2 (T2), MCT8 and MCT10 may also transport MIT and DIT. COS1 cells were transiently transfected with human MCT8, MCT10, LAT1 or LAT2, and incubated for 5-60 minutes at 37° C with 10 nM ^{125}I-labeled T3, T4, rT3 or T2, or 10 μM MIT or DIT in PBS. LAT1 and LAT2 expression were evaluated by RT-qPCR. We found that MCT8 transports iodothyronines with preference for T4~T3>rT3~T2, while MCT10 showed preference for T3~rT3>T4~T2. Uptake of MIT and DIT by control COS1 cells transfected with empty vector was strongly inhibited by the specific LAT inhibitor BCH. Both LAT1 and LAT2 facilitated MIT and DIT uptake. However, COS1 cells expressed LAT1, but not LAT2. Therefore, endogenous uptake of MIT and DIT by COS1 cells was largely mediated by LAT1. Both in the absence and the presence of exogenous LAT1 or LAT2, transfection of COS1 cells with MCT8 or MCT10 resulted in a marked reduction in cellular accumulation of MIT and DIT. These results suggested that MCT8 and MCT10 primarily facilitated cellular efflux of MIT and DIT. Export of MIT by MCT8 was inhibited by T4 and T3, but this was not observed for DIT. We demonstrated effective cellular uptake of MIT and DIT by LAT1 and LAT2, and effective cellular efflux of these iodotyrosines by MCT8 and MCT10.

INTRODUCTION

Thyroid hormone (TH) is involved in different physiological processes, such as neuronal differentiation, metabolism and growth (1). TH is synthesized in the lumen of the thyroid gland, where tyrosine residues of thyroglobulin are iodinated by thyroid peroxidase (TPO) resulting in the formation of monoiodotyrosine (MIT) and diiodotyrosine (DIT) residues. Subsequently, MIT and DIT are coupled by TPO to form T4 and T3 (2).

TH is activated or inactivated by deiodinases which convert T4 to T3 (D1, D2) or degrade T4 to rT3 (D1, D3), rT3 to 3,3'-T2 (D1, D2) and T3 to T2 (D1, D3) (3,4). Most actions of T3 are mediated by nuclear TH receptors (TRs) which regulate the transcription of target genes (1,2). As deiodinases and TRs are localized intracellularly, TH metabolism and action is dependent on transport across the plasma membrane. This transport is mainly driven by TH transporters rather than diffusion (5,6)

Different TH transporters have been described of which monocarboxylate transporter 8 (MCT8) is most extensively studied. The importance of TH transporters has been demonstrated by the identification of mutations in MCT8 which result in severe psychomotor retardation and abnormal thyroid parameters: the Allan-Herndon-Dudley syndrome (AHDS) (7,8). These patients display high serum T3 and low circulating T4 levels. This constellation of thyroid function tests is partially explained by increased liver and kidney D1 activities, that stimulate the conversion of T4 to T3, as well as diminished TH secretion from the thyroid gland (9-11). The impaired psychomotor development is explained by strongly diminished TH uptake into the brain (12-15).

So far MCT8 is shown to be a specific transporter of iodothyronines (16,17). However, it also has been hypothesized that MCT8 can transport other biologically active substrates, that could be important in human physiology. This seems likely in view of the characteristics of the highly homologous monocarboxylate transporter 10 (MCT10), which also transports aromatic amino acids in addition to iodothyronines (18). The hypothesis of alternative ligands for MCT8 is also supported by a patient with ID carrying a mutation in MCT8, whose mutation does not affect TH transport capacity when tested *in vitro* (19).

It is very important to identify alternative substrates for MCT8, to get further insights in the pathophysiology of AHDS and to develop possible new treatment strategies. The aim of our study is to explore the transport the structure-activity relationship of the transport of different iodothyronines (T4, T3, rT3, 3,3'-T2) and iodotyrosines (MIT, DIT) by MCT8 and MCT10. This was done by studying the uptake of the different substrates by COS1 cells transfected with human MCT8 or MCT10. The transport of MIT and DIT was also studied in cells transfected with the human L-type amino acid transporters LAT1 or LAT2 alone or in combination with MCT8 or MCT10.

MATERIALS AND METHODS

Constructs

The cloning of MCT8.pcDNA3, MCT10.pcDNA3, FLAG-LAT1.pcDNA3 and FLAG-LAT2.pcDNA3 has been described previously (16,18,20). The mouse CD98-pcDNA3 plasmid was kindly provided by Dr. Gerd Krause. The cloning of MCT1 has been described previously (21).

Cell culture and transfection

COS1 cells were cultured at 37 °C and 5% CO_2 in 75 cm^2 flasks with DMEM/F12 (Life Technologies, Bleiswijk, The Netherlands [NL]) supplemented with 9% heat-inactivated fetal bovine serum (Sigma Aldrich, Zwijndrecht, NL), 1% penicillin-streptomycin, and 100 nM Na_2SeO_3. At confluence, cultured cells were split and seeded in 24-well dishes. At 70% confluence, cells were transiently transfected using X-treme GENE 9 Transfection Reagent (Roche Diagnostics, Almere, NL) according to the manufacturer's protocol.

For uptake studies, COS1 cells were transiently transfected in triplicate with 50 ng MCT8, MCT10 or MCT1 plasmid, or 50 ng LAT1 or LAT2 plus 50 ng CD98 plasmid. In some experiments, 50 ng MCT8 or MCT10 plasmid was combined with 50 ng LAT1 or LAT2 plus 50 ng CD98 plasmid. Empty pcDNA3 vector was always added to bring the total amount of plasmid to 200 ng in each well. Previous experiments showed stable transfection efficiency of these transporters. Empty pcDNA3 vector was always added to bring the total amount of plasmid to 200 ng.

Uptake experiments

Two days after transfection, cells were washed with assay buffer (Dulbecco's phosphate-buffered saline (DPBS)+ Ca^{2+}/Mg^{2+} + 0.1% D-glucose + 0.1% BSA) and incubated for 30 minutes at 37 °C with 10 nM (50,000 cpm) [^{125}I]T4, [^{125}I]T3, [^{125}I]rT3 or [^{125}I]3,3'-T2 or 10 μM (50,000 cpm) [^{125}I]MIT or [^{125}I]DIT in 0.5 ml assay buffer. For inhibition studies, we incubated the cells for 30 minutes with 10 μM (50,000 cpm) [^{125}I]MIT or [^{125}I]DIT together with 1-100 μM T4 or T3 or 1 mM of the selective LAT inhibitor 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH). After incubation, cells were washed with assay buffer and lysed with 0.1 M NaOH. The role of amino acid (AA) exchange in the transport of MIT and DIT was studied by pre-incubating the cells for 1h with PBS or DMEM/F12. After this period the uptake assay was performed as previously described in the presence of PBS.

Radioactivity in the lysate was measured in a γ-counter. All radioactive compounds were prepared as previously described (22). Nonradioactive iodothyronines were obtained from Henning (Berlin, Germany) and MIT and DIT from Sigma Aldrich, (Zwijndrecht, NL).

RNA extraction and RT-qPCR of LAT transporters in COS1 cells

Total RNA cells was extracted from COS1 cells using High Pure RNA Isolation kit (Roche Diagnostics), according to the manufacturer's instructions. RNA (1 μg) was reversely transcribed using Transcriptor High Fidelity cDNA Synthesis kit (Roche Diagnostics). Quantitative real-time PCR was performed using qPCR Core kit for SYBR® Green (Eurogentec, Maastricht, NL). GAPDH was used as housekeeping gene for normalization of mRNA expression. PCR primers are listed in Table 1.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (Version 5, Graphpad Software Inc., San Diego, USA). All results are presented as the means ± SEM of at least 2 independent experiments performed in triplicate. Where appropriate, we used one-way or two-way ANOVA followed by the Bonferroni's posttest.

Table 1 Primers used for qRT-PCR.

GAPDH_Fw	AATGACCCCTTTATTGAC
GAPDH_Rv	TCCACGACGTACTCAGCGC
LAT1_Fw	GAAGGGTGATGTGTCCAATCT
LAT1_Rv	GCAAAGAGGCCGCTGTATAA
LAT2_Fw	CATCGTAGGGAACATCATCGG
LAT2_Rv	GAGTTCAGCATAGCAGAGGG

RESULTS

Transport of iodothyronines and iodotyrosines by MCT8 and MCT10

The homologous structures of the iodothyronines T4, T3, rT3 and 3,3'-T2 and the iodotyrosines MIT and DIT were presented in Figure 1A. Transport of these compounds by MCT8 and MCT10 was studied by incubating transiently transfected COS1 cells for 30 minutes with ^{125}I-labeled iodothyronines (10 nM) or iodotyrosines (10 μM). Expression of MCT8 and MCT10 increased T4 uptake 2.25 and 1.84 fold, T3 uptake 1.53 and 2.23 fold, rT3 uptake 1.69 and 3.42 fold, and 3,3'-T2 uptake 1.17 and 1.49 fold, respectively (Figure 1B).

Interestingly, transfection with MCT8 and MCT10 induced a significant reduction in cellular MIT and DIT accumulation, suggesting that these transporters predominantly facilitated the efflux of these substrates (Figure 1B). We also performed an identical experiment using MCT1 as a control, which did not transport TH (unpublished data). For all substrates, cells transfected with MCT1 showed similar uptake as control cells transfected with empty vector (EV), indicating that the decreased accumulation of MIT and DIT in cells transfected with MCT8 or MCT10 was not caused by reduced expression of endogenous MIT and DIT uptake transporters (data not shown).

Endogenous MIT and DIT transport by LAT1 and LAT2

We have previously demonstrated that the L-type amino acid transporters LAT1 and LAT2 effectively facilitated cellular uptake of MIT and DIT (20). To study the contribution of endogenous LAT transporters to MIT and DIT uptake in COS1 cells, we determined the effect of the specific LAT inhibitor BCH on iodotyrosine uptake. BCH significantly decreased uptake of both MIT (Figure 2A) and DIT (Figure 2B) by control cells, which strongly supported an important role of LATs in the endogenous uptake of MIT and DIT in COS1 cells.

Effects of MCT8 and MCT10 on cellular uptake of MIT and DIT by LAT1 and LAT2

Figure 3 showed that transfection of COS1 cells with LAT1 or LAT2 resulted in a marked increase in MIT and DIT uptake. To further investigate the efflux of MIT and DIT by MCT8 and MCT10, we analyzed transport of MIT and DIT in cells co-transfected with MCT8 or MCT10 plus LAT1 or LAT2. We observed a significant decrease in cellular MIT and DIT accumulation in the co-transfected cells compared to cells expressing LAT1 or LAT2 alone (Figure 3A-H). These findings presented further evidence that MIT and DIT taken up by LAT1 or LAT2 are indeed exported by MCT8 or MCT10.

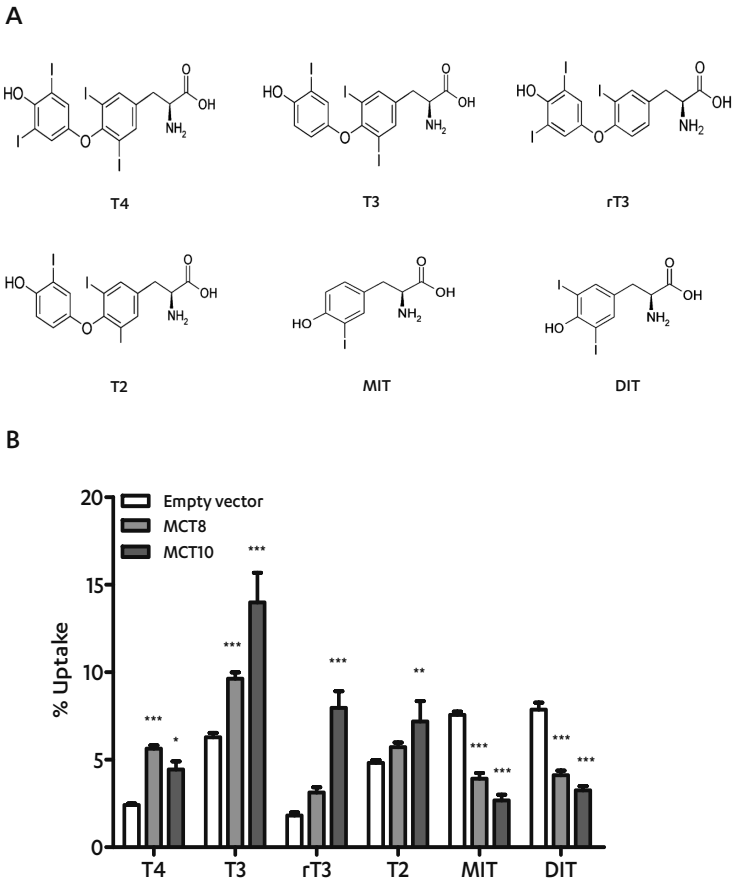

Figure 1 Substrates for the thyroid hormone transporters MCT8 and MCT10. **A** Chemical structures of iodothyronines and iodotyrosines. **B** Uptake of different substrates for 30 minutes (T4, T3, rT3, 3,3'-T2, MIT, DIT) in COS1 cells transfected with MCT8 and MCT10. The figure corresponds to representative data of 3 distinct experiments. *P<0.05; **P<0.01; ***P<0.001; versus empty vector.

Expression of endogenous LAT1 and LAT2 in COS-1 cells

The expression of LAT1 and LAT2 in COS1 cells was determined by real-time PCR. As shown in Figure 4, LAT1 was highly expressed by COS1 cells, whereas no expression of LAT2 was observed. Thus, LAT1 appeared to be largely responsible for endogenous MIT and DIT uptake by COS1 cells.

Export of MIT and DIT by hMCT8 and hMCT10

To get further insight in the transport characteristics of MCT8 and MCT10 towards iodotyrosines, we studied the time course of MIT and DIT uptake. Interestingly, transport of MIT and DIT in COS1 cells showed different patterns. Uptake of MIT by control cells increased during the first 10 minutes, followed by a steep decrease in cellular MIT accumulation to only one-third of the peak level after 60 minutes of incubation. (Figure 5A). This decrease in cellular

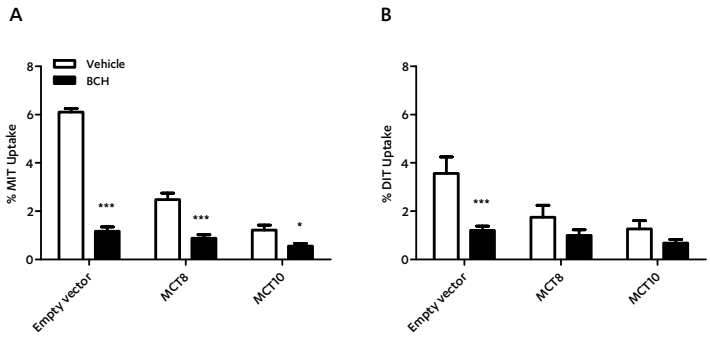

Figure 2 The involvement of LAT transporters in the endogenous uptake of MIT and DIT in COS1 cells. **A** MIT uptake after 30 minutes of time in the presence or absence of BCH (1 mM) in COS1 cells. **B** DIT uptake after 30 minutes in the presence or absence of BCH (1 mM) in COS1 cells. ***P<0.001 versus BCH. **C** LAT1 and LAT2 mRNA levels in COS1 cells. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as internal control. Results are presented as 2e-ddCt.

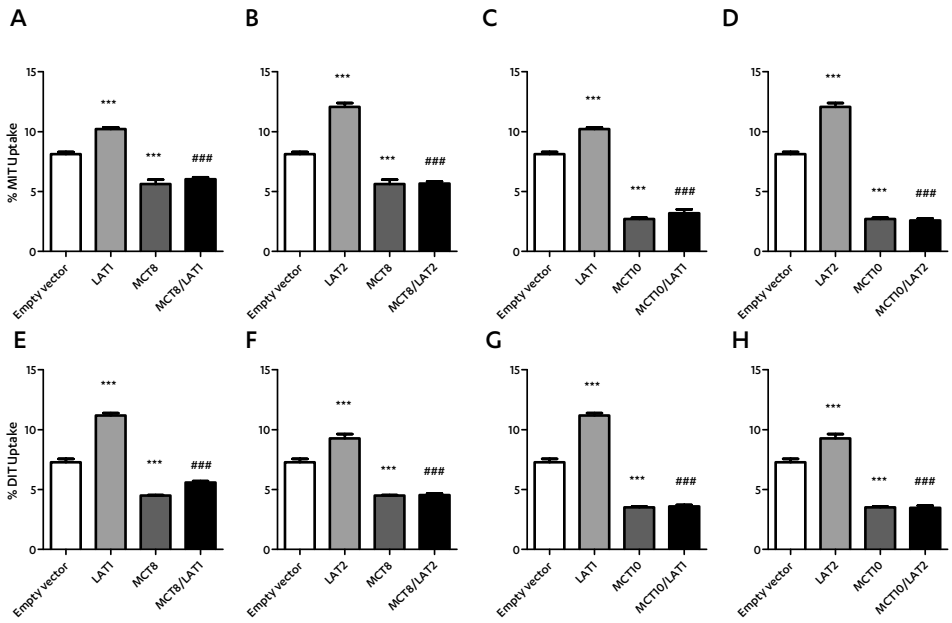

Figure 3 Accumulation of MIT and DIT in COS1 cells co-expressing MCT8 or MCT10 and LAT1 or LAT2. **A,B,C,D** MIT uptake for 30 minutes in COS1 cells transfected with LAT1 or LAT2, MCT8 or MCT10, and co-transfected with LAT1, LAT2, MCT8 or MCT10. **E-F-G-H** DIT uptake for 30 minutes in COS1 cells transfected with LAT1 or LAT2, MCT8 or MCT10, and co-transfected with LAT1, LAT2, MCT8 or MCT10. CD98 was co-transfected with LAT. ***P<0.001 versus empty vector; ###P<0.001 LAT1, LAT2 versus MCT8, MCT10, LAT1, LAT2.

content of MIT could be explained by rapid metabolism of this substrate. We tested this hypothesis by analyzing the generation of iodide or other products by UPLC of the medium. However, we did not observe any formation of metabolites upon incubation of the cells with

MIT (Supplemental Figures 1A and 2A). Cells transfected with MCT8 or MCT10 showed the highest MIT uptake at the shortest incubation time tested (5 minutes), followed by a rapid decline. At all time points, accumulation of MIT was lower in cells that expressed MCT8 or MCT10 than in control cells transfected with EV.

Uptake of DIT by cells transfected with EV increased until 30 minutes of incubation, followed by a modest decrease at 60 minutes (Figure 5B). As for MIT, DIT was found to be completely stable during the 60 minutes incubation (Supplemental Figure 1B, 2B). Again, at all incubation times, accumulation of DIT in cells expressing MCT8 or MCT10 was lower than in control EV cells. Similar results were obtained using H4 glioma cells (data not shown). These findings support the idea that MCT8 and MCT10 predominantly facilitated the cellular efflux of MIT and DIT.

MIT and DIT are differently transported in COS-1 cells

One possible explanation for the decrease in MIT uptake over time is that, as LAT is an obligatory exchanger, it needs AA inside the cells to be exchanged for MIT. As our assay was performed

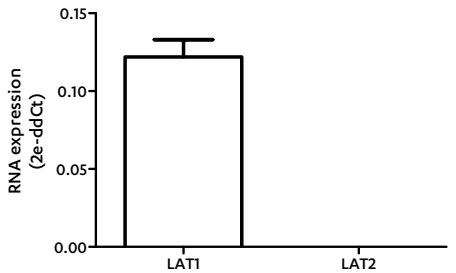

Figure 4 Expression of LAT1 and LAT2 transporters in COS1 cells. LAT1 and LAT2 mRNA levels in COS1 cells. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as internal control. Results are presented as 2e-ddCt.

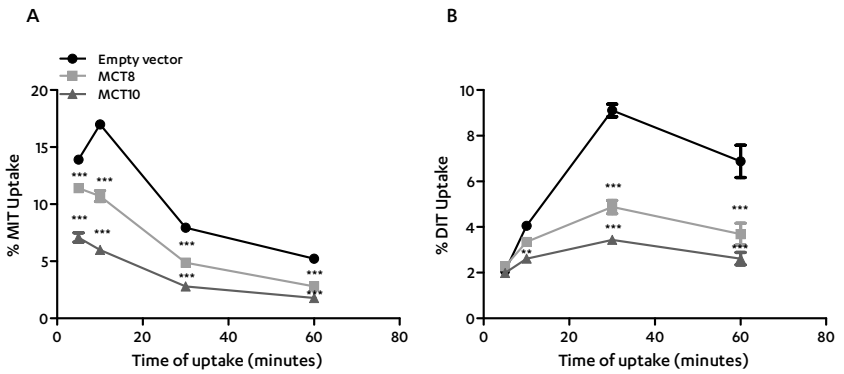

Figure 5 Time-course of MIT and DIT accumulation in COS1 cells transfected with MCT8 and MCT10. **A** MIT uptake for 5, 10, 30 and 60 minutes. ***P<0.001 versus empty vector. **B** DIT uptake for 5, 10, 30 and 60 minutes. ***P<0.001 versus empty vector. The figure corresponds to representative data of 3 distinct experiments.

in PBS, we hypothesized that after 10 minutes, less AA were present to be exchanged, resulting in decreased MIT uptake. To test this hypothesis, we pre-incubated cells transfected with EV for 1h in the presence of PBS or DMEM/F12 and after that we performed the time course uptake assay for MIT in PBS. As can be observed in Figure 6A, the pre-incubation with PBS immediately decreased the MIT uptake, while the DMEM/F12 only showed little decrease within the time. Pre-incubation with PBS did not interfere with DIT transport (Figure 6B). These results demonstrated that transporters with exchange abilities facilitate MIT transport, but none were involved in the endogenous uptake of DIT.

Influence of T3 and T4 on MIT and DIT uptake and efflux

As iodothyronines were expected to compete with MIT and DIT transport by MCT8 and MCT10 and possibly also by endogenous transporters, we studied the effects of T4 or T3 on cellular accumulation of MIT and DIT. Interestingly, co-incubation with 1-100 μM T4 did not affect uptake of MIT by EV cells (Figure 7A), suggesting that T4 did not inhibit MIT transport by endogenous transporters in COS1 cells. Accumulation of MIT in MCT8-expressing cells increased at higher levels of added T4, up to the level of MIT uptake by EV cells, suggesting that T4 inhibited efflux of MIT by MCT8. However, T4 did not affect the low MIT uptake in MCT10-expressing cells.

T3 showed a bi-phasic effect on MIT uptake by COS1 cells transfected with EV, MCT8 or MCT10 (Figure 7B). At 1 and 10 μM, T3 induced a marked increase in MIT uptake by MCT8-expressing cells and a modest increase in MIT uptake by cells transfected with EV or MCT10. At 100 μM, T3 profoundly lowered MIT uptake by COS1 cells, irrespective of transfection with EV, MCT8 or MCT10, suggestion inhibition of MIT uptake by endogenous transporters in COS1 cells.

Both T4 and T3 produced dose-dependent inhibition of DIT uptake by cells transfected with EV, MCT8 or MCT10. Near-complete inhibition was observed at 10 μM T3 and at 100 μM T4 (Figure 7C-D). These findings suggested that T4 and T3 inhibit DIT uptake by endogenous transporters in COS1 cells.

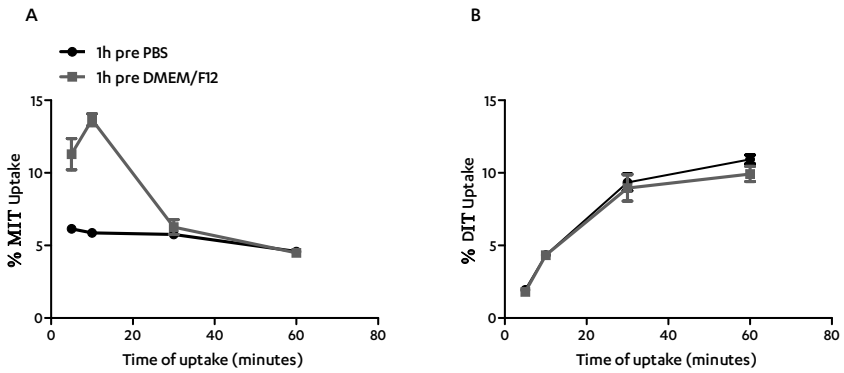

Figure 6 Time-course of MIT and DIT endogenous accumulation in COS1 cells. **A** MIT uptake for 5, 10, 30 and 60 minutes after 1h pre-incubation with PBS or DMEM/F12. **B** DIT uptake for 5, 10, 30 and 60 minutes after 1h pre-incubation with PBS or DMEM/F12. The figure corresponds to representative data of 3 distinct experiments.

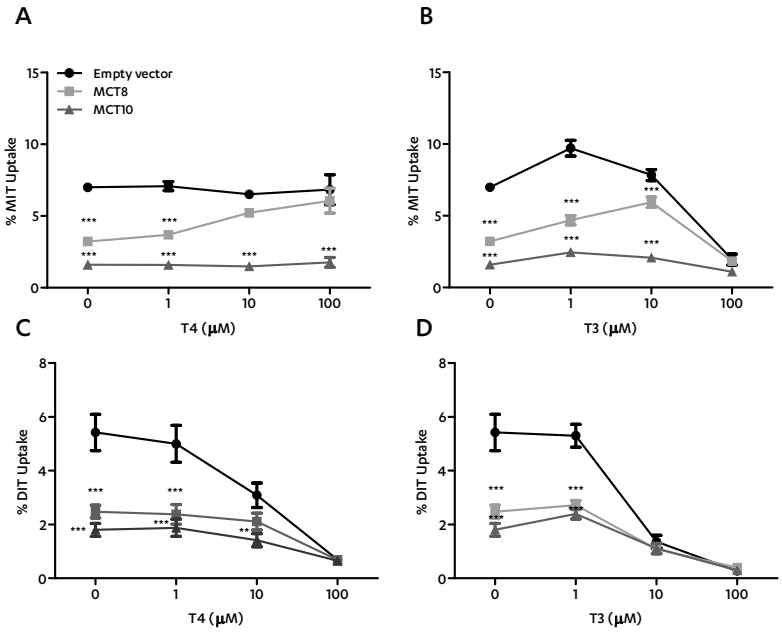

Figure 7 Effects of T3 and T4 on MIT and DIT uptake and efflux. **A** MIT uptake for 30 minutes with increasing concentrations of T4 (1, 10, 100 µM). **B** MIT uptake for 30 minutes with increasing concentrations of T3 (1, 10, 100 µM). **C** DIT uptake for 30 minutes with increasing concentrations of T4 (1, 10, 100 µM). **D** DIT uptake for 30 minutes with increasing concentrations of T3 (1, 10, 100 µM). The figure corresponds to representative data of 3 distinct experiments. ***P<0.001 versus empty vector.

DISCUSSION

The discovery of MCT8 as a TH transporter a decade ago, has profoundly changed our understanding of cellular TH regulation. That MCT8 is an important TH transporter is underscored by the severe neurological disorder (AHDS) caused by MCT8 mutations (7,8,15). *Mct8* KO mice showed strongly impaired T3 transport into the brain (12,23,24). However, for some patients, the phenotype cannot be totally explained by the reduction in T3 transport (19). Therefore, it has been hypothesized that MCT8 could also transport other biologically active substrates. This may seem likely in view of the fact that MCT10, which is highly homologous to MCT8, also transported aromatic amino acids in addition to iodothyronines (19). Since T4 and T3 are iodotyrosine derivatives (Figure 1A), we tested the hypothesis that MCT8 and MCT10 could also transport the iodotyrosines MIT and DIT.

Our findings suggested that uptake of MIT and DIT by control cells was largely mediated by the L-type amino acid transporter LAT1. MIT and DIT uptake by control COS1 cells was potently inhibited by the LAT inhibitor BCH. Both LAT1 and LAT2, but not LAT3, LAT4 or LAT5, were capable of transporting MIT and DIT. However, only LAT1 but not LAT2, was significantly expressed by COS1 cells. As a consequence, LAT1 appeared to be primarily responsible for MIT and DIT uptake by control COS1 cells, although we could not exclude the contribution of other transporters.

After initial uptake of MIT, we noted a marked reduction in accumulation of this substrate in COS1 cells with increasing the incubation time, irrespective of transfection with EV, MCT8 or MCT10. The uptake phase was prolonged with DIT as the substrate and there was only a modest decrease in cellular DIT concentration with a longer incubation period. Our findings excluded that the loss of cellular MIT and DIT with continued incubations was due to the cellular metabolism of these substrates. A more likely explanation was offered by studies indicating that LAT1 is an obligate exchanger (25-27), implying that the cellular uptake of substrates, such as MIT and DIT, required the efflux of intracellular substrates. Before the uptake experiments, cells were cultured in the presence of DMEM/F12, which contained high concentrations of AA. This implied that at the start of the experiments, cells contained high concentrations of AA substrates for LAT1, affording a large supply of efflux substrates to drive the uptake of MIT and DIT. During the course of the uptake experiments, cells became depleted of these intracellular AA through efflux by uniporters, resulting in a diminished uptake of extracellular substrates such as MIT and DIT. This hypothesis was supported by our findings that the pre-incubation of COS1 cells with PBS before the uptake experiment, resulted in a marked reduction in the initial uptake of MIT and DIT. This is supporting our explanation for the biphasic nature of the uptake of in particular MIT by COS1 cells.

In the present report, we demonstrated for the first time that MCT8 and MCT10 facilitated cellular efflux of MIT and DIT. We observed a significant decrease in accumulation of MIT and DIT in COS1 cells transfected with MCT8 or MCT10, both in the presence or absence of transfected LAT1 or LAT2. Transport of MIT and DIT was differently modulated in the presence of T3 and T4. Endogenous uptake of MIT was markedly inhibited 100 μM T3, but not by T4. However, uptake of DIT was strongly, and dose-dependently, reduced in the presence of both 10-100 μM T4 or T3.

The lack of effect of up to 100 μM T4 on MIT uptake by control cells may be explained by equal inhibition of uptake and efflux by endogenous transporter(s). In particular T4, but also T3, inhibits the efflux of MIT mediated by MCT8, but the iodothyronines had less effect on MIT efflux mediated by MCT10. Also, cellular efflux of DIT by MCT8 or MCT10 appeared to be affected less by T4 and T3 than DIT uptake by endogenous transporters.

In this study we also demonstrated that in addition to T4 and T3, human MCT8 also significantly facilitated cellular uptake of rT3, but it showed less activity towards 3,3'-T2 as the substrate. Therefore, the iodothyronine substrate preference of MCT8 was T4>T3~rT3>3,3'-T2. Among the iodothyronine substrates, MCT10 appeared to be most active in the cellular uptake of rT3 followed by T3, T4 and 3,3'-T2. The physiological relevance of the preferred transport of rT3 by MCT10 remains to be investigated.

What could be the physiological role of MCT8 and MCT10 in the export of iodotyrosines? MIT and DIT are iodotyrosine intermediates in the biosynthesis of T4 and T3 in the thyroid gland (28). Excess MIT and DIT released from thyroglobulin is deiodinated in the thyroid by DEHAL1 to reutilize the iodide for TH synthesis. MCT8 is highly expressed in thyroid follicular cells and appears to play an important role in the thyroidal secretion of TH (12,23). Thyroidal release of MIT and DIT would reduce the exposure of the iodotyrosines to DEHAL1 in follicular cells and, hence, decrease the efficiency of iodide retrieval. However, DEHAL1 is also highly expressed in liver and kidney where it may be involved in the deiodination of MIT and DIT taken up from

the blood by LAT transporters (29,30). The role of MCT10 in the thyroid is less clear as this transporter is expressed in the thyroid of mice, but not of humans (31).

With our data we could design a schematic, and very simplified overview, of the presented transport characteristics of MCT8, MCT10, LAT1 and LAT2 for all iodothyronines and iodotyrosines (Figure 8). The substrate preference of MCT10 is T3~rT3>T4~T2, while MCT8 showed to be a better transporter for T4 and T3, with no significant transport of T2 or rT3. Besides that, both MCT8 and MCT10 can export the iodotyrosines MIT and DIT, with MCT10 being more effective than MCT8. We have shown that LAT transporters are involved in the uptake of iodotyrosines, and probably LAT1 was the endogenous transporter in our cell system. It is possible that other transporters were also involved in MIT and DIT uptake, as we observed, mainly for DIT, that a transporter lacking exchange abilities must be involved. Further studies must be performed to identify other potential transporters.

If efflux of MIT and DIT by MCT8 has any contribution to the phenotype of patients with AHDS, remains to be clarified in further studies. Our study demonstrated that MCT8 is able to transport substrates beyond iodothyronines. These findings encourage us to further explore the possible transport of other compounds by MCT8 as well as the role of these non-iodothyronine substrates in the pathophysiology of AHDS.

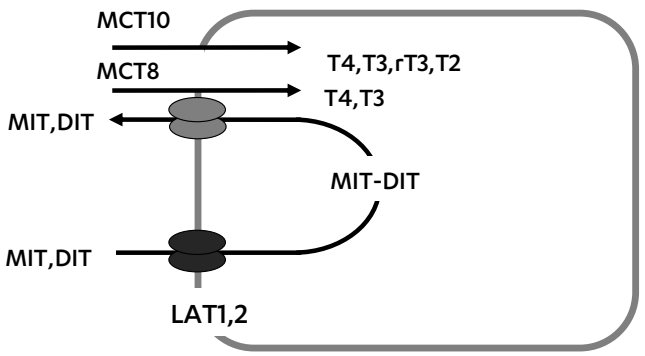

Figure 8 Schematic overview of MIT and DIT transport by MCT8 or MCT10 and LAT1 or LAT2. MCT8 and MCT10 participate in MIT and DIT export while LAT1 and LAT2 are involved in the uptake of iodotyrosines.

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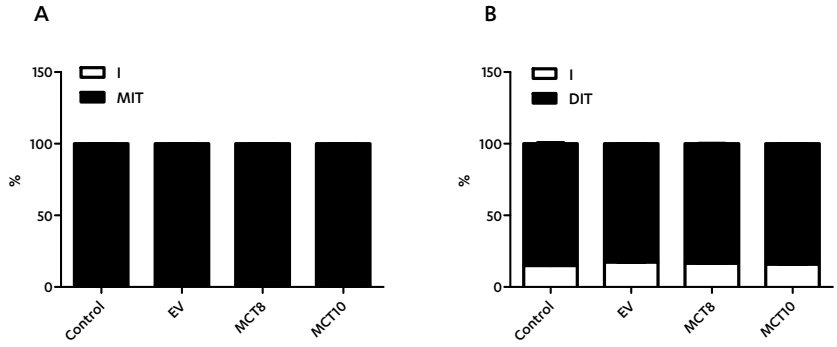
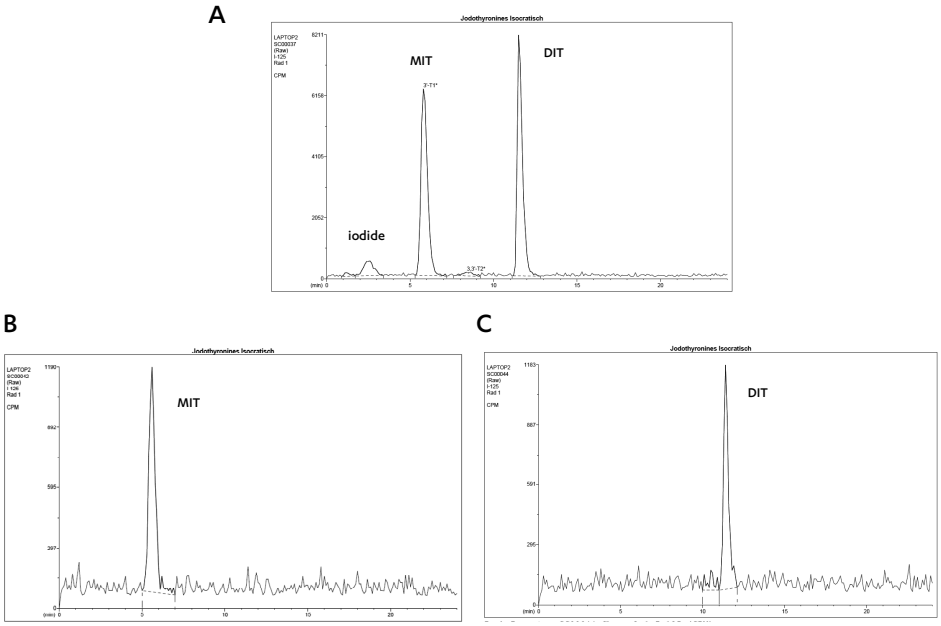


Supplemental Figure 1 Separation of MIT or DIT and iodide, using a Dowex colunm. COS1 cells were incubated for 60 minutes in the presence of MIT or DIT and hereafter, the medium was removed for analysis. **A** Medium of cells incubated for 60 minutes with MIT. **B** Medium of cells incubated for 60 minutes with DIT.



Supplemental Figure 2 Separation of MIT or DIT and iodide using UPLC. COS1 cells were incubated for 60 minutes in the presence of MIT or DIT, and the medium was removed for analysis. **A** Example of MIT and DIT metabolism to iodide, using MIT and DIT. **B** Analysis of metabolites in medium of cells incubated for 60 minutes with MIT. **C** Analysis of metabolites in medium of cells incubated for 60 minutes with DIT.

Chapter 6

PHYSIOLOGICAL CONCENTRATIONS OF THYROXINE STIMULATE THE TRANSCRIPTION OF THYROID HORMONE RESPONSIVE GENES

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ABSTRACT

Thyroid hormone (TH) mediates its action by binding to nuclear T3 receptors (TRs), which regulate the transcription of target genes. T3 is generally regarded as the active hormone, based on the higher binding affinity compared to T4. However, it has been shown that T4 can induce similar conformational changes in the TR. To study direct transcriptional T4 effects, we depleted SHSY5Y cells of TH and incubated them with T3 or T4. Microarray analysis showed a similar response in gene expression after treatment with T3 or T4, with *ENPP2*, *KLF9* and *HR* as prominent examples. Although 1 nM T3 is more potent than 1 nM T4 to stimulate expression, they induce similar responses at higher concentrations. Inhibition of transcription and translation suggested that T4 can regulate gene expression at the transcriptional level directly. These results from cellular studies complement previous studies that T3 is the main bioactive hormone, but T4 can exert transcriptional effects.

INTRODUCTION

Thyroid hormone (TH) is the common name for thyroxine, 3,3',5'5'-tetraiodothyronine (T4) and 3,3',5'-triiodothyronine (T3) and these molecules are important for the development and metabolism of virtually all tissues. TH mediates its action by binding to nuclear T3 receptors (TRs) which regulate the transcription of target genes (1,2). TRs bind to TH response elements (TREs) in the promoter regions of target genes, thereby regulating gene transcription by the attraction or release of corepressors and coactivators (3,4). T3 is known as the active hormone, based on receptor binding studies showing that T3 binds with higher affinity (10-100x) to the TR than T4 (5-9). T4 is regarded as the prohormone and is converted to T3 by the activating deiodinases type 1 and 2 (D1 and D2), which are expressed in different tissues and are known to regulate local TH metabolism (10,11).

That TH could exert biological, non-genomic effects has only recently been recognized (12-15). Farwell *et al* showed that T4 and rT3 could regulate actin polymerisation and D2 activity in the developing brain via non-transcriptional ways (12). Also, 3,3'-T2 en 3,5-T2 showed to effect mitochondrial energy metabolism and in modelling studies TH analogues could bind to the extracellular part of the integrin αvβ3, which is a cell surface structure protein (13,15,16). Binding of TH to αvβ3 can influence cell proliferation via mitogen activated protein kinases. Furthermore, nongenomic TH signaling was linked to a previously uncharacterized membrane-bound receptor that is generated by translation initiation from an internal methionine of TRα, which produces a transcriptionally incompetent protein (17). TH signaling through this receptor stimulated a pro-proliferative and pro-survival program by increasing the intracellular concentrations of calcium, nitric oxide (NO), and cyclic guanosine monophosphate (cGMP). It has also been demonstrated that the TH-TRβ complex can exert non-genomic actions in the cytosol by inducing the transcription of *hypoxia-inducible factor* (*HIF-1*α) and it's target genes, which has important roles in cellular glucose metabolism (18).

There are arguments that support the hypothesis that T4 may exert direct transcriptional effects. First, biological effects at the receptor level are not only dependent on binding affinity, but also on availability of the hormones. Serum FT4 levels circulate at 4 times higher concentrations than serum FT3 levels. (1,7,19,20). Second, based on the cellular repertoire of TH transporters and deiodinases, the intracellular T3 and T4 concentrations may differ from serum concentrations. Third, it has been shown that T4 can induce conformational changes in the TR similar to those induced by T3 (21). The group of Sandler *et al* observed that despite the less compact nature of the TR-T4 complex, maximally effective doses of T4 were as effective as those of T3 in stimulating the association of coactivators and the release of corepressors in a GST pull-down assay (7). Fourth, a second binding site for THs was recently identified in the ligand binding domain of the TR in which T4 showed to have a higher binding affinity over T3 (19). However, the biological relevance of this second binding site remains to be elucidated.

Although there are indications that T4 could be a direct transcriptional modulator, no cell culture studies have been performed to directly test this hypothesis (22). In the present study we made a first attempt to explore the possibility if T4 can modulate gene transcription using a cell model devoid of D1 and D2.

MATERIALS AND METHODS

Cell culture

SHSY5Y cells were cultured at 37 C° and 5% CO_2 in 75 cm^2 flasks with DMEM/F12 (Life Technologies, Bleiswijk, The Netherlands) supplemented with 9% heat-inactivated fetal bovine serum (Sigma Aldrich, Zwijndrecht, The Netherlands) and penicillin-streptomycin. At confluence, cultured cells were split and seeded in 6-well dishes. After 24 hours the medium was replaced by serum free DMEM/F12 with 0.1% BSA to deplete the cells of thyroid hormones. After 48 hours the cells were incubated for 2, 6, 12, 24 or 48 hours in similar medium supplemented with 0, 1, 10 or 100 nM of T3 or T4, with or without 5 µg/ml actinomycin D (Sigma Aldrich, Zwijndrecht, The Netherlands), 25 µg/ml cycloheximide (Sigma Aldrich, Zwijndrecht, The Netherlands) or 50 µg/ml iopanoic acid. When the incubation time was finished, cells were washed with Dulbecco's phosphate buffered saline (d-PBS) and harvested in 200 µl of the same buffer.

RNA isolation and microarray analysis

RNA was isolated using the RNAeasy isolation kit (Roche, Woerden, The Netherlands). 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 RNA integrity number >8 and were all used for subsequent labeling. RNA (3-5 µg) from all samples 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 GeneChips were visually inspected for irregularities. The R package AffyQC-report was used for quality control and indicated high quality and overall comparability of 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 data file 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 2log 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.

Quantitative-PCR (qPCR)

cDNA was synthesized using 1.0 µg of RNA and TaqMan RT reagent (Roche, Woerden, The Netherlands). SYBR Green I (Eurogentec, Maastricht, The Netherlands) was used as the detector dye for qPCR of the differentially expressed thyroid hormone responsive genes

(*ENPP2-HR-KLF9/BTEB*). The primer sequences are presented in Supplemental Table 1. mRNA levels are corrected for the house-keeping gene *Cyclophilin A* and displayed relative to the wells incubated with a matching vehicle.

Deiodinase activity and radioimmunoassay (RIA)

Metabolite production due to endogenous deiodinase activity of the SHSY5Y cells was measured using an intact cell metabolism experiment, in which the cells were incubated for 24 h with 10 nM ^{125}I-labeled T4, T3 or rT3, as described previously (23). Before incubation of the cells, T3 and T4 levels of the medium supplemented with either 10 nM T3 or T4 were measured by a RIA (24).

Luciferase assay

Transcriptional activity of TRα1 and TRβ1 receptors was determined using the Dual-Glo Luciferase kit (Promega, Leiden, NL), as described before (25). JEG3 cells (a human choriocarcinoma cell line) were transfected with 100 ng pdV-L1 luciferase-renilla reporter construct together with 100 ng TRα1 or TRβ1 receptor. The luciferase activity was normalized to the renilla activity to adjust for transfection efficiency.

Statistical analysis

The results are the mean of at least 2 representative experiments and are expressed as mean ±SE. The changes of mRNA levels after T3 or T4 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*) and relative to incubation with the vehicle. Statistical analysis was carried out in GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA) using the Student's t-test for unpaired observations. The results of the luciferase assays are presented as the means ±SEM of at least three or more independent experiments, carried out in triplicate. Statistical significance was determined using two-way ANOVA followed by a Bonferroni post test.

RESULTS

To address the specific effects of T3 and T4 in cultured SHSY5Y cells, it is important to avoid cellular production of T3 from T4 by endogenous deiodination. Therefore, we studied the neuronal cell line SHSY5Y which is devoid of D1 and D2 activity and is known to exhibit only D3 activity (26). This was validated in an intact cell metabolism experiment, in which SHSY5Y cells were incubated for 24 h with 10 nM ^{125}I-labeled T4, T3 or rT3. The results indicated deiodination of T4 and T3 by D3 expressed in SHSY5Y cells, but no conversion of T4 to T3 (Supplemental Figure 1). Furthermore, we measured T3 and T4 levels by RIA of the medium supplemented with either 10 nM T3 or T4 before incubation of the cells. While we measured similar amounts of added T3 and T4, no T3 (lower detection limit is 0.1 nM) was detected in the T4-containing medium (data not shown). Together, these data indicated that no T3 was present in T4-containing culture medium.

To assess the transcriptional effects of T3 and T4, we performed genome-wide expression profiles on SHSY5Y cells treated with 10 nM T3, 10 nM T4 and vehicle. Therefore, we selected

probe sets for analysis, which showed a >1.5-fold change in gene expression on T3 exposure. At a false discovery rate of 0.05, we identified 31 such probe sets representing 24 unique genes and 2 non-annotated Affymetrix IDs. Importantly, nearly all genes in this list were also similarly regulated by T4. Figure 1A shows the selected probe sets as a hierarchical clustering, clearly indicating that both T3 and T4 regulated gene expression. In general, effects on gene expression were less pronounced with T4 than with T3, although selected genes appear to be regulated similarly by T3 and T4. Of the 24 genes regulated by T3 and T4 in the microarray analysis, 3 appeared to be well-known TH responsive genes (*ENPP2-KLF9-PLD5*). A search in literature showed that some of the other genes like *ITGB5* and *TMOD1* are also under positive control of T3, which supported our data (27,28). We used *ENPP2* and *KLF9* to confirm the transcriptional stimulation of T3 and T4 by qPCR and these results correlated well with the microarray data (Figure 1B-C). In addition, we verified the effect of T3 and T4 on the well-known T3-responsive gene *Hairless* (*HR*) (Figure 1D). This gene was not detected by the microarray, presumably because it did not reach the preset threshold due to the lower expression of this transcript (Supplemental Table 2). *HR* was highly and significantly induced by both T3 and T4. As expected the neuronal cell line SHSY5Y showed a very pronounced expression of *THRA*, encoding TRα1 and an almost undetectable expression of *THRB*, encoding TRβ1 (Supplemental Figure 2).

Next, we studied the dose dependence of the effects of T4 and T3 on *ENPP2*, *KLF9* and *HR* expression. Although the effects that we observed are specific and unique per gene, in general at lower concentrations (1 nM) T3 is more potent than T4 to induce expression of these genes (Figure 2).

To investigate the time course of the effects of T4 and T3 on the expression of *ENPP2*, *KLF9* and *HR*, SHSY5Y cells were incubated for 2, 6, 12 or 48 hours with 10 nM T4 or T3. All genes showed an increased expression after 6 hours of incubation with T3 and T4 and for *KLF9* this effect was even already significant after 2 hours for both substrates (Figure 3). Figure 3 also indicates a maximal response after 12 h of incubation for all genes.

To confirm that the observed effects resulted from a direct transcriptional T4 effect, we incubated the cells with T4 or T3 together with the transcription-blocker actinomycin D (29) or the protein-synthesis blocker cycloheximide (30). Actinomycin D prevents the increase in expression of target genes (Figure 4), suggesting that the effects of T3 and T4 are mediated at the transcriptional level. After co-incubation of the cells with T4 or T3 and cycloheximide, we still observed similar effects of T4 and T3 on the transcription of target genes (Figure 4). This suggests that the effect of T4 occurs indeed directly at the transcriptional level, instead of being dependent on a protein component in this regulatory pathway. Cycloheximide increased the expression of all genes in the untreated cells and cells incubated with T3 and T4 increased. This phenomenon is described before, as cycloheximide inhibits the synthesis of enzymes that degrade RNA, thereby increasing RNA stability (31).

As a last approach we treated the cells with 10 nM T3 or T4 with or without 50 µg/ml iopanoic acid to completely block all deiodinase activity (32) and observed an identical response in gene expression after treatment with T3 or T4 (data not shown).

To assess the possible differential response of the TR isoforms to T4, we measured the transcriptional activity of TRα1 and TRβ1 after incubation with increasing concentrations of

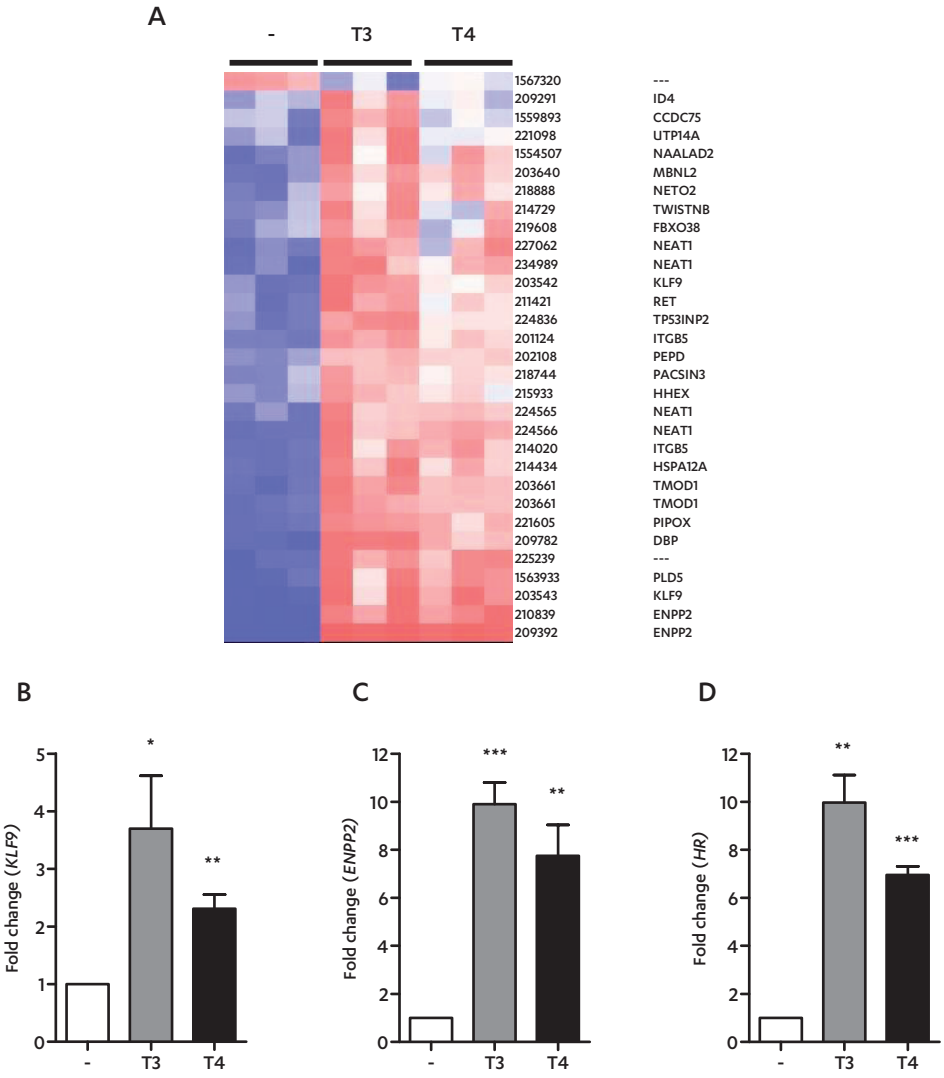

Figure 1A Genome wide expression profiles on SHSY5Y cells incubated for 24h with vehicle, 10 nM T3 or T4. Gene expression levels: red, up-regulated genes compared to the geometric mean; blue, down-regulated genes compared to the geometric mean. The colour intensity correlates with the degree of change. **B-D** Effects of incubation of SHSY5Y cells for 24h with a vehicle, 10 nM T3 or T4 on **(B)** *KLF9*, **(C)** *ENPP2*, **(D)** *HR* mRNA expression using qPCR. Significance represents cells incubated with T3 or T4 compared to the vehicle for all genes. **P* <0 .05; ***P* <0.01; ****P* <0.001.

T3 and T4, using a luciferase assay. We used JEG3 cells, because of their adequate transfection efficiency and their expression of D3, similar to SHSY5Y cells. We showed that, in line with the work of Schroeder *et al.*, T4 is transcriptionally active on both receptors, although TRα1 is more responsive to T4 compared to TRβ1 (Figure 5).

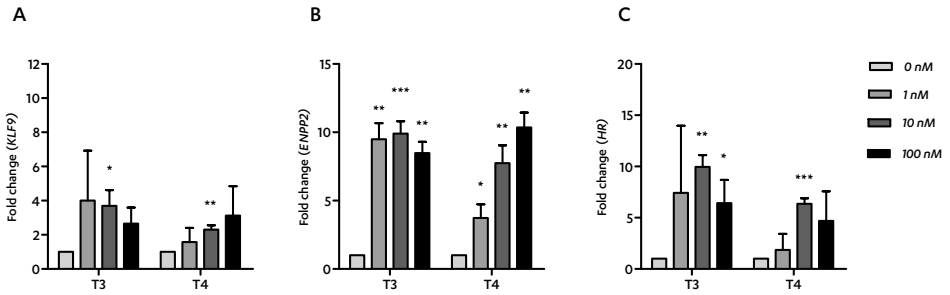

Figure 2 A-C Dose-dependent effects of incubation of SHSY5Y cells for 24h with 0-1-10-100 nM T3 or T4 on **(A)** *KLF9*, **(B)** *ENPP2*, **(C)** *HR* mRNA expression using qPCR. Significance represents cells incubated with 1-10-100 nM of T3 or T4 compared to the vehicle (0 nM). **P* <0 .05; ***P* <0.01; ****P* <0.001.

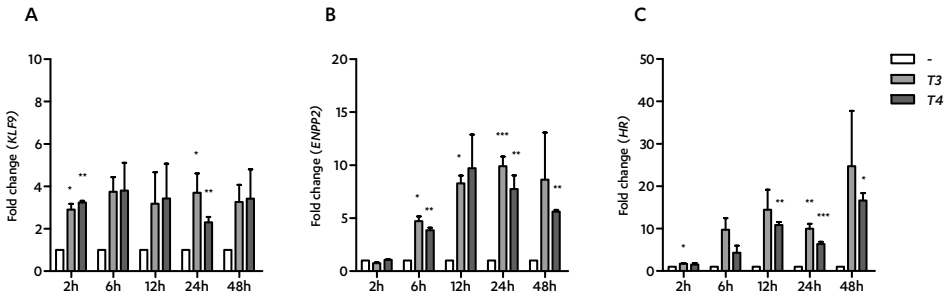

Figure 3 A-C Effects of different incubation times on total RNA content of SHSY5Y cells incubated with vehicle, 10nM T3 or T4 on **(A)** *KLF9*, **(B)** *ENPP2*, **(C)** *HR* mRNA expression using qPCR. Significance represents cells incubated with T3 or T4 compared to the vehicle at all time points. **P* <0 .05; ***P* <0.01; ****P* <0.001.

DISCUSSION

The common wisdom that holds that only T3 is the bioactive form of TH is mainly based on receptor binding studies (5,6). There are multiple arguments that could support the idea of T4 as a direct transcriptional stimulator. However, no studies have been performed to explore the possibility that T4 could directly stimulate transcription of TH responsive target genes.

In this study, we compared the effects of incubation with physiological concentrations of T3 *versus* T4 in SHSY5Y cells on the mRNA expression of TH responsive genes. In an explorative approach, using microarray analysis, we observed that genes were similarly induced by 10 nM T3 and T4 (Figure 1A). We selected 3 TH responsive genes to perform quantitative PCRs on treated SHSY5Y cells and we could indeed confirm a significant increase in mRNA expression of TH responsive target genes (*ENPP2*, *KLF9*, *HR*) after incubation with low concentrations (1 nM) T3 and T4 (Figure 1B-D and Figure 2). The effects were already observed after 2 or 6 hours of incubation time (Figure 3). The results after treatment with actinomycin D and cycloheximide supported that the effects observed after treatment with T4 are at the transcriptional level (Figure 4).

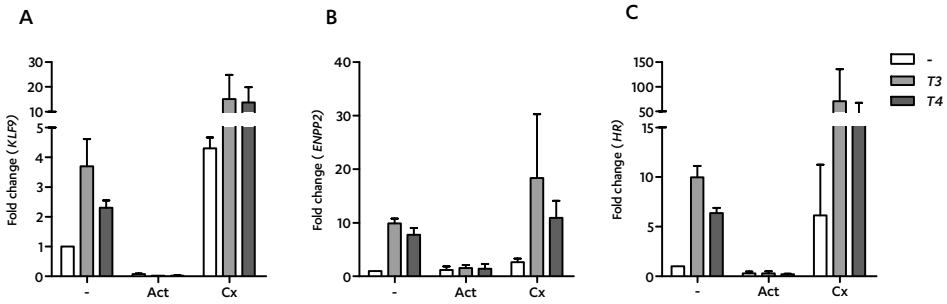

Figure 4A-C Effects of 24h incubation with actinomycin D (5 mg/ml) and cycloheximide (25 mg/ml) on SHSY5Y cells co-incubated with vehicle, 1 nM T3 or T4 on **(A)** *KLF9*, **(B)** *ENPP2*, **(C)** *HR* mRNA expression using qPCR. Significance represents untreated cells (vehicle or 10 nM of T3,T4) compared to cells incubated with actinomycin D or cycloheximide. **P* <0 .05; ***P* <0.01; ****P* <0.001.

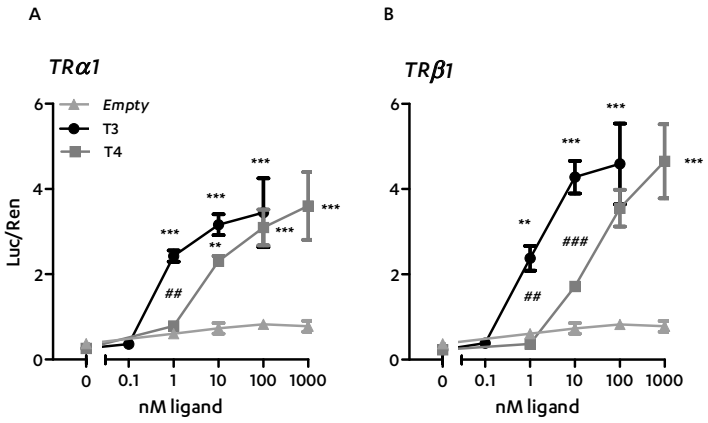

Figure 5A-B Effects of 0-0.1-1-10-1000 nM T3 or T4 on the transcriptional activity of TRα1 (A) and TRβ1 (B) in JEG3 cells using a luciferase assay. The luciferase activity was normalized to the renilla activity to adjust for transfection efficiency. Significance represents empty vector compared to cells treated with T3 or T4 *** or T3 compared to T4 ###. **P* <0 .05; ***P* <0.01; ****P* <0.001.

We used various complementary experiments to rule out that endogenous present T3 or deiodination of T4 to T3 is not responsible for the increase in expression of target genes in T4 treated cells (Supplemental Figure 1). Also incubation with iopanoic acid did not change our results.

Globally, most genes appear somewhat less sensitive to T4 as compared to T3. However, it is important to realize that T4 and T3 have different binding capacities to the BSA used in our incubation medium. As T4 has a higher binding affinity to BSA (~5x) compared with T3, the transcriptional effects of T4 are probably underestimated (33).

Our studies suggest that T4 can exert transcriptional effects in SHSY5Y cells. Our data are well in line with other studies, which show T4 can induce conformational changes in the TR similar to those induced by T3 (21) and by the work of Schroeder *et al* in which they

described the ability of both TRα1 and TRβ1 to respond to T3 and T4. TRα1 was generally more responsive and they also showed the recruitment of coactivators by T4, especially to TRα1 (22). This supports our findings in SHSY5Y cells which mainly express TRα1. Also recently, a second binding site in the TR was identified with a higher binding affinity for T4 compared to T3 (19), however the biological importance of this second binding site needs to be further investigated. To test the hypothesis that T4 can stimulate transcription of target genes *in vivo,* it would be very interesting to study thyroidectomised D1/D2 double KO mice supplied with T3 or T4.

Although our data suggest that T4 indeed can stimulate the expression of TH sensitive genes, we should keep in mind that these effects can be tissue or even cell specific. This is underscored by the study of Freitas *et al* who showed that the expression of TH sensitive genes in neurones is dependent on T4 to T3 conversion by the type 2 deiodinase in the glial compartment (34). However, the expression of *ENPP2* was already doubled with low concentrations of T4 in neurons alone. Also, in various other studies iopanoic acid was used to show that the effects of T4 at the pituitary level require local conversion to T3 in rats (35,36). Iopanoic acid blocks all deiodinase activity and incubation with this agent prevented the decrease in thyroid stimulating hormone (TSH) release and the activation of growth hormone (GH) synthesis only after administration of T4 and not T3. Most likely some tissues, like brown adipose tissue (37) or parts of the brain, are dependent on local conversion of T4 to T3, whereas in other tissues T4 is able to exert direct transcriptional effects.

In this study, we made a first attempt to show that in addition to T3, also T4 exerts transcriptionally effects on TH target genes. Our studies exploiting a cell line expressing TRα1, that is devoid of D1 and D2, largely complement and confirm other non-cellular studies that T3 is the main bioactive hormone, but T4 can exert transcriptional activity under certain conditions.

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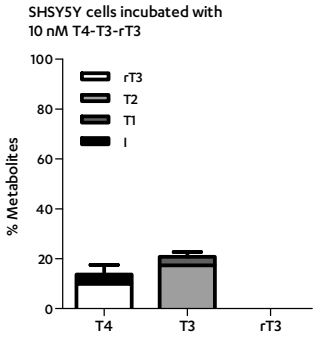
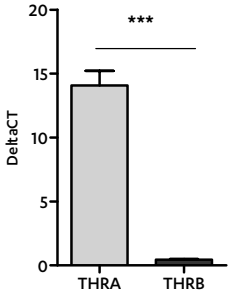


Supplemental Figure 1 Intact cell metabolism of 10 nM T4, T3 and rT3 after 24h incubation in SHSY5Y cells. Metabolism is shown as percentage metabolites in the incubation medium. Results are the means ± SEM of 2 experiments. **P* <0 .05; ***P* <0.01; ****P* <0.001.



Supplemental Figure 2 mRNA levels of *TR*α and of *TR*β in untreated SHSY5Y cells. **P* <0 .05; ***P* <0.01; ****P* <0.001.

Supplemental Table 1 Primer sequences.

Gene	Primer Sequence (5'-3')
ENPP2	FW:ACTCCGTGAAGGCAAAGAGA REV:CAAGATCCGGAGATGTTGGT
HR	FW:TGACTGCCACCTGCTTTATGC REV:GCCACCTTCACTGCTTGGAA
KLF9-BTEB	FW:TTACAGAGTGCATACAGGTGAACG REV:CTCGTCTGAGCGGGAGAACT
TRα1	FW: GCTGTGCTGCTAATGTCAACAGA REV:CGATCATGCGGAGGTCAGT
TRβ1	FW:CCAAGTTCCACACATGATTTAATGA REV:TGGACAGTGCTTCGGTTTGTC

Supplemental Table 2 Ct values (± SD) of KLF9-ENPP2-HR expression after 24h of incubation with 10 nM of T3 or T4.

	KLF9	ENPP2	HR
T3	26.98 (± 4.98)	26.68 (±2.39)	31.14 (± 2.72)
T4	28.02 (± 4.73)	27.19 (± 2.25)	32.14 (± 2.49)

Chapter 7

GENDER DIFFERENCES IN AGING-RELATED SUPPRESSION OF THYROID HORMONE SIGNALLING

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To be submitted

ABSTRACT

Background

Recently, we disclosed a novel link between aging and diminished TH signalling by demonstrating both decreased D1 activity and increased D3 activity in different models of premature aging, including *Csb*$^{m/m}$*/Xpa*$^{-/-}$ DKO mice and naturally aging mice. This may be a response to survive stressful conditions. It is well known that there are sex-differences in aging and lifespan, which are most likely based on complex associations between TH signalling, aging and possibly the influence of gonadal hormones. In this study we will therefore focus on gender-related differences in TH suppression in various models of aging.

Materials and Methods

In 15-days-old prematurely aging male and female *Csb*$^{m/m}$*/Xpa*$^{-/-}$ double knock-out (DKO) mice (maximum age: 21 days), age-matched controls and wild-type (WT) naturally aging male and female animals (13, 52 and 104-week-old), we quantified serum and tissue TH levels, liver and kidney D3 and D1 activities, and expression of hepatic T3 responsive genes.

Results

In the DKO mice, we observed lower serum and tissue TH levels, an increase in liver D3 activity and expression, associated with a decrease in D1 and a hypothyroid liver gene expression profile compared to age-matched controls. There was no difference between males and females. In livers of male naturally aged mice we observed increased D3 and decreased D1 activity in parallel with decreased hepatic TH content. However, in female naturally aging mice, we observed a trend towards an increased TH state over time, accompanied by a decrease in D3 activity and an increase in D1 activity.

Conclusion

In prematurely aging mice, TH signalling is equally attenuated during aging in both sexes. Naturally aging male mice showed similar changes as in accelerated aging, but naturally aging female mice displayed an opposite trend. TH signalling changed upon aging, although gender may contribute to a differential pattern in various models.

INTRODUCTION

Despite intense research, the molecular basis of the aging process is still unresolved. However, the causative role of stochastic DNA damage in aging is widely accepted (1). DNA is continuously damaged by chemicals like reactive oxygen species (ROS) produced by the body's own metabolism (2). A whole repertoire of DNA repair systems is present to counteract such damage and to preserve vital genetic information (3). Nevertheless, a fraction of DNA damage will escape repair, which consequently accumulates and ultimately will result in cellular dysfunction and abnormal DNA metabolism. This process triggers a survival response in which the energy equilibrium will shift from growth and proliferation to preservation (4). The causative role of DNA damage in aging is shown in humans with defective DNA repair, who have features of premature aging. Also, mice with various defects in the transcription-coupled nucleotide excision repair system (TC-NER), as models for human progeroid syndromes, show accelerated aging (5-7). These DNA repair deficient mice are used to model the normal aging process (8,9) and they show many true features of normal aging and, interestingly, display a dampening of the growth hormone (GH)/IGF1 axis.

Thyroid hormone (TH) is important for metabolism of almost all tissues and it is known for decades that TH signalling and aging are closely linked. The thyroid mainly produces the prohormone T4, which is converted to the bioactive form T3 in the peripheral tissues by the deiodinases D1 and D2. The deiodinase D3 degrades T4 and T3 to receptor-inactive metabolites (10,11). TH mediates its action by binding to nuclear T3 receptors (TRs) which regulate the transcription of target genes, thereby regulating body metabolism (12).

In humans, an age-dependent increase in serum thyroid stimulating hormone (TSH) and decrease in (free) T3 is observed, whereas (free) T4 remains unchanged (13-17) and some evidence suggests that in older individuals lowering of TH levels may be beneficial (18-20). The importance of endocrine physiology in aging and lifespan is also illustrated by dwarf mutant mice with pituitary dysfunction (suppressed GH and TH) resulting in a prolonged lifespan (21). In addition, the lifespan of mice carrying mutations in endocrine signalling pathways such as *Igf-receptor* KO or Klotho-overexpressing mice, is increased (22,23). Therefore, we recently explored the possible mechanisms underlying changes in TH signalling during aging using various accelerated aging TC-NER-deficient mice (including $Csb^{m/m}/Xpa^{-/-}$ DKO mice) and naturally aging wild-type (WT) mice (Visser et al. Submitted to PLoS One 2015). Diminished TH signalling was observed in normal and accelerated aging. Not only a decrease in D1 activity, but also a strong induction of D3 was observed in livers of naturally and accelerated aged animals.

It is well known that there are sex-differences in aging and lifespan, with women living longer than men and it is also known that TH levels are different in man and women throughout life (13). These differences are most likely based on complex associations between TH signalling, aging and possibly the influence of gonadal hormones (24-32). Furthermore, the females in $Csb^{m/m}/Xpa^{-/-}$ DKO group appeared to be in much better shape compared to the males, although their lifespan was not different. However, no studies have investigated gender differences in the attenuated TH state during aging. Therefore, in the present study we explored the possible influence of sex on TH homeostasis in different models of aging.

MATERIALS AND METHODS

Animals

The generation and characterization of TC-NER-deficient *Csb$^{m/m}$/Xpa$^{-/-}$* mice has been previously described (7). All mice were bred in a pure C57BL/6J genetic background. Animals were kept on a regular diet and housed at the Animal Resource Center (Erasmus University Medical Center) and the National Institute of Public Health and the Environment (RIVM), which operate in compliance with the "Animal Welfare Act" of the Dutch government, using the "Guide for the Care and Use of Laboratory Animals" as its standard. *Csb$^{m/m}$/Xpa$^{-/-}$* mice have a lifespan of 3-4 weeks. As required by Dutch law, formal permission to generate and use genetically modified animals was obtained from the responsible local and national authorities. All animal studies were approved by an independent Animal Ethical Committee (Dutch equivalent of the IACUC). All experiments were conducted in accordance with NIH and local animal care guidelines.

Serum and tissue T4 and T3 concentrations

Serum T4 and T3 levels were determined by radioimmunoassay as previously described (33). Tissue T4 and T3 content was measured by radioimmunoassay after extraction, as previously described (34).

Deiodinase activities

Tissues were homogenized on ice in 10 volumes of 0.1 M phosphate (pH 7.2), 2 mM EDTA, containing 1 mm DTT, using a Polytron (Kinematica, Lucerne, Switzerland). D1 activities were determined by incubation of 0.1 μM rT_3 (including 200,000 cpm [3',5'-^{125}I]rT_3) for 30 min at 37 C with 200x diluted tissue homogenate in 0.1 M phosphate (pH 7.2), 2 mM EDTA, 10 mm DTT (PED10). D3 activities were determined by incubation of 1 nM T_3 (including 200,000 cpm [3'-^{125}I] T_3) for 60 min at 37 C with 10x diluted tissue homogenate in 0.1 M PED10. After incubation, 100 μl of incubation medium was added to 125 μl ice-cold ethanol (pH 2.3) and incubated for 30 minutes on ice. After centrifugation, 125 μl supernatant was mixed with 100 μl 0.1% TFA in water. Metabolites were separated by UPLC as described previously for the HPLC method (35).

RT-qPCR

cDNA was synthesized using 1 μg RNA and Superscript™IIReverse Transcriptase (Life Technologies, Breda, The Netherlands). SYBR Green I (Eurogentec, Maastricht, The Netherlands) was used as detector dye for qRT-PCR of all genes. Primer sequences were previously provided (Visser et al. PLoS One 2015 under review). The housekeeping gene *RPS9* was used for normalization.

Statistical analysis

The results are the mean of at least N=4/group (males and females) and are expressed as mean ±SE. Statistical analysis was carried out in GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, California). All results in the accelerated aging *Csb$^{m/m}$/Xpa$^{-/-}$* DKO mice (serum and tissue TH levels and deiodinase activities) are presented relative to the WT males.

In naturally aging mice all data is presented relative to males age 13-weeks. This way we could compare gender and phenotype directly. Statistical significance of the differences between WT, DKO, male and female animals were determined using two-way ANOVA followed by Bonferroni post test. We used the Student's t-test for unpaired observations in conditions where we compared only WT *vs*. DKO samples (Supplemental Figure 1).

RESULTS

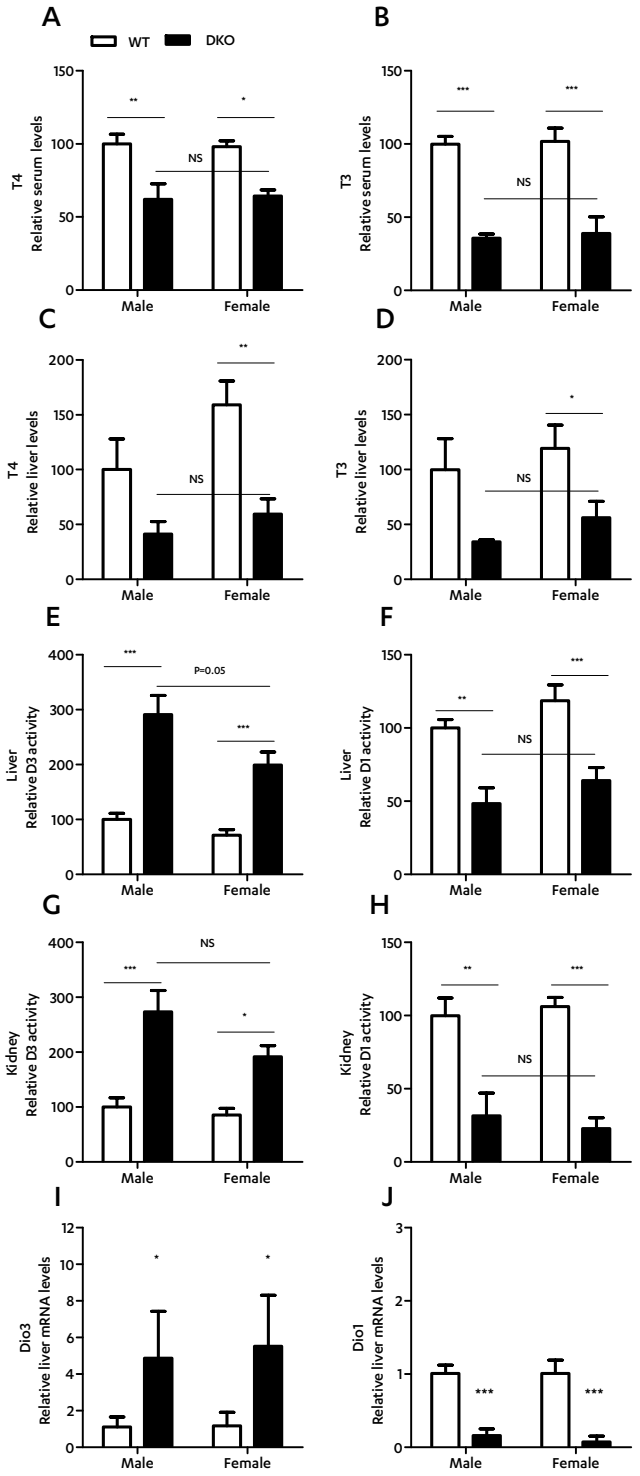
First, we confirmed our previous findings showing that D1 activity was decreased and D3 activity was increased in livers and kidneys of 15-day-old *Csb*$^{m/m}$*/Xpa*$^{-/-}$ DKO mice compared to age-matched controls (Visser et al. Submitted to PLoS One 2015) (Supplemental Figure 1). In this study, a new cohort of WT and DKO mice were used and all experiments were carried out again. Within the groups, the man and female samples were analyzed separately.

TH signalling in DKO (*Csb*$^{m/m}$*/Xpa*$^{-/-}$) mice

Next, we measured TH concentrations in serum samples of 15-day-old DKO male and female mice. The females in *Csb*$^{m/m}$*/Xpa*$^{-/-}$ DKO group appeared to be in much better shape compared to the males, although their lifespan was not different. Both T4 and T3 were significantly decreased to a similar extent in male (T4: WT 100±13%, DKO 62± 21% and T3: WT 100±11%, DKO 36± 6%) and female (T4: WT 98±5%, DKO 64± 8% and T3: WT 102±18%, DKO 39± 23%) mutant mice (Figure 1A,B). Representative approximate absolute serum, tissue and deiodinase values are presented in Supplemental Table 1. Because systemic TH levels not necessarily parallel peripheral tissue levels, we also measured T4 and T3 concentrations in liver samples (36). Both T4 and T3 liver content decreased in the DKO males (T4: WT 100±23%, DKO 41± 23% and T3: WT 100±23%, DKO 34± 4%) and females (T4: WT 160±43%, DKO 60± 28% and T3: WT 119±42%, DKO 56± 30%) although liver T4 and T3 levels were not significantly different in male and female DKO animals (Figure 1C,D). We observed a stronger decrease in liver T4 content in DKO females compared to males, as female control animals displayed higher T4 levels in the liver (Figure 1C). These effects occurred in the absence of obvious cachexia or illness in these DKO animals.

To evaluate peripheral deiodination, we measured D3 and D1 activities in liver and kidney tissue samples. Both liver and kidney D3 activities increased in the DKO mice. The increase in liver and kidney D3 activity was higher in male DKO animals (liver: WT 100±29%, DKO 291± 97%, kidney: WT 100±34%, DKO 274± 78%) compared to DKO females (liver: WT 73±28%, DKO 199± 60%, kidney: WT 85±24%, DKO 192± 40%) (Figure 1E,G). D1 activities in both tissues were decreased to a similar level in both male (liver: WT 100±15%, DKO 48± 30%, kidney: WT 100±15%, DKO 31± 31%) and female (liver: WT 119±33%, DKO 64± 18%, kidney: WT 106±13%, DKO 23± 14%) DKO mice (Figure 1F,H).

As a complementary approach, we measured mRNA levels of *Dio1* and *Dio3* using RT-qPCR. In line with the altered deiodinase activities, we observed a significant increase in the expression of *Dio3* and subsequently a significant decrease in *Dio1* in both male and female DKO mice (Figure 1I,J). These results suggested that the changes in D3 and D1 activities are mainly regulated at the transcriptional level.


A
WT
DKO
T4
Relative serum levels
B
T3
Relative serum levels
C
T4
Relative liver levels
D
T3
Relative liver levels
E
Liver
Relative D3 activity
P=0.05
F
Liver
Relative D1 activity
G
Kidney
Relative D3 activity
H
Kidney
Relative D1 activity
I
Dio3
Relative liver mRNA levels
J
Dio1
Relative liver mRNA levels
Male
Female
NS

Finally, we quantified a panel of known mouse T3-responsive genes in the liver. In general, most genes that are normally suppressed in hypothyroidism, such as *Thrsp, Fmo, Nudt7, Prodh, Slc22a7, Elov3* and *Stat5b,* showed a decreased expression in the DKO animals (Figure 2A,B). Subsequently, *Cyp4a10*, that is normally induced in hypothyroidism was higher expressed, which confirmed a decrease in TH signalling in these prematurely aging animals. In this set, all genes were equally changed in both male and female DKO mice (Figure 2A,B). Together, in the prematurely aging mice, TH signalling was attenuated to a similar extent in males and females.

TH signalling in naturally aging mice

Next, we studied 13, 52, and 104 - weeks-old WT animals as models for normal aging. Serum T4 levels were stable in both male (13-wk: 100±25%, 52-wk: 98±118%, 104-wk: 93±8%) and female (13-wk: 124±48%, 52-wk: 92±20%, 104-wk: 118±20%) mice over time (Figure 3A). Serum T3 levels were increased at 52 weeks in male mice and serum T3 levels were not different between 104-weeks-old male (13-wk: 100±38%, 52-wk: 188±36%, 104-wk: 137±30%) and female (13-wk: 126±39%, 52-wk: 106±31%, 104-wk: 154±41%) animals (Figure 3B).

Interestingly, while hepatic T4 and T3 content decreased in males (T4, 13-wk: 100±21%, 52-wk: 118±22%, 104-wk: 71±25% and T3, 13-wk: 100±28%, 52-wk: 81±5%, 104-wk: 51±12%) with increasing age, in females we observed an increase in both T4 (13-wk: 93±25%, 52-wk: 113±21%, 104-wk: 173±62%) and T3 (13-wk: 76±10%, 52-wk: 83±6%, 104-wk: 151±103%) levels (Figure 3C,D). The renal T4 and T3 levels remained the same in the naturally aging mice in both genders (Figure 3E,F).

Next, we studied D3 and D1 activities in liver samples. As expected, we observed an increase in D3 activity and a slight decrease in D1 activity in male (D3, 13-wk: 100±41%, 52-wk: 87±20%, 104-wk: 158±50% and D1, 13-wk: 100±33%, 52-wk: 101±19%, 104-wk: 74±51) normal aging animals (Figure 3G,H). In female livers, anticipated from the increase in T4 and T3 content, we observed an opposite trend, with significant decreased D3 (13-wk: 243±57%, 52-wk: 183±114%, 104-wk: 126±42) and significant increased D1 (13-wk: 108±14%, 52-wk: 138±39%, 104-wk: 187±54%) levels, when we compared the youngest with the oldest animals (Figure 3G,H). Similar opposite patterns in D1 activities were obtained in male (13-wk: 100±43%, 52-wk: 59±26%, 104-wk: 8±16%) and female (13-wk: 295±122%, 52-wk: 170±54%, 104-wk: 488±212%) kidney samples (Figure 3I). Kidney D3 activities could not be reliably assessed due to very low expression levels.

Expression levels of hepatic *Dio3* did not change in male naturally aging mice, whereas *Dio1* expression was only increased at 52-weeks (Figure 3J,K). As expected from our earlier results, *Dio3* expression decreased and *Dio1* mRNA increased over time in female naturally aging mice and these data suggested that the changes in D3 and D1 activities are mainly regulated at the transcriptional level (Figure 3J,K).

◄ **Figure 1 A-B** Serum T4 (A) and T3 (B) levels in 15-day-old WT and $Csb^{m/m}/Xpa^{-/-}$ male en female mice. **C-F** T4 (C), T3 (D) concentrations and D3 (E), D1 (F) activity in livers of 15-day-old WT and DKO male en female mice. **G-H** D3 (G) and D1 (H) activity in kidneys of 15-day-old WT and DKO male en female mice. Significance represents WT *versus* DKO in males or females and WT *versus* WT and DKO *versus* DKO in males or females. **I-J** D3 (I) and D1 (J) mRNA expression in livers of 15-day-old male and female WT and DKO mice. Significance represents WT *versus* DKO in males or females for the different genes. **P* <0 .05; ***P* <0.01; ****P* <0.001.

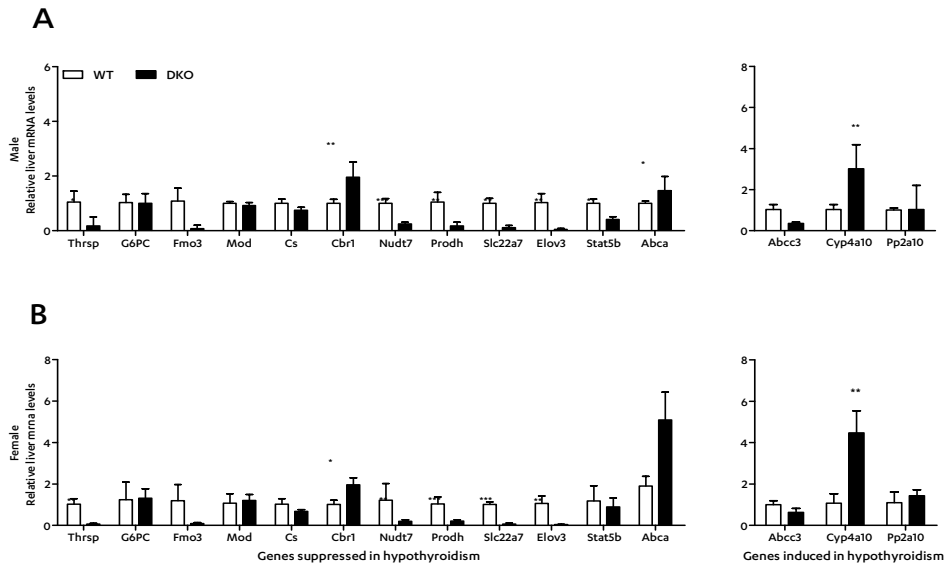


Figure 2 A-B Expression profiling of a set of known T3-responsive genes in 15-day-old DKO males (A) and females (B) compared to age-matched controls. Significance represents WT *versus* DKO in males or females for the different genes. **P* <0 .05; ***P* <0.01; ****P* <0.001.

Finally, we profiled known mouse T3-responsive genes in the livers of naturally aging mice. In females we observed an increase in *Thrsp* and *Mup2*, as expected from the increased liver and kidney T4 content and decreased D3 liver activity (Figure 4B). In males *Cyp4a10* and *Abcc3* increased, which are genes that are normally induced during hypothyroidism, which confirmed a hypothyroid state in male naturally aging mice (Figure 4A).

DISCUSSION

Although the intimate link between thyroid parameters and aging is known for many years, the underlying mechanisms are poorly understood. Recently, we disclosed a novel link between aging and TH signalling by demonstrating both decreased D1 activity and increased D3 activity in different models of premature aging, including $Csb^{m/m}/Xpa^{-/-}$ DKO mice and naturally aging mice (Visser et al. Submitted to PLoS One 2015). The following scenario was presented to explain these changes. Persistent DNA damage triggers a general stress response including induction of D3, which prevents T4 from being activated and will decrease T3 concentrations. This will diminish TH action, which was confirmed by attenuated T3 responsive genes in the liver transcriptome. It has been hypothesized that these mechanisms could be beneficial in terms of aging and clinicians should be very careful to treat older subjects with subclinical hypothyroidism.

It is well known that there are sex-differences in aging and lifespan, with women living longer than men and it is also known that TH levels are different in man and women throughout life (13). These differences are most likely based on complex associations between TH signalling, aging

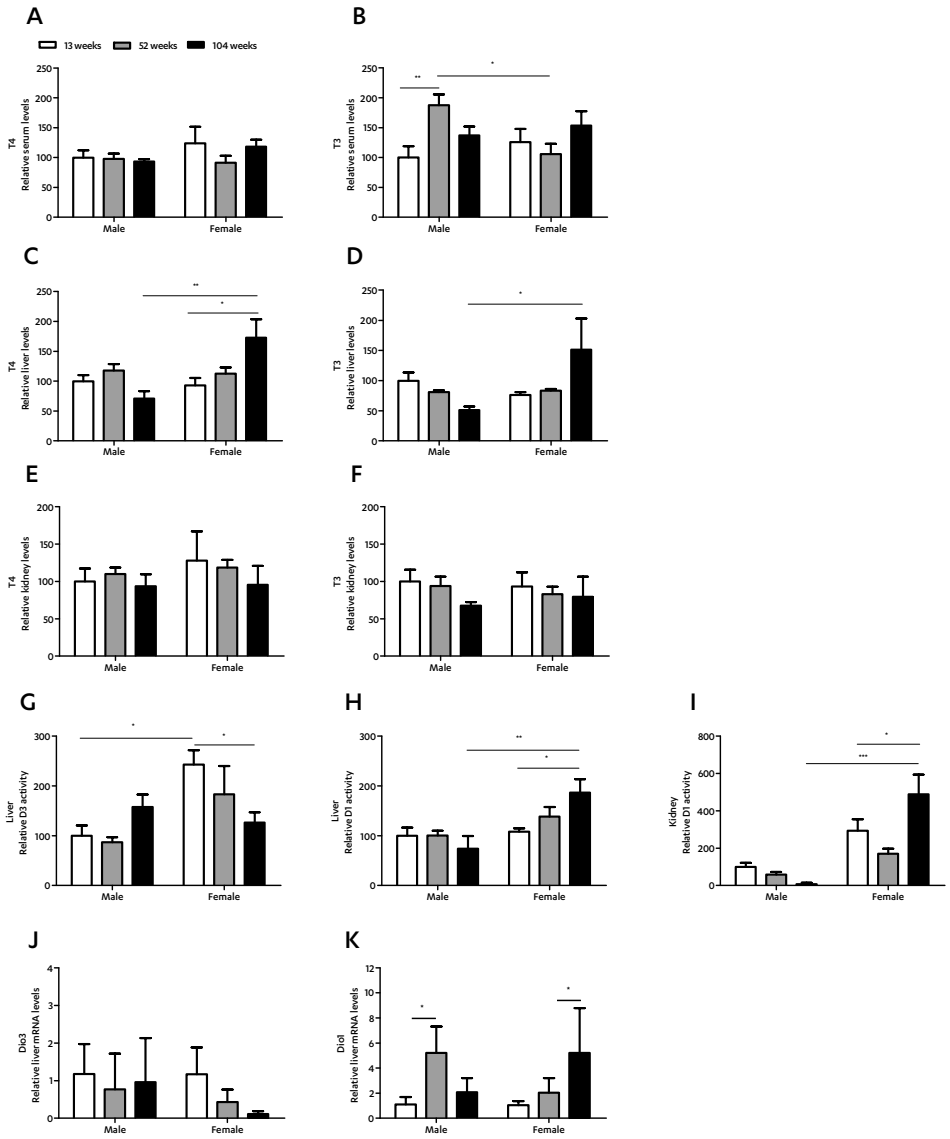


Figure 3 A-B Serum T4 (A) and T3 (B) levels in 13-, 52-, and 104-week-old naturally aging male en female mice. **C-F** T4 (C), T3 (D) concentrations in livers and T4 (E), T3 (F) concentrations in kidneys of 13-, 52-, and 104-week-old naturally aging male en female mice. **G-I** D3 (G) and D1 (H) activity in livers and D1(I) activity in kidneys of 13-, 52-, and 104-week-old naturally aging male en female mice. Significance represents the difference between the various age categories in males and in females separately and the difference in age categories between males and females. **J-K** D3(J) and D1 (K) mRNA expression in livers of 13-, 52-, and 104-week-old naturally aging male and female mice. Significance represents the difference between the various age categories in males and in females separately for the different genes. **P* <0 .05; ***P* <0.01; ****P* <0.001.

and possibly the influence of gonadal hormones (24-32). However, no studies have explored if sex-differences exist in changed TH signalling during aging. In the present study, we investigated TH signalling with a focus on liver and kidney of male and female prematurely aging (15-days-old) *Csb*$^{m/m}$/*Xpa*$^{-/-}$ DKO mice as well as naturally aging (13, 52 and 104-weeks-old) mice.

The findings in the DKO mice perfectly replicated previous findings (Visser et al. Submitted to PLoS One 2015). Thus, decreased TH levels in serum and liver as well as increased hepatic D3 and decreased hepatic D1 expression and activity were observed in mutant mice of both genders. The lifespan in males and females was not different and it is important to note that these effects occurred in the absence of obvious cachexia or illness in the DKO group.

Identical experiments were performed in naturally aging 13, 52 and 104-weeks-old WT animals. In male samples we confirmed the changes in TH state and metabolism reported previously, although less pronounced than in DKO mice. Thyroid state appeared to be regulated differently in naturally aging females than in naturally aging male WT mice, and male and female mutant mice. We observed a trend towards higher TH serum and tissue levels paralleled by a significant decrease in D3 and increase in D1 expression and activity in older females (Figure 3).

Both male and female prematurely aging and male naturally aging mice showed suppressed TH signaling. However, female normal aging mice showed a trend in opposite direction. In our previous study, findings in naturally aged mice mimicked the results in the prematurely aged animals. However, the aging WT group only consisted of male animals. There are several possible explanations for the observation that TH regulation in female WT aging mice is different from male WT aging mice and from *Csb*$^{m/m}$/*Xpa*$^{-/-}$ mice.

First, it is well known that males and females can respond differently to exogenous stressors like pain and caloric restriction (37-39). In aging male rats, decreased TH signalling and D1 activity was observed (40), while Correa da Costa *et al*. showed significantly decreased

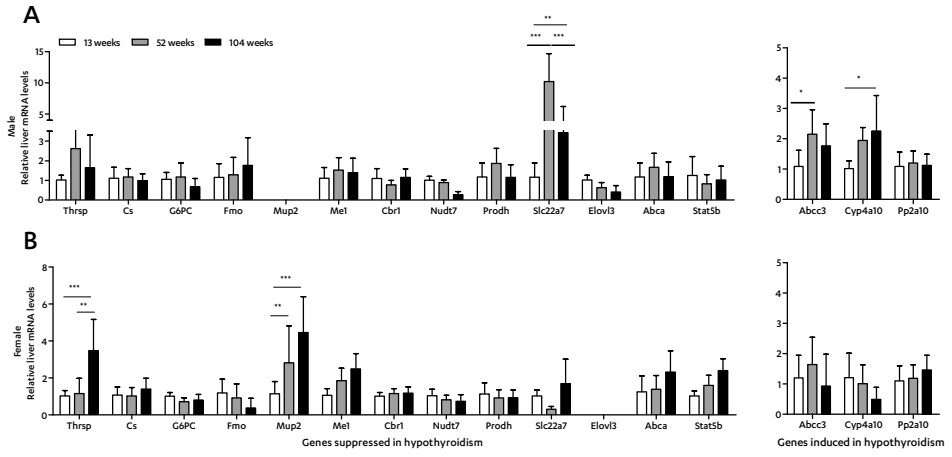


Figure 4 A-B Expression profiling of a set of known T3-responsive genes in 13-, 52-, and 104-week-old naturally aging males (A) and females (B). Significance represents the difference between the various age categories in males and in females separately for the different genes. **P* <0 .05; ***P* <0.01; ****P* <0.001.

T3 levels in male and significant increased liver D1 activity in female aging rats (41). The opposite effects on TH state in male *versus* female naturally aging mice could be due to the influence of sex hormones. In literature the influence of sex steroids on TH function is under debate. Some studies show that liver D1 activity in male rats is decreased after castration and increased after administration of testosterone (24,28). On the other hand, the data on female liver D1 activity after ovariectomy and estradiol administration is controversial (27,28,30,31). Future studies should reveal the complex associations between TH signalling, aging and the influence of gonadal hormones and their binding globulines (24-32).

Second, aging is severely accelerated in the $Csb^{m/m}/Xpa^{-/-}$ mice. The lifespan of these animals could be too short to result in differences between male and female mice which are normally seen during the WT aging process. Also, aging-related pathological processes appear much more severe in the mutant animals than in naturally aging animals, and this may overwhelm sex-related differences in TH homeostasis during normal aging.

Third, the oldest naturally aging mice we used in our studies are 104-weeks-old, although mice have a lifespan of up to three years. It is possible that older (e.g. 130-weeks) female mice resemble the attenuated TH state in old male mice. However, 130-week-old mice are a heavily selected subpopulation and any results in this group should be interpreted with care.

Fourth, whether normal male or female mice live longer could be dependent on the genetic background of the animals. The suppressed thyreotropic axis in male WT mice is congruent with the observation that in WT C57BL/6J mice males outlive the females, although there could be other explanations as well.

Altogether, our data confirmed attenuated TH signalling in $Csb^{m/m}/Xpa^{-/-}$ and naturally aging male mice. However, gender influenced the changes in TH signalling during aging, as we observed opposite trends in male and female naturally aging mice. This reinforced the notion that animal studies in one sex should be interpreted with caution and not extrapolated easily to the other sex. Furthermore, it has been shown before, that there are similarities and differences in the human and rodent model for aging (42), which suggest that extrapolating our data to the human situation would be challenging.

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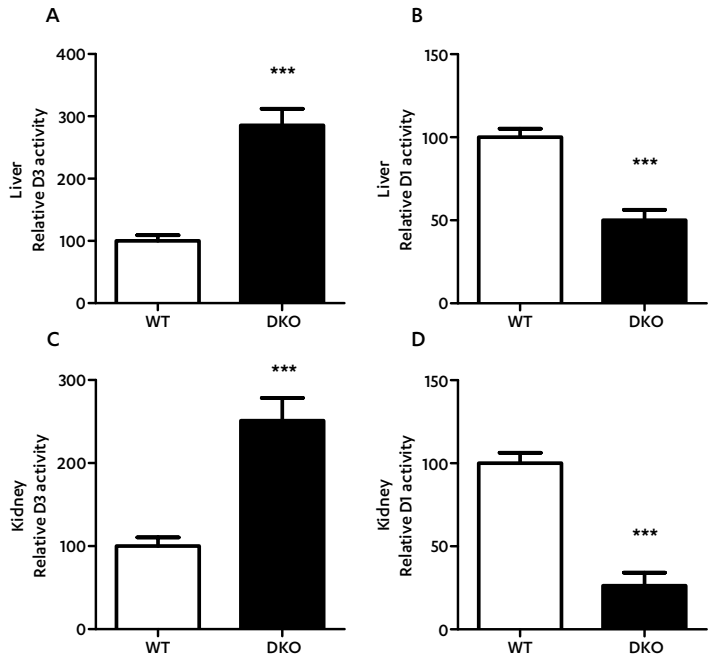

Supplemental Figure 1 A-D D3 (A) and D1 (B) activity in livers and D3 (C) and D1 (D) activity in kidneys of 15-day-old WT and DKO mice. Significance represents WT *versus* DKO. *P <0 .05; **P <0.01; ***P <0.001.

Supplemental Table 1 Representative approximate absolute serum, tissue and deiodinase values

Prematurely aging animals	**Male WT 15-days-old**
T4 serum	~100 nmol/L
T3 serum	~1.5 nmol/L
T4 liver	~35 pmol/g
T3 liver	~6 pmol/g
D3 liver	~3 fmol/mg/min
D1 liver	~15 pmol/mg/min
D3 kidney	~0.4 fmol/mg/min
D1 kidney	~15 pmol/mg/min
Normally aging animals	**Male 13 weeks old**
T4 serum	~40 nmol/L
T3 serum	~0.7 nmol/L
T4 liver	~30 pmol/g
T3 liver	~7 pmol/g
T4 kidney	~15 pmol/g
T3 kidney	~5 pmol/g
D3 liver	~0.2 fmol/mg/min
D1 liver	~14 pmol/mg/min
D1 kidney	~3 pmol/mg/min

Chapter 8

ASSOCIATION OF ANTIEPILEPTIC DRUG USAGE, TRACE ELEMENTS AND THYROID HORMONE STATUS

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ABSTRACT

Background

Sufficient levels of thyroid hormone (TH) and trace elements (copper (Cu), selenium (Se)) are important for normal development of the brain, while abnormalities can result in neurocognitive impairment. The use of antiepileptic drugs (AEDs) has also been shown to influence serum TH and trace element levels. However, the relationship between AEDs, THs and trace elements has not yet been studied directly. We explored the interaction between AEDs with TH and trace element levels.

Methods

898 participants from the Thyroid Origin of Psychomotor Retardation (TOP-R) study, a nation-wide cohort study in the Netherlands, designed to investigate thyroid parameters in subjects with intellectual disability (ID), had data available on serum Se, Cu, TSH, FT4, T3, reverse T3, T4 and thyroxine binding globuline (TBG) and 401 subjects were on AED treatment. Participants that used thyroid interfering medication (N=40) and/or that were TPOAb positive (N=24) were excluded. Differences in trace elements according to medication usage was investigated using ANOVA, associations between trace elements and thyroid parameters were analysed using (non-)linear regression models.

Results

Study participants were not deficient in any of the trace elements analyzed. AED (carbamazepine, valproate, phenytoin) usage was negatively associated with serum Se and showed compound-specific associations with Cu levels. After correction for drug usage, Se was positively associated with TSH levels (β±SE; 0.0008 ±0.0003, *P*=0.03), negatively associated with FT4 levels (-0.0212 ±0.0065, *P*=0.001) and positively with T3 levels (0.0028 ±0.0005, *P*<0.001). There was a positive association between Cu and T4, T3 and rT3, which was largely dependent on TBG levels (0.0022 ±0.0002, *P*<0.001).

Conclusions

The subjects with ID did not display profound deficiencies in TH or trace element levels. Commonly prescribed AEDs were associated with serum Se and Cu levels, while serum Se and Cu were also associated with thyroid parameters. Further studies on the underlying mechanisms and potential clinical importance are warranted.

INTRODUCTION

Thyroid hormone (TH) is important for the normal development and energy metabolism of almost all tissues (1). The thyroid gland pre-dominantly secretes the inactive pro-hormone T4, which is locally converted to the active form T3 by selenium (Se)-dependent enzymes of the family of iodothyronine deiodinases (2). T3 mediates its major effects by binding to nuclear TH receptors (TRs) which regulate the transcription of target genes (3). Abnormalities in TH status are commonly found among endocrine disorders (4) at varying prevalences according to the group of patients that is being studied (5,6).

Essential trace elements, like Se and copper (Cu) are micronutrients that are present in low concentrations in the human body and are dependent on a sufficiently high dietary intake. Trace elements are essential for many enzymatic reactions and are therefore important for proper functioning of biochemical pathways and the endocrine system (7).

Sufficient levels of both TH and trace elements are important for normal development of the brain (8). Abnormalities in TH signalling can give rise to neurocognitive impairment, as illustrated by subjects with mutations in the gene encoding the TH transporter monocarboxylate transporter 8 (*MCT8*) (9,10) or in the TH receptor *THRA* (encoding TRα) (11-14). Adequate levels of Cu are also important for normal development of the brain, as Cu is important in energy metabolism, anti-oxidative defence and the production of neurotransmitters (15). Also, it was shown that TH or Cu deficiencies resulted in similar defects during rodent cerebral cortical development (16). The brain appears to preserve Se levels during Se shortage, as rats on a Se deficient diet did not display any neurological symptoms, because of only slightly reduced brain Se levels (17). However, mice lacking Selenoprotein P (SelP), which is an important Se carrier, showed very low brain Se levels together with movement disorders and occasional seizures (18).

Only few data on the relationship between TH and Se and Cu is currently available. It was shown in rats, that adequate Se in nutrition, supports TH synthesis and metabolism and protects the thyroid gland from damage by excessive exposure to reactive chemicals (19). Furthermore, in humans, high levels of Cu were associated with higher levels of both T3 and T4 (20). As a corollary, Bastian *et al*. showed in different rat studies that fetal deficiency of Cu resulted in impaired TH-regulated brain gene expression (21-23). While these studies suggested an interaction of the trace elements Se and Cu and TH during brain development, its relevance for humans is unknown.

In the Thyroid Origin of Psychomotor Retardation (TOP-R) study, thyroid parameters of subjects with intellectual disability (ID) were extensively profiled (24). In this cohort it has been shown that TH profiles in subjects without antiepileptic drugs (AEDs) were comparable with the general population. However, AEDs were strongly associated with decreased T4, FT4, T3 and rT3 levels which is in agreement with other studies (25-27). It has been speculated that the changed thyroid parameters in patients that use AEDs can be explained by an influence of AEDs on binding proteins (28), a stimulation of hepatic degradation or conjugation of TH (29) or an altered peripheral deiodinase activity (24). Interestingly, AEDs have been associated with changes in serum levels of Se and Cu (24,30-34). However, the complex relationship between AEDs, THs, Se and Cu has never been directly studied.

We used the TOP-R study to analyze the associations between AEDs, Se, Cu, and thyroid parameters.

MATERIALS AND METHODS

TOP-R Study

The TOP-R study is a nation-wide cohort study in the Netherlands, which was designed to investigate thyroid parameters in subjects with intellectual disability (ID). The study population has been described in detail before (24). The study was approved by the medical ethics committee of the Erasmus University Medical Centre. Written informed consent was obtained from the legally authorized representatives. Within the TOP-R cohort, data on thyroid parameters and trace elements was available in 898 out of 946 subjects of which additional information on thyroid medication usage (antithyroid drugs and L-thyroxine replacement therapy) was available in 806 subjects and on AEDs usage in 786 subjects. Full case analyses according to this data did not change the results (data not shown). After exclusion of subjects that used thyroid interfering medication (N=40) and/or TPOAb positive individuals (cut-off >60 IU/ml; N=24), 834 subjects were included in one or more analyses.

Measurements of thyroid status, Se and Cu

Serum samples were stored frozen at −20 °C. Serum T4, FT4, and TSH were measured by chemiluminescence assays (Vitros ECI Immunodiagnostic System; Ortho-Clinical Diagnostics Inc., Rochester, NY, USA). T3 was measured using an in-house RIA and rT3 using a commercial RIA (Immunodiagnostic Systems, Scottsdale, AZ, USA). TBG and TPO-antibodies were determined by immunoassay (Immulite 2000, Siemens, Breda, the Netherlands). Se and Cu concentrations were determined by total reflection X-ray fluorescence (TXRF) spectroscopy (35). The method was validated with a Seronorm standard (Sero AS, Billingstad, Norway). Briefly, all samples were diluted 1:1 in a Gallium-containing solvent for standardization. The analysis was performed in duplicate and the results of each sample differed by less than 20% for both Se and Cu. In every measurement run, a human control serum was included, allowing the calculation of an intra-assay coefficient of variation of 7% (Se) and 8% (Cu) and an inter-assay coefficient of variation of 12% (Se), and 18% (Cu), respectively.

Statistical analyses

To satisfy model assumptions, TSH levels were logarithmically transformed. The association between trace elements and thyroid parameters was investigated using ordinary least squares linear regression models with restricted cubic splines utilizing three to five knots. Figures show back-transformed axis values for TSH and all associations were adjusted for sex, age and relevant medication usage (covariates set to mean levels or most appearing category). The analyses were adjusted for medication usage when a drug was associated with a thyroid function parameter in a backward linear regression analysis, utilizing a cut-off of $P<0.15$. In addition, a variable for missing data on AED usage was added but did not reach the threshold for any thyroid parameters.

We investigated differences in the ratio of T3 to rT3 using linear regression analyses with a product term of the independent thyroid function parameter and trace element. Subsequently, to allow for non-linear associations/interactions, a sensitivity analysis was performed by adding quadratic terms and/or a product term of the trace element or thyroid function variables, these

were maintained according to *P*-values or changes in R^2. Interaction figures show the associations between thyroid function parameters according to low (red line with 95% CI) or high (blue line with 95% CI) trace element values (rounded number of 10th percentile or 90th percentile, respectively).

All statistical analyses were performed using R Statistical Software v 3.03 (1) (package rms or visreg) or Statistical Package of Social Sciences version 21.0 for Windows (IBM SPSS statistics, Chicago, IL, USA).

RESULTS

Descriptive statistics of the study population are shown in Supplemental Table 1. First, we studied the association between AED usage and serum Se and Cu levels (Figure 1). Carbamazepine or valproate usage was associated with lower serum Se levels (Figure 1A, 1B). Usage of carbamazepine or phenytoin was associated with higher serum Cu levels and valproate usage with lower serum Cu levels (Figure 1C, 1D, 1E). Other AED usage was not associated with serum Se or Cu levels, and there was no association between the daily dosage of AEDs and Se, Cu or thyroid hormone levels (data not shown). Thus, commonly prescribed AEDs affect serum Se and Cu levels in a compound-specific way.

Next, we studied associations between serum Se and Cu levels and thyroid parameters. The associations between serum Se levels and thyroid parameters are shown in Figure 2. After correction for drug usage, Se was positively associated with TSH levels (β±SE; 0.0008 ±0.0003, *P*=0.03), negatively associated with FT4 levels (linearly for Se below 125: -0.0212 ±0.0065, *P*=0.001) and positively with T3 levels (linearly for Se below 125: 0.0028 ±0.0005, *P*<0.001; Figure 2B, 2C). Since all three deiodinases are selenoproteins, we also investigated possible effects of trace elements on peripheral deiodinase activity. As a proxy for peripheral TH deiodination, we studied the association between serum Se, Cu and T3/rT3 ratio. Serum Se was positively associated with the T3/rT3 ratio (0.0122 ±0.0036, *P*<0.001; Supplemental Figure 1A).

Next, the associations between serum Cu levels and thyroid parameters were studied. Cu levels were positively associated withT3, rT3 and T4 levels (Figure 3A, 3B, 3C). Also, a particularly strong association was observed between Cu and thyroxine-binding globulin (TBG) (0.0022 ±0.0002; *P*<0.001; Figure 3F). As estrogens may influence TBG levels, we also investigated sex differences. The association between Cu and TBG was stronger amongst women, as compared to men (*P*interaction=0.0007; Supplemental Figure 2). After correction for TBG, the positive associations between serum Cu and T3, rT3 and T4 levels disappeared (Supplemental Figure 3). Serum Cu levels were not associated with changes in the T3/rT3 ratio (Supplemental Figure 1B).

DISCUSSION

TH and the trace elements Se and Cu are important for normal neurocognitive development and abnormal brain development may increase AED usage. The relationship between TH, Se, Cu and AED is currently unclear. In this study, we analyzed this relationship in the TOP-R cohort, in which Se and Cu, as well as thyroid parameters were determined. Our analyses indicated

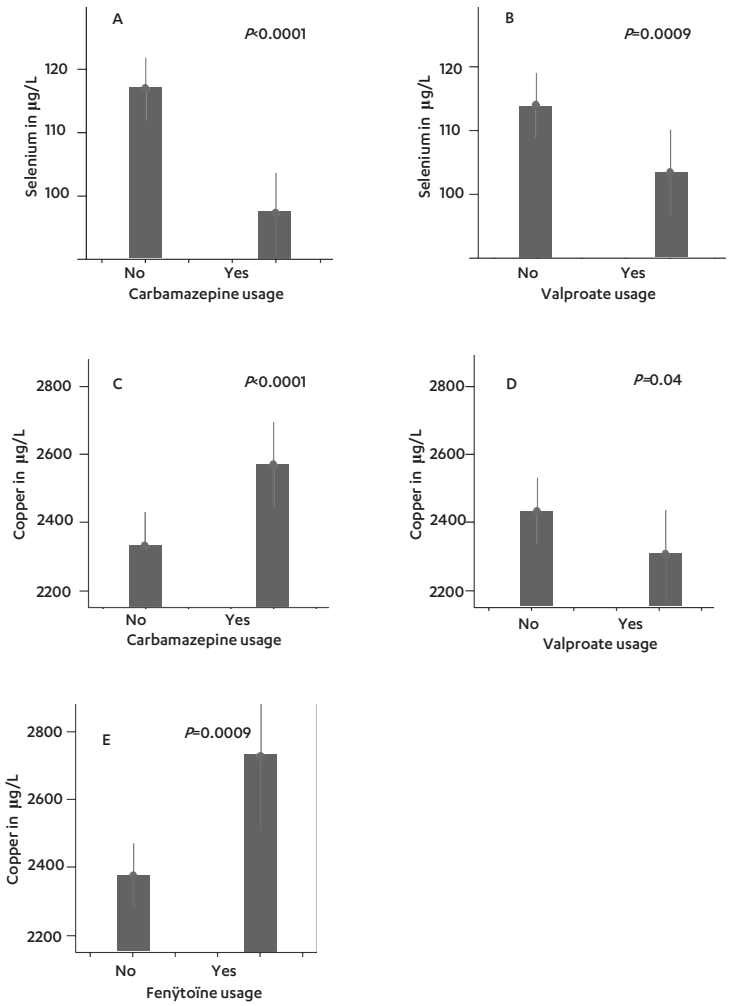

Figure 1 Graphs show the association between AED usage and serum selenium or copper and as predicted mean (gray dot) and 95% confidence interval (gray line). In addition, there was no dose dependent effect of AED usage for Figure A-E (*P*=0.16, *P*=0.32, *P*=0.57, *P*=0.40 and *P*=0.62, respectively). All analyses were performed in subjects with AED usage data available after exclusion of subjects with thyroid medication usage or TPOAb positivity and were adjusted for sex and age.

that serum Se levels were associated with TSH, FT4 and T3 levels, while serum Cu levels were associated with T3, rT3 and T4, via changes in TBG levels.

In this cohort of patients with ID, Se levels were significantly lower in patients that use valproate or carbamazepine, whereas phenytoin and carbamazepine usage was associated with increased Cu levels (Figure 1). Many studies described effects of AEDs on trace element levels like Se and Cu, although the effects of different classes of AEDs were neither consistent in magnitude nor in direction (30-33,36-38). There are several potential reasons for these inconsistencies,

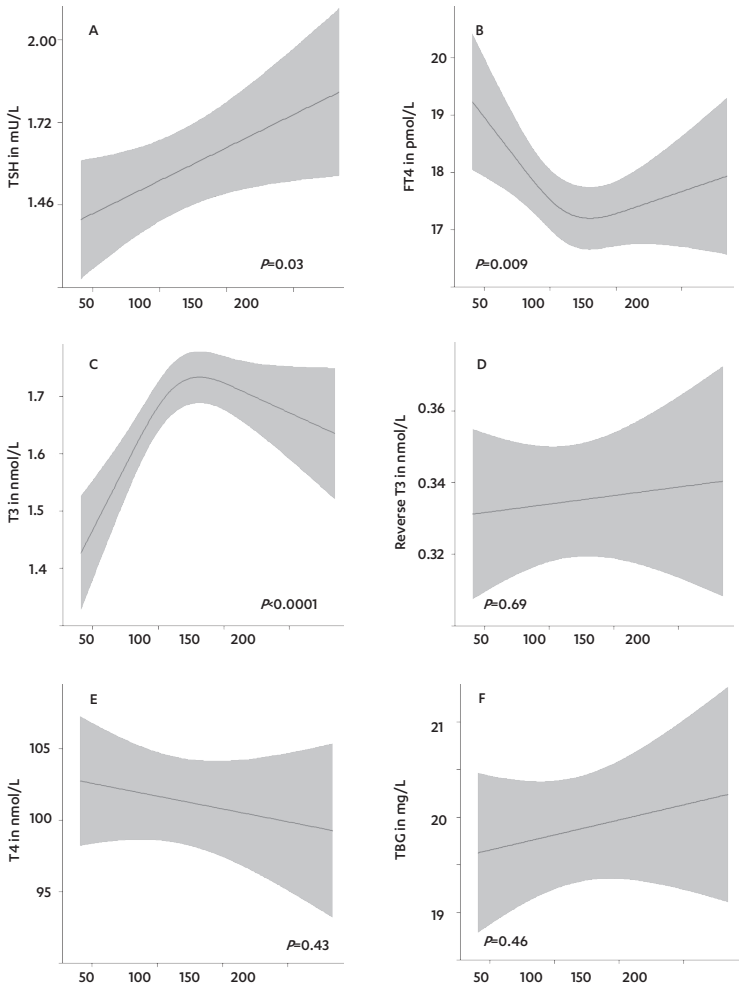

Figure 2 Graphs show the association between serum selenium and thyroid function parameters as predicted mean (blue line) and 95% confidence interval (grey are). All analyses were performed after exclusion of subjects with thyroid medication usage or TPOAb positivity and were adjusted for sex, age and antiepileptic drug usage and TBG levels.

ranging from dosage and duration of AED usage, age, health and nutritional status of the patients to baseline trace element concentrations which differ profoundly in different geographical areas as well as the small number of subjects studied (39). Due to the observational nature of this study, it is very difficult to draw any conclusions on the causative or mechanistic features of these results. It is very well possible, although speculative, that AEDs interfere with the transport, metabolism or excretion of trace elements, as is described for TH (24-29). Furthermore although, the generalizability of our findings may be limited, it is important to be aware of confounders if Se or Cu levels are analyzed in subjects on commonly prescribed AEDs. Depending on the baseline level of the population, this may cause a physiologically meaningful disbalance.

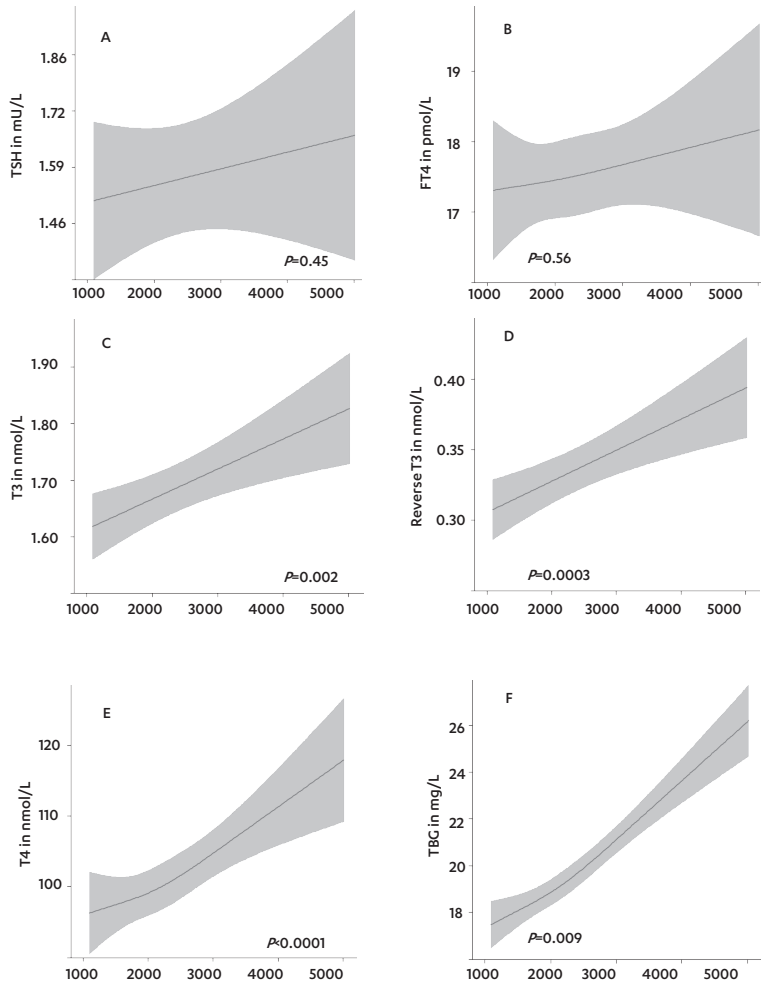

Figure 3 Graphs show the association between serum copper and thyroid function parameters as predicted mean (blue line) and 95% confidence interval (grey are). All analyses were performed after exclusion of subjects with thyroid medication usage or TPOAb positivity and were adjusted for sex, age and antiepileptic drug usage, but not for TBG levels.

Se is a trace element that is incorporated in selenocysteine, which is required for the normal production of selenoproteins (40-42). In the last decades, accumulating evidence has shown that Se plays an essential role in TH biosynthesis and metabolism as well as in normal thyroid function (43,44). The thyroid gland contains the highest Se concentration among human tissues due to the expression of several selenoproteins that are important in the maintainance of normal TH metabolism (deiodinases) and the protection of thyroid cells against oxidative damage such as glutathione peroxidases (44,45). If Se is limiting, lower levels of selenoproteins are synthesized which may potentially disturb peroxide-dependent iodination of thyroglobulin in the thyroid, thyrocyte defence systems and TH activation and inactivation (19,42,46). In this study, we showed

that serum Se was positively associated with TSH, inversely associated with FT4, and positively associated with T3 levels and the T3/rT3 ratio, as a proxy for peripheral deiodination (Figure 2, Supplemental Figure 1). This is also reminiscent of the constellation of critical illness, where a Se deficit often parallels a low T3 syndrome. These results are in line with several studies in rats, in which Se deficiency decreased liver and kidney D1 activities in combination with modest alterations in thyroid parameters (increased T4 and decreased T3 levels) (47-51). However, the majority of studies with human subjects on the interaction of Se and TH yielded inconsistent results (52). Our results indicated that even low-normal Se levels may already impact negatively on the TH state. However, net effects of limiting Se availability for the expression of the three deiodinase isoenzymes in different human tissues is difficult to predict, as hierarchical principles control the expression of the selenoenzymes with organ-specific preferences (53).

Cu is necessary in many metabolic processes and plays an important role in endogenous anti-oxidative defence mechanisms (54). Studies of Bastian *et al.* in mice showed that low levels of Cu were associated with a decreased TH state, which interfered with normal brain development (21-23). Similarly, serum ceruloplasmin and Cu levels have recently been opposed as direct biomarkers of TH signalling (55). In line with these studies, we observed strong positive associations between serum Cu and T3, rT3 and T4 levels (Figure 3). However, we were first to describe that these effects are totally driven by TBG (Supplemental Figure 3). Indeed, FT4 levels are not affected by Cu state. After correction for TBG, TH parameters were not significantly associated with serum Cu anymore, suggesting that the effects of Cu on thyroid function are mediated via changes in TBG. This may be explained by the fact that Cu may directly affect hepatic TBG expression, secretion or turnover. TBG is the main transport protein for TH in serum and many substances are known to influence TBG concentrations, such as estrogens, androgens, glucocorticoids and heroin (56,57). Most extensively studied are estrogens, which increased TBG levels by slowing its clearance via the liver and therefore increasing its half-life (56). In addition, estrogens have also been shown to increase serum Cu levels, which is most likely driven by an increase in ceruloplasmin, the transport protein of Cu (58-62). The association of Cu with TBG was stronger in women, which is consistent with a positive effect of Cu on TBG levels via estrogens, although this did not fully explain our observations.

Our study has strengths and limitations. The strength of this study is that the TOP-R cohort contains one the largest number of subjects with available data on serum Se, Cu, extensive profiling of TH parameters and commonly prescribed AEDs. It is important to note that the generalizability of this study is limited, as the patients in the cohort are all diagnosed with unexplained ID. Another potential limitation is the observational nature of the study, which precludes the analysis of causality of the detected associations. Still, many of our findings itself, and the direction of causality are supported by results from *in vivo* animal studies.

Together, our analyses indicate that the commonly prescribed AEDs carbamazepine and valproate affect serum Se and Cu levels. Furthermore, while Se levels may partially affect TH signalling via modifying the expression of deiodinases, the effects of Cu on thyroid parameters may be primarily driven by its effects on TBG. Future research is needed to explore the underlying mechanisms of the observed associations in the current study and to investigate to what extent trace elements are a risk factor for the development of thyroid diseases.

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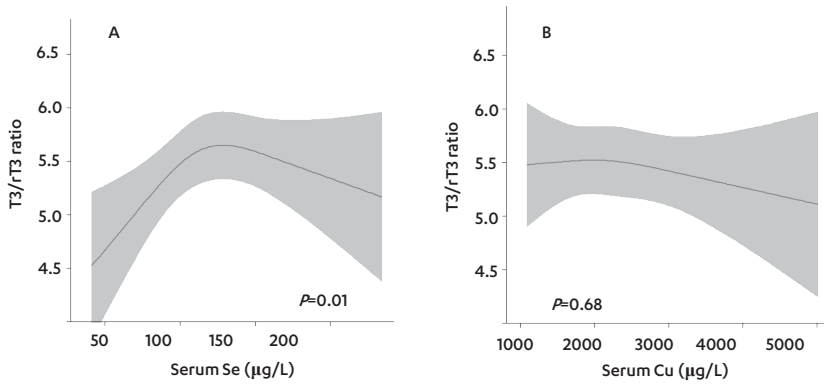
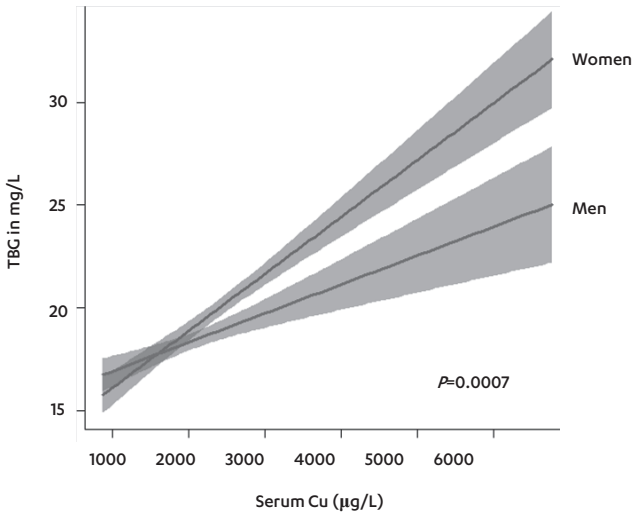
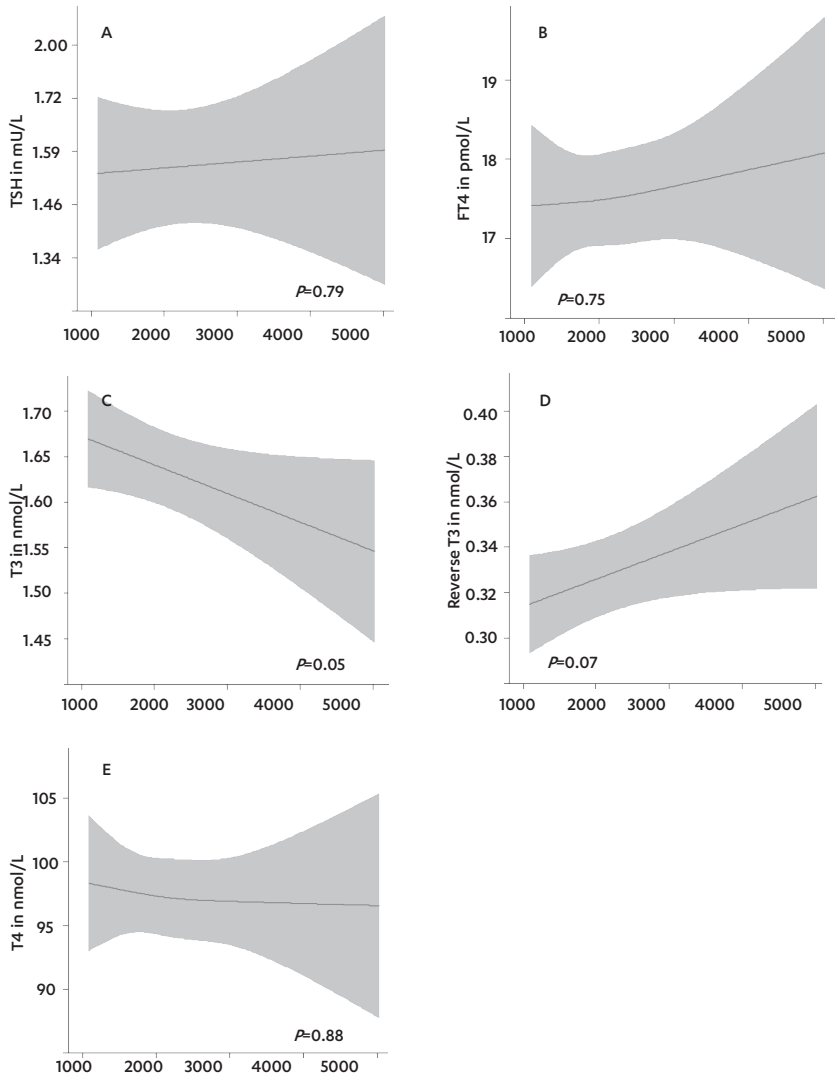


Supplemental Figure 1 Graphs show the association between serum trace elements and the ratio of T3/reverse T3 as predicted mean (blue line) and 95% confidence interval (grey are). All analyses were performed after exclusion of subjects with thyroid medication usage or TPOAb positivity and were adjusted for sex, age and antiepileptic drug usage.



Supplemental Figure 2 Graphs show the association between serum copper and TBG stratified by sex as predicted mean (blue or red line) and 95% confidence interval (blue or red are). All analyses were performed after exclusion of subjects with thyroid medication usage or TPOAb positivity and were adjusted for age and antiepileptic drug usage.



Supplemental Figure 3 Graphs show the association between serum copper and thyroid function parameters as predicted mean (blue line) and 95% confidence interval (grey are). All analyses were performed after exclusion of subjects with thyroid medication usage or TPOAb positivity and were adjusted for sex, age and antiepileptic drug usage and TBG levels.

Supplemental Table 1 Descriptive statistics of 834 subjects from the TOP-R cohort.

	Median or percentage	(95% range)
Age (years)	48	(16- 75)
Gender (%men)	54.7	
TSH (mU/L)	1.57	(0.36 – 5.31)
FT4 (pmol/L)	16.7	(10.4 - 27.4)
T3 (nmol/L)	1.64	(1.01 – 2.45)
rT3 (nmol/L)	0.30	(0.13 – 0.61)
T4 (nmol/L)	91	(51 – 144)
TBG (mg/L)	18.2	(11.7 – 29.5)
Selenium (µg/L)	111	(58 – 199)
Copper (µg/L)	2085	(1194 – 3951)
Carbamazepine (% users)	20.4	
Valproate "	19.8	
Fenobarbital "	7.0	
Fenytoïne "	5.3	
Lamotrigine "	4.7	
Frisium "	2.4	
Keppra "	2.3	
Rivotril "	1.7	
Oxcarbamazepin "	1.3	
Topirate "	1.1	
Vigabitrine "	0.6	
Gabapentin "	0.2	
Unknown "	17.6	

Descriptive statistics of study population are shown after exclusion of individuals that used thyroid interfering medication (N=40) or TPOAb positive individuals (cut-off >60 IU/ml; N=24).

Chapter 9

GENERAL DISCUSSION

CELLULAR MECHANISMS OF THYROID HORMONE HOMEOSTASIS

The title of this thesis covers the whole world of local thyroid hormone (TH) transport, metabolism and action. Normal TH homeostasis is dependent on the intracellular availability of TH, as the genomic actions of TH are mediated by nuclear TH receptors (TRs) bound to promoter elements of TH responsive genes. The intracellular availability of TH, is dependent on adequate function of transporter proteins and deiodinating enzymes that convert T4 to the active form T3, as well as degradation of T3. Although we know that all described key players are important for normal TH homeostasis, many issues are still unresolved. The work in this thesis presents studies in which key players of TH homeostasis, in health and disease, are investigated. First, we discussed the impact of genetic variation in the type 2 deiodinase (D2). Next, new insights in the TH transport characteristics of the L-type amino acid transporters (LAT) 1-5 and monocarboxylate transporters (MCT) 8 and 10 were described. After that we discussed the ability of T4 to stimulate transcription of TH responsive genes and we explored gender differences in the age-related suppression of TH signalling. Finally, the associations between TH and trace elements were discussed. In this chapter, the relevance of the performed studies will be discussed and future research strategies will be explored.

Genetic variation in D2

TH is very important for the normal development of various tissues, including the brain (1,2). Cellular TH levels are importantly controlled by D2 and D3 (3) and it is known that D2 generates as much as 80% of cerebral T3 levels (4,5). As a consequence, it has been hypothesized that mutations in D2 may give rise to neurocognitive impairments in humans. The TOP-R study was designed to discover novel syndromes of TH resistance in patients with intellectual disability (ID) in key players of TH homeostasis.

In **chapter 2** we described the identification and functional consequences of 2 novel heterozygous mutations (c.11T>A; p.L4H and c.305C>T; p.T102I) in the coding sequence of D2 identified in this cohort. The functional consequences of these novel genetic variants were studied using cell lysates or in intact cells expressing D2 under natural conditions. Neither the L4H nor the T102I variant differed in activity compared to WT D2. This suggests that the D2 mutations identified in our cohort do not underlie the neurocognitive impairment in these patients and that these apparently harmless variations in D2 are not a cause of severe ID. This is in contrast to several MCT8 mutations, which were identified in the TOP-R study and are causally linked to ID (15). We also studied the prevalence of the commonly occurring T92A D2 SNP in this selection of patients which appeared to be comparable with previous studies (6-12). This observation suggests that this SNP is not directly related to ID. However, the T92A SNP has been associated with impaired psychological well-being (6) in general and during T4 replacement therapy in hypothyroid patients with a normalized TSH. This genetic variant was also associated with a preference for LT3/LT4 combination therapy above LT4 monotherapy in hypothyroid patients (6). It has been observed that hypothyroid patients on LT4 replacement therapy can have reduced quality of life, despite normal serum TH levels (13). Although speculative, this may be explained in part by the influence of genetic variation in D2 on local conversion of T4 to T3

in the brain. The same could hold true for the newly identified variations in D2. Although they appear not to be related to a specific phenotype, it cannot be excluded that these variants exert effects under different conditions. For example, based on the importance of D2 in influencing local T3 levels, it can be speculated that patients harboring these variants, who are dependent on treatment with exogenous T4 (e.g. in primary hypothyroidism), respond differently than non-carriers. If the newly identified variants interfere with D2 function they may play a role in the level of efficacy of LT4 treatment (6). Subsequently, in a recent study, unique modifications in the cellular transcriptome have been identified in human brains homozygous for the T92A-D2 polymorphism, that are independent of TH signalling. Furthermore, a cellular model revealed that T92A-D2 protein exhibits a longer half-life and, as opposed to normal D2, can be found in the Golgi apparatus. Its presence in the Golgi disrupted basic cellular functions and increased pre-apoptosis. Interestingly, these findings were also observed in other brain degenerative diseases (14). Altogether, our data provide evidence of the existence of rare but apparently harmless genetic variants of D2. This field of next generation sequencing is rapidly expanding, leaving us with numerous variants in different genes without having knowledge about possible pathogenic effects. Thus, it is important to establish the functional consequences of such variants.

In D2, the SECIS element is crucial to introduce Sec and the presence of this amino acid is important for normal enzymatic activity (15,16). **Chapter 3** described the identification of two heterozygous mutations in the SECIS element of D2 (c.5703C>T and c.5730A>T). These mutations were not localized in the 3 groups of highly conserved nucleotides that are known to be most important for SECIS element functionality (15). Various *in vitro* assays suggested that the 5703C>T mutation did not interfere with normal D2 activity and most likely this mutation can be described as a rare, harmless variant. However, the 5730A>T mutation showed 75% decreased SECIS element read-through, using a luciferase reporter assay and changing nucleotide 5730 from A to C showed an even more pronounced decrease in the luciferase activity, compared to the patient's mutation. Therefore, the nucleotide at position 5730 in the SECIS element appears to be important for SECIS element read-through. However, we could not demonstrate any deleterious effect on D2 activity after introducing both mutations. We will perform genome sequencing on DNA of the index patient and her relatives to search for mutations in critical genes involved in SECIS element processing and to identify possible other genetic causes of neurological impairment.

How are we going to find new pathogenic mutations in D2? As hypothyroid patients on LT4 replacement therapy, with variations in D2, show lower quality of life despite normal TH levels, it would be interesting to search for mutations in these non-responders. Also, the presence of variations in D2 was linked to a higher risk on bipolar mood disorder (17), which makes this group of patients an interesting target. Furthermore, D2 activity fulfils a vital role in bone mineralization and strength (18). *Dio2* KO mice showed to have brittle bones with increased fracture vulnerability, due to increased mineralization and low bone turnover (19). In contrast, the T92A-D2 SNP was associated with decreased total hip mineral density (20). It would therefore be interesting to search for DIO2 mutations in patients that are diagnosed with osteoporosis or brittle bones. *Dio2* KO mice showed defective auditory function, which could be an extra argument to search for D2 mutations in patients suffering from deafness in combination with other symptoms (21).

Finally, D2 increases in brown adipose tissue (BAT) after cold exposure (22). Mice with targeted disruption of the *Dio2* gene, become hypothermic because of impaired BAT thermogenesis, despite normal T3 plasma levels. This leads to the suggestion that D2 mutations may be found in patients with metabolic disorders with defects in maintaining their body temperature.

TH transport characteristics of LAT1-5

Evidence that LAT1 and LAT2 transport TH is scarce, since most studies show indirect evidence of inhibited LAT-mediated uptake of amino acids by iodothyronines (23-27). The possibility that LATs may participate in the transport of TH in the brain may have important consequences for understanding the neurological phenotype of patients with MCT8 mutations. Therefore, we have studied the characteristics of iodothyronine transport by LAT1 and LAT2 in detail. Furthermore, we explored the possibility of iodothyronine transport by the recently identified LAT3, LAT4 and LAT5.

In **chapter 4,** we confirmed that LAT1 facilitated intracellular accumulation of various iodothyronines (T4, T3, rT3, 3,3'-T2) and we measured net uptake of T3 and 3,3'T2 by LAT2. We also showed for the first time that monoiodotyrosine (MIT) is taken up into the cells by LAT1 and LAT2. Next, we showed that the expression of LAT3 or LAT4 did not affect transport of T4 and T3 but resulted in decreased cellular accumulation of 3,3'-T2 and MIT. LAT5 did not facilitate transport of any substrate. MIT is a iodinated tyrosine molecule, that is present within thyroglobulin in the thyroid follicle and is used to form T4 and T3.

In our studies, LAT1 facilitated intracellular accumulation of T4 and T3 and this transporter may therefore be important for normal uptake of TH in various tissues. It has been suggested that LAT2 facilitates T3 transport in neurons in the prenatal and perinatal period, as in neonatal *Mct8* KO mice, local hyperthyroidism and expression of TH target genes in cerebral cortex are prevented by additional deletion of Lat2 (28). However, our data indicate that LAT2 is a relatively poor T4 and T3 transporter, which could be possibly, at least partly, explained by insufficient levels of intracellular amino acids levels available for exchange. Given the net accumulation of T4 and T3 by LAT1 and its expression in the mouse brain, we suggest that LAT1 may be another compensatory TH transporter in Mct8 deficient mice (26).

LAT1 and LAT2 transport the receptor inactive metabolites 3,3'-T2 and MIT. In contrast to LAT2, LAT1 is not expressed in the liver, kidney or thyroid, where these compounds are mainly metabolized by D1 and DEHAL1 (29-31). However, next to transcriptional effects, iodothyronines may also exert non-genomic biological effects (29,32-34). It has been shown that T4 and rT3 regulate actin polymerization and D2 activity in the developing brain via non-transcriptional ways (32). Also, 3,3'-T2 can influence mitochondrial energy metabolism (34,35). However, the exact role of these transporters in the uptake of receptor inactive metabolites, needs to be elucidated.

The uptake of MIT in control cells, showed a marked decline over time. It is possible that MIT uptake by COS1 cells is driven by the exchange with intracellular amino acids, since LAT1 and LAT2 are obligatory exchangers (36-40). The physiological role of intracellular amino acids as driving force for the uptake of TH by LAT1 needs to be elucidated.

In contrast to LAT1 and LAT2, we were also first to describe efflux of 3,3'-T2 and MIT by LAT3 and LAT4. A physiological role of LAT3 and LAT4 is expected in the placenta, where these transporters

could mediate efflux of amino acids from the syncytiotrophoblast to the fetus (41). Furthermore, a zebrafish morphant lacking Lat3 function showed collapsed glomeruli and disruption of glomerular permeability, suggesting that LAT3 may play a crucial role in the development and maintenance of podocyte structure and function (42). Also recently, it was observed that LAT4 facilitated the uptake and efflux of amino acids (43) and *Lat4* KO mice showed intrauterine and postnatal growth retardation, low amniotic amino acid concentrations and premature death after 9 days. As described for LAT1 and LAT2, these transporters could have a role in the non-genomic effects of metabolites. Furthermore, the efflux of 3,3'T2 and MIT by LAT3 and LAT4 into the bloodstream could be helpful to transport this substrates to the liver or thyroid for the re-utilization of iodide. Altogether, the physiological role of iodothyronine export by LAT3 and LAT4 *in vivo* remains to be elucidated, for example by measuring TH levels in the *Lat3* KO zebrafish model and the *Lat4* KO mouse.

New substrates for MCT8 and MCT10

Mutations in the TH transporter MCT8 result in a severe phenotype with psychomotor retardation; the Allan-Herndon-Dudley syndrome (AHDS) (44,45). The pathogenic mechanism involves defects in TH transport in the brain during critical stages of brain development (46-48). However, it has not been excluded that MCT8 also transports biologically relevant substrates other than TH. This may also seem likely in view of the characteristics of the highly homologous MCT10 transporter, which also transports in particular aromatic amino acids in addition to iodothyronines (49-51).

Chapter 5 described that both MCT8 and MCT10 effectively facilitate cellular efflux of MIT and diiodotyrosine (DIT), which are iodotyrosine intermediates in the biosynthesis of T4 and T3 in the thyroid gland (52). Our data suggested, that in our cell system, the uptake of MIT and DIT is mainly mediated by LAT1 followed by the efflux of these substrates by MCT8 and MCT10. This also explains the marked decline in MIT (and less marked decrease of DIT) concentration over time in cells transfected with empty vector, MCT8 or MCT10, as LAT1 is known to be an exchanger. During the incubation time, our medium is deplete of amino acids and not enough intracellular amino acid substrates are left over time to sustain the necessary exchange with MIT by LAT1.

What could be the physiological role of MCT8 and MCT10 in the export of iodotyrosines? Excessive MIT and DIT released from thyroglobulin, is deiodinated in the thyroid by DEHAL1 to reutilize the iodide for TH synthesis. MCT8 is highly expressed in thyroid follicular cells and appears to play an important role in the thyroidal secretion of TH (46,53). Thyroidal release of MIT and DIT would reduce the exposure of the iodotyrosines to DEHAL1 in follicular cells and, hence, decrease the efficiency of iodide retrieval. However, DEHAL1 is also highly expressed in liver and kidney where it may be involved in the deiodination of MIT and DIT taken up from the blood by LAT transporters (29,30). The role of MCT10 in the thyroid is less clear as this transporter is expressed in the thyroid of mice, but not of humans (54). We can also hypothesize that within the thyroid, MIT and DIT are not only released by exocytosis from fagolysosomes, as LATs are also present in these structures (55).

Interestingly, MIT also appears to be a potent inhibitor of tyrosine hydroxylase, an important enzyme for the biosynthesis of dopamine (56). MIT is taken up by cells through the L-type amino acid transporters LAT1 and LAT2, which are highly expressed in the blood-brain barrier and central neurons (26,57,58). If the MIT or DIT efflux from neurons is impaired by MCT8

mutations, the consequent increase in MIT accumulation may result in a significant inhibition of dopamine synthesis (Figure 1). Therefore, further studies should be performed to determine the effects of MCT8 mutations on dopamine homeostasis in the brain.

If MCT8 is able to transport other substrates besides TH and its metabolites, this could possibly have important consequences for pathophysiology, diagnosis and treatment of the AHDS. For example, new ligands could create new diagnostic possibilities to screen newborns for the disease and possibly introduce new therapeutic strategies to substitute the missing substrate.

Stimulation of TH responsive genes by T4

The common wisdom that holds that only T3 is the bioactive form of TH is mainly based on receptor binding studies (59,60). There are multiple arguments that support the idea of T4 as a direct transcriptional stimulator. However, no studies have been performed to explore the possibility that T4 could directly stimulate transcription of TH responsive target genes.

In **chapter 6**, we made a first attempt to explore the possibility if T4 can modulate gene transcription using a cell model and our data suggest that T4 can exert transcriptional effects in SHSY5Y cells. Our data are well in line with other studies, which show T4 can induce conformational changes in the TR similar to those induced by T3 (61) and by the work of Schroeder *et al* in which they described the ability of both TRα1 and TRβ1 to respond to T3 and T4. TRα1 was generally more responsive to T4, possibly explained by the recruitment of coactivators by T4 (62).

Although our data suggest that T4 indeed can stimulate the expression of TH sensitive genes, we should keep in mind that these effects can be tissue or even cell specific. This is for example underscored by the observation that the expression of TH sensitive genes in neurones is dependent on T4 to T3 conversion by the type 2 deiodinase in the glial compartment (63). Most likely some tissues, like brown adipose tissue (64) or parts of the brain, are dependent on local conversion of T4 to T3, whereas in other tissues T4 may be able to exert direct transcriptional effects.

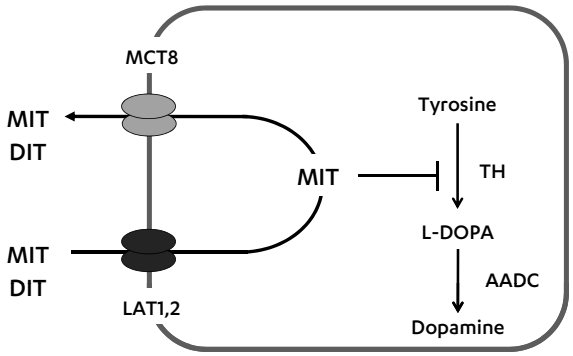


Figure 1 Schematic overview of MIT and DIT uptake and action in the brain. MIT and DIT are transported across the plasma membrane by LAT1 and LAT2. MIT is a potent inhibitor of tyrosine hydroxylase (TH), which is responsible for catalysing conversion of tyrosine to L-DOPA. L-DOPA is subsequently converted to dopamine by aromatic amino acid decarboxylase (AADC). If the efflux from neurons is impaired by MCT8 mutations, the consequent increase in MIT accumulation may result in a significant inhibition of dopamine synthesis.

To further explore the hypothesis that T4 can stimulate transcription of target genes, it would be interesting to test the transcription of TH responsive genes in cells that only express T4 transporters or to study thyroidectomised *Dio1/Dio2* double KO mice supplied with T3 or T4.

Gender-related differences in the suppression of TH signalling during aging

Although the intimate link between thyroid parameters and aging is known for many years, the underlying mechanisms are poorly understood. Recently, we disclosed a novel link between aging and TH signalling by demonstrating both decreased D1 activity and increased D3 activity in different models of premature aging, including *Csb*$^{m/m}$*/Xpa*$^{-/-}$ DKO mice and normal aging mice (Visser et al. Submitted to PLoS One 2015).

It is well known that there are sex-differences in aging and lifespan, with women living longer than men. It is also known that TH levels are different in man and women throughout life (65). These differences are most likely based on complex associations between TH signalling, aging and the possible influence of gonadal hormones (66-74). However, no studies have explored if sex-differences exist in changed TH signalling during aging.

In **chapter 7**, we focussed on TH signalling in liver and kidney samples of male and female naturally aged (13-, 52-, and 104-week-old animals) as well as prematurely aged 15-day-old *Csb*$^{m/m}$*/Xpa*$^{-/-}$ DKO mice. In the *Csb*$^{m/m}$*/Xpa*$^{-/-}$ DKO mice, both genders responded equally to DNA damage, with similar changes in TH levels, deiodinase activities and consequent gene expression changes. In male naturally aging samples we could confirm the changes in TH state and metabolism as reported previously. However, thyroid state appeared to be regulated differently in normal aging females as compared with normal aging male WT or mutant mice. We observed a trend towards higher TH serum and tissue levels paralleled by a significant decrease in D3 and increase in D1 expression and activity in older females. The opposite effects on TH state in male *versus* female naturally aging mice can due several possible mechanisms.

First, the opposite effects on TH state in male *versus* female naturally aging mice can be due to the influence of sex hormones. In literature the influence of sex steroids on TH function is under debate. Some studies show that liver D1 activity in male rats is decreased after castration and increased after administration of testosterone (66,70). On the other hand, the data on female liver D1 activity after ovariectomy and estradiol administration is controversial (69,70,72,73). Future studies should reveal the complex associations between TH signalling, aging and the influence of gonadal hormones and their binding globulines (66-74).

Second, aging is severely accelerated in the *Csb*$^{m/m}$*/Xpa*$^{-/-}$ mice. The lifespan of these animals could be too short to result in differences between male and female mice which are normally seen during the WT aging process.

Third, the oldest naturally aging mice we used in our studies are 104-weeks-old. It is possible that older (e.g. 130-weeks) female mice resemble the attenuated TH state in old male mice.

Fourth, whether normal male or female mice live longer could be dependent on the genetic background of the animals.

Altogether, our data confirmed attenuated TH signalling in *Csb*$^{m/m}$*/Xpa*$^{-/-}$ and naturally aging mice. However, gender influences the changes in TH signalling during aging, as we observed opposite trends in male and female naturally aging mice. This reinforces the notion that animal studies in one sex should be interpreted with caution and not extrapolated easily to the other sex. Furthermore, it has been shown before, that there are similarities and differences in the human and rat model for aging (75), which suggest that extrapolating our data to the human situation is challenging. Finally, in this study, we only observed changed D3 and D1 activities in liver and kidney samples. We should keep in mind that these effects could be cell or tissue specific.

Associations between TH, trace elements and antiepileptic drugs (AEDs)

TH and the trace elements Se and Cu are important for normal neurocognitive development and abnormal brain development may increase AED usage (76-81). The relationship between TH, Se, Cu and AED is currently unclear. In **chapter 8**, we analysed this relationship in the TOP-R cohort, in which Se and Cu, as well as thyroid parameters were determined. Our analyses indicated that the commonly prescribed AEDs carbamazepine and valproate affect serum Se and Cu levels. Carbamazepine or valproate usage was associated with lower serum Se levels. Usage of carbamazepine or phenytoin was associated with higher serum Cu levels and valproate usage with lower serum Cu levels. Other AED usage was not associated with serum Se or Cu levels and there was no association between the daily dosage of AEDs and Se, Cu or thyroid hormone levels. Next, we showed that serum Se was positively associated with TSH, inversely associated with FT4, and positively associated with T3 levels and the T3/rT3 ratio, as a proxy for peripheral deiodination. We also observed strong positive associations between serum Cu and T3, rT3 and T4 levels. While low normal Se levels may partially affect TH signalling in a negative way via modifying the expression of deiodinases, the negative effects of low Cu levels on thyroid parameters may be primarily driven by its effects on TBG. Future research is needed to explore the underlying mechanisms of the observed associations in the current study and to investigate to what extent deficient or excessive trace element levels are a risk factor for the development of thyroid diseases.

Concluding remarks

Thyroid hormone homeostasis is a complex system, in which many key players are involved. In the last decades, the identification of mutations in these key players helped us to learn about the normal and abnormal physiology of the thyroid, TH transport, metabolism and action. Today, many gaps are still remaining and more studies are necessary to complete the knowledge on this small, but powerful organ. It is exciting that many more players in the control of local TH signalling are still out there to be discovered. In the next years, new mutations in deiodinases, transporters and receptors, using exome and whole genome sequencing, will be indentified. It is important to find these mutations and make connections to the patients' phenotypes. The ultimate goal of these analysis is to offer more or better treatment options.

Cellular Mechanisms of Thyroid Hormone Homeostasis, one step closer in unravelling the mysterious thyroid hormone system.

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Chapter 10

SUMMARY
SAMENVATTING
LIST OF PUBLICATIONS
CURRICULUM VITAE
PHD PORTFOLIO
DANKWOORD

SUMMARY

Thyroid hormone (TH) is important for the normal development and metabolism of virtually all tissues, including the brain. This is illustrated by congenital hypothyroidism, which if untreated, can give rise to severe neurological and cognitive problems. In humans, the thyroid gland mainly secretes the inactive hormone T4 and in a lesser extent the bioactive form T3. Normal TH homeostasis is dependent on the intracellular availability of TH, as the genomic actions of TH are mediated by nuclear TH receptors (TRs) bound to promoter elements of TH responsive genes. The intracellular availability of TH, is dependent on adequate function of transporter proteins and deiodinating enzymes that convert T4 to the active form T3. Changes in any of the previously described key players of TH homeostasis can alter local TH signalling. This is illustrated by mutations in the TH transporter MCT8, which results in a severe neurological and endocrine disorder, including intellectual disability (ID), motor abnormalities, and altered TH parameters. The work in this thesis presents studies in which various key players of TH homeostasis in health and disease are investigated.

Chapter 1 provided a general background of normal TH physiology. It briefly introduces TH in health and disease and the biosynsthesis of TH. More attention was paid to cellular TH transport, metabolism by deiodinases and nuclear TH receptor action. **Chapter 1** ended with the objective and outline of this thesis. In **chapter 2** and **chapter 3**, we reported on the identification and functional implications of mutations in the type 2 deiodinase (D2) of patients with unexplained intellectual disability (ID). **Chapter 2** described the existence of two heterozygous mutations (c.11T>A; p.L4H and c.305C>T; p.T102I) in the coding sequence of D2, that were also found in family members without ID and in various genetic databases. The identified mutations in D2 were extensively functionally analyzed using various *in vitro* techniques and no harmful effect was found. These data suggested the existence of rare but apparently harmless genetic variants in the coding sequence of D2.

In **chapter 3** we also identified two mutations (c.5703C>T and c.5730A>T) in the SECIS element of D2, which is necessary to introduce selenocysteine and this amino acid is known to be important for normal activity of D2. Sequence analysis of family members revealed several carriers. Extensive tests with different *in vitro* D2 assays did not show differences between WT and 5703C>T SECIS mutant. Our data provide evidence that nucleotide 5730 seems to be important for normal SECIS element read-through. However, we could not demonstrate any deleterious effect on D2 activity. At present, we cannot conclude that the phenotype of ID is related to the presence of this mutation. Therefore, whole genome sequencing is currently performed on DNA of the index patient and her relatives to search for mutations in critical genes involved in SECIS element processing and to identify possible other causes of neurological impairment.

In **chapter 4** we confirmed the cellular accumulation of all iodothyronines in the presence of LAT1 and net uptake of T3 and 3,3'-T2 by LAT2. LAT1 and LAT2 also showed uptake of monoiodotyrosine (MIT). Expression of LAT3 or LAT4 did not affect transport of T4 and T3, but resulted in decreased cellular accumulation of 3,3'-T2 and MIT. LAT5 did not facilitate transport of TH. Altogether, our data highlighted novel functions for LAT1-4 in the transport of iodothyronines and showed that LAT1 and LAT2 facilitated mainly uptake of TH, whereas LAT3 and LAT4 were exporters. Further studies are necessary to explore the physiological role of these transporters *in vivo*.

Chapter 5 explored the transport of alternative substrates by the TH transporter MCT8. MCT8 and MCT10 transported the inactive metabolites MIT and diiodotyrosine (DIT) out of the cells, which is the first evidence that the specific TH transporter MCT8 also accepted other substrates. If the efflux of MIT and DIT, especially by MCT8, has any contribution on normal TH physiology or in patients with mutations in MCT8, remains to be elucidated.

In **chapter 6**, we showed that T4 is able to stimulate expression of TH responsive genes at the transcriptional level. This data showed that in addition to T3 also T4 can exert transcriptional effects. It can be speculated that in specific tissues and under certain conditions, T4 is able to regulate gene expression.

In **chapter 7**, we confirmed the recent finding that TH signalling is attenuated during aging. In 15-day-old *Csb*$^{m/m}$*/Xpa*$^{-/-}$ DKO mice we observed lower serum and tissue TH levels, an increase in liver D3 activity and expression, paralleled by a decrease in liver D1 activity and expression compared to age matched controls. In this work, we focused on gender differences in the changed TH signalling during aging. There was no difference between males and females in the 15-day-old *Csb*$^{m/m}$*/Xpa*$^{-/-}$ DKO mice. In livers of male naturally aged mice we observed decreased D1 and increased D3 activity in parallel with decreased hepatic TH content. However in female normal aging mice, we observed a trend towards an increased TH state during aging, accompanied by a decrease in D3 activity and an increase in D1 activity. Altogether, our data confirmed attenuated TH signalling in *Csb*$^{m/m}$*/Xpa*$^{-/-}$ and naturally aging mice. Gender is likely to influence the changes in TH signalling during aging, as we observed opposite trends in male and female normal aging mice. This reinforces the idea that animal studies in one sex should be cautiously interpreted and extrapolated.

In **chapter 8**, we described the complex relationship between AEDs, THs and the trace elements Se and Cu. Se levels were significantly lower in patients that use valproate or carbamazepine, whereas phenytoin and carbamazepine usage showed increased Cu level. Next, we showed that lower levels of Se are associated with increased FT4, decreased T3 levels and a decreased T3/rT3 ratio. We also reported positive associations between serum Cu and T3, rT3 and T4 levels. However, we are the first to describe that these effects were totally driven by TBG. Future research should explore the underlying mechanisms of the observed associations in the current study and investigate to what extent trace elements are a risk factor for thyroid disease entities.

In **Chapter 9**, the observations that were presented in this thesis were discussed in view of the current literature and possible implications of the findings were explored. The work in this thesis: *'Cellular mechanisms of thyroid hormone homeostasis'* included different key players in TH homeostasis and expanded our knowledge on the subject, however many gaps in our understanding are still present. Future directions for new research are provided in light of all the work in this thesis.

SAMENVATTING

Schildklierhormoon (SKH) wordt geproduceerd door de schildklier en dit orgaan gelegen in de hals scheidt voornamelijk het prohormoon T4 en in mindere mate het bio-actieve hormoon T3 af. SKH is belangrijk voor de normale ontwikkeling van bijna alle weefsels in het lichaam, waaronder ook de hersenen. Wanneer een tekort aan SKH optreedt tijdens de ontwikkeling van een foetus, kan dit aanleiding geven tot ernstige verstandelijke en motorische beperkingen. SKH is daarnaast belangrijk voor vele processen in vrijwel alle organen, met name de energiehuishouding en warmteproductie. Een verminderde werking van de schildklier leidt tot een tekort aan SKH in het bloed en dit heet hypothyreoïdie. Dit wordt gekenmerkt door klachten als moeheid, gewichtstoename, koude-intolerantie en obstipatie. Een overschot aan SKH in het bloed geeft aanleiding tot hyperthyreoïdie en veroorzaakt symptomen als gewichtsverlies, gejaagdheid en warmte-intolerantie. De biologische effecten van SKH vinden plaats in de celkern, waar de zich T3 receptoren zich bevinden. Het beschikbare, actieve T3 bindt zich aan deze receptoren en op zijn beurt zal dit complex binden aan T3 responsieve elementen (TREs) die de transcriptie van SKH gevoelige genen activeren of juist onderdrukken. Omdat deze T3 receptoren zich in de cel bevinden zijn een aantal andere processen noodzakelijk om de intracellulaire T3 beschikbaarheid te reguleren. Aangezien SKH goed oplost in vet en de plasma membraan met name uit vetten bestaat, is lange tijd gedacht dat SKH de plasma membraan vanzelf passeerde. Het is echter in de laatste jaren steeds meer duidelijk geworden dat specifieke transporters in de plasma membraan nodig zijn. Het echte belang van deze transporters werd duidelijk toen mutaties (fouten) in een belangrijke schildkliertransporter (MCT8) bij mensen bleek te leiden tot ernstige verstandelijke beperkingen en afwijkende SKH waardes in het bloed. Naast de transporters wordt de T3 concentratie in de cellen beïnvloed door dejodases. Type 1 en type 2 dejodase zetten in verschillende weefsels het prohormoon T4 om in de actieve variant T3. Het type 3 dejodase heeft met name een inactiverende functie. Samenvattend wordt de concentratie van T3 in de cellen gereguleerd door transporters en dejodases om uiteindelijk te kunnen binden aan T3 receptoren in de celkern. Hierdoor wordt de energiehuishouding te gereguleerd via de transcriptie van SKH gevoelige genen.

In normale omstandigheden zijn de transporters, dejodases en receptoren goed op elkaar ingespeeld en kunnen zij heel nauwkeurig de hoeveelheid van het SKH in cel aanpassen naar de behoefte van het lichaam. Wanneer zich echter defecten voordoen in een van deze schakels, kan dit resulteren in ziekteprocessen. In dit proefschrift worden experimenten beschreven, waarin diverse schakels binnen de SKH homeostase in gezondheid en ziekte zijn bestudeerd.

Hoofdstuk 1 geeft een indruk van de normale SKH fysiologie. In het kort wordt de rol van SKH in gezondheid en ziekte beschreven en de productie van SKH in de schildklier wordt uitgelegd. Vervolgens wordt er dieper ingegaan op de belangrijkste schakels binnen SKH homeostase: transporters, dejodases en receptoren. Als laatste worden de doelstellingen en hoofdlijnen van dit proefschrift beschreven.

In de volgende twee hoofdstukken van dit proefschrift beschrijven we de zoektocht naar mutaties (fouten) in het type 2 dejodase (D2) in een cohort dat bestaat uit mensen met onverklaarde verstandelijke en motorische beperkingen (TOP-R cohort). D2 zet T4 om naar

de actieve vorm T3 en komt in hoge mate voor in de hersenen. Om die reden hebben we de hypothese gevormd, dat mutaties in D2 aanleiding zouden kunnen geven tot verstandelijke en motorische beperkingen. Uiteindelijk hebben we vier mutaties gevonden in D2 en de effecten op de functionaliteit van deze D2 worden beschreven in **hoofdstukken 2** en **3.** De eerste twee mutaties bevinden zich in dat het deel van het gen dat wordt overgeschreven naar messenger RNA (mRNA). Dit mRNA wordt vertaald naar aminozuren en een groeiende keten aminozuren vormen samen het D2 eiwit. Nadat we de mutaties hadden gevonden bij de patiënten, hebben we ook hun familieleden onderzocht. Uiteindelijk hebben we deze mutaties ook kunnen aantonen in familieleden die geen verstandelijke beperking hebben. In **hoofdstuk 2** hebben we het effect van de mutaties op D2 activiteit onderzocht en we hebben geen negatieve invloed kunnen aantonen. Daarom hebben we geconcludeerd dat de verstandelijke beperking bij de patiënten niet door deze mutaties wordt veroorzaakt. De volgende twee geïdentificeerde mutaties bevinden zich in het SECIS element van D2. Dit element is noodzakelijk om het cruciale aminozuur selenocysteine (Sec) in de groeiende aminozuurketen in de bouwen. Opnieuw hebben we deze mutaties kunnen aantonen in familieleden zonder beperkingen. In **hoofdstuk 3** laten we zien dat de eerste mutatie in het SECIS element geen nadelige invloed heeft op D2 activiteit. Het testen van de tweede mutatie laat normale D2 activiteit zien, alleen in tegenstelling tot dit resultaat, hebben we wel een verminderde werking van het SECIS element geobserveerd. Omdat we nu niet met zekerheid kunnen zeggen of deze mutatie iets te maken heeft met de stoornis, willen we uitsluiten of er nog andere mutaties bij deze patiënt aanwezig kunnen zijn die de verstandelijke beperking zouden kunnen verklaren. Tevens kan een andere mutatie die inwerkt op het SECIS element, samen met de door ons gevonden mutatie, de werking van het SECIS element nadelig beïnvloeden.

L-Type aminozuur tranporters (LATs) zijn een familie van transporters die met name aminozuren transporteren. Daarnaast is aangetoond dat twee LAT familieleden, LAT1 en LAT2, ook SKH kunnen opnemen. Er is hier echter weinig direct onderzoek naar verricht. In **hoofdstuk 4** tonen we aan dat LAT1 alle vormen van SKH en metabolieten (T4-T3-rT3-T2-MIT) kan transporteren en dat LAT2, naast het actieve T3, ook het transport van T2 en MIT faciliteert. Recent zijn drie nieuwe leden van de LAT familie geïdentificeerd: LAT3, LAT4 and LAT5. LAT3 en LAT4 transporteren ook aminozuren. In **hoofdstuk 4** beschrijven we tevens dat LAT3 en LAT4 de concentratie van T2 en MIT in de cellen verlaagt, wat kan worden verklaard door transport van binnen naar buiten de cel (export). LAT5 transporteert geen enkele vorm van SKH. Deze experimenten laten nieuwe functies zien voor LAT1-4. De fysiologische rol in SKH homeostase moet nog verder worden uitgezocht.

MCT8 staat bekend als een specifieke SKH transporter. Het is echter niet uitgesloten dat MCT8 ook andere substraten kan transporteren. Zeker, omdat MCT10, die lid is van dezelfde familie, ook het transport van aminozuren kan faciliteren. Tot op heden zijn nog geen andere substraten voor MCT8 aangetoond. In **hoofdstuk 5** beschrijven we export van monoiodotyrosine (MIT) en diiodotyrosine (DIT) door MCT8 en MCT10. MIT en DIT zijn aminozuurderivaten die worden gebruikt in de opbouw van SKH in de schildklier. Waarom de export van MIT en DIT door MCT8 en MCT10 belangrijk is in het schildklierhormoonmetabolisme, moet verder

worden bestudeerd. Het is ook interessant om verder onderzoek te voeren naar de rol van deze moleculen in patiënten met mutaties in MCT8.

Zoals al is besproken, verloopt de actie van SKH via receptoren, die de transcriptie van SKH gevoelige genen activeren of juist onderdrukken. Het wordt algemeen aangenomen dat T3 het actieve SKH is, echter recente onderzoeken tonen aan dat ook T4 een complex kan vormen met de SKH receptor. In **hoofdstuk 6** tonen we aan dat T4 ook in staat is om de transcriptie van SKH gevoelige genen te stimuleren. Dit geeft aan dat naast T3, ook T4, relevante functies kan hebben met betrekking tot de transcriptie van genen en onder bepaalde condities genexpressie zou kunnen reguleren.

Recent hebben we aangetoond dat er een direct verband bestaat tussen DNA schade, veroudering en verminderde werking van het SKH. Minder SKH betekent minder energieverbruik, wat beschermend kan werken tijdens veroudering. In verschillende muismodellen (vroegtijdig verouderende progeria muizen door defecten in het herstel van DNA schade en normaal verouderende muizen) hebben we verlaagde waardes van SKH in het bloed gevonden, die veroorzaakt wordt door een aanpassing in het type 1 en het type 3 dejodase. Het is niet bekend of geslacht een rol speelt in deze bevindingen. In **hoofdstuk 7** bevestigen we opnieuw dat er tijdens veroudering sprake is van een demping in SKH actie. In de progeria muizen hebben we geen verschil gevonden tussen mannetjes en vrouwtjes. Ook in de mannelijke normaal verouderende muizen vinden we lage SKH waardes in serum door aangepaste werking van de dejodases, echter in normaal verouderende vrouwtjes vinden we een omgekeerd effect, met toegenomen SKH waardes. Uiteindelijk lijkt geslacht dus een effect te hebben tijdens veroudering, maar niet in alle muismodellen. Het is daarom belangrijk om rekening te houden met het geslacht bij de interpretatie van resultaten in verschillende muismodellen.

Sporenelementen, als koper of selenium, zijn slechts in zeer kleine hoeveelheden in het lichaam aanwezig en moeten via de voeding worden ingenomen. Het is bekend dat SKH, maar ook sporenelementen belangrijk zijn voor de normale ontwikkeling van de hersenen. Een tekort aan SKH of sporenelementen kan aanleiding geven tot verstandelijke beperkingen. Mensen met een verstandelijke beperking gebruiken veelvuldig medicijnen tegen epilepsie: anti-epileptica. Het is echter ook bewezen dat anti-epileptica de waardes van SKH en sporenelementen kan aanpassen. Deze complexe relatie tussen SKH, sporenelementen en anti-epileptica is nog nooit onderzocht. In **hoofdstuk 8** beschrijven we de associaties die we hebben gevonden tussen bovengenoemde determinanten, in patiënten met een verstandelijke beperking uit het eerder genoemde TOP-R cohort. Het gebruik van anti-epileptica beïnvloed de concentraties van selenium en koper in het bloed. Daarnaast hebben we geobserveerd dat selenium en ook koper de waardes van SKH kan veranderen. Veder onderzoek is noodzakelijk om de invloed van sporenelementen op het ontstaan van schildklierziektes op te helderen.

In **hoofdstuk 9** bediscussiëren we alle observaties in dit proefschrift in het licht van de huidige literatuur en de gevolgen van de resultaten worden uiteengezet. Het werk in dit proefschrift omvat diverse belangrijke schakels binnen het SKH homeostase en heeft onze kennis vergroot. Er zijn echter nog vele onderdelen die verder uitgediept dienen te worden. Nieuwe ideeën voor verder onderzoek worden aangedragen in het kader van dit proefschrift.

LIST OF PUBLICATIONS

Zevenbergen C, Klootwijk W, Peeters RP, Medici M, de Rijke YB, Huisman SA, Goeman H, Boot E, de Kuijper G, de Waal KH, Meima ME, Larsen PR, Visser TJ, Visser WE. *Functional analysis of novel genetic variation in the thyroid hormone activating type 2 deiodinase*. The Journal of Clinical Endocrinology and Metabolism. 2014;99:E2429-E2436.

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CURRICULUM VITAE

Chantal Zevenbergen was born on November 28th, 1983 in Rotterdam. After completing secondary school at Develstein College in Zwijndrecht, she started her study of Medicine at the University of Amsterdam. After obtaining her Bachelors degree, she continued her study at the Erasmus University Rotterdam. In July 2010 she received her medical degree and started as a resident of Internal Medicine at the Maasstad Hospital in Rotterdam. In July 2011 she started a new adventure in the thyroid laboratory of Prof. Dr. Ir. Theo Visser and the results of her 4 years of PhD research are presented in this thesis. In May 2015 she started her training residencies in Internal Medicine at the Sint Franciscus Gasthuis, Rotterdam under supervision of Dr. Rietveld. October 15th 2015 she married Wilco Nap after 15 years of being together.

PHD PORTFOLIO

Name PhD Student:	Chantal Zevenbergen
Erasmus MC Department:	Internal Medicine-Thyroid Laboratory
Research School:	MolMed
PhD Period:	July 2011-April 2015
Promotor:	Prof. dr. ir. T.J. Visser
Copromotor:	Dr. W.E. Visser

Courses	Year	Workload
The Ensembl Workshop	2011	2 days
Course on radioactive rules, protection and hygiene	2011	3 days
Course on confocal imaging	2011	1 day
The Basic course Rules and Organization for Clinical researchers	2012	5 days
The workshop on Photoshop and Illustrator CS5	2013	1 day
Conferences	**Year**	**Workload**
Internal Medicine Science days, Antwerp, Belgium	2012	2 days
Annual Molecular Medicine Day, Rotterdam, The Netherlands	2012	1 day
36 th Annual Meeting of the European Thyroid Association, Pisa, Italy	2012	5 days
Internal Medicine Science days, Antwerp, Belgium	2013	2 days
Dutch Endocrine Meeting, Noordwijkerhout, The Netherlands	2013	2 days
Dutch Thyroid Club, Amsterdam, The Netherlands	2013	1 day
Annual Molecular Medicine Day, Rotterdam, The Netherlands	2013	1 day
37 th Annual Meeting of the European Thyroid Association, Leiden, The Netherlands	2013	5 days
Internal Medicine Science days, Antwerp, Belgium	2014	2 days
Dutch Endocrine Meeting, Noordwijkerhout, The Netherlands	2014	2 days
Dutch Thyroid Club, Amsterdam, The Netherlands	2014	1 day
11 th International workshop on resistance to thyroid hormoneb and thyroid hormone action, Madrid, Spain	2014	3 days
38 th Annual Meeting of the European Thyroid Association, Santiago de Compostela, Spain	2014	5 days
Presentations	**Year**	**Type**
Functional analysis of newly discovered mutations in the thyroid hormone activating type 2 deiodinase. Annual Molecular Medicine Day, Rotterdam, The Netherlands	2012	Poster
Functional analysis of newly discovered mutations in the thyroid hormone activating type 2 deiodinase. 36 th Annual Meeting of the European Thyroid Association, Pisa, Italy	2012	Oral
Functional analysis of newly discovered mutations in the thyroid hormone activating type 2 deiodinase. Internal Medicine Science days, Antwerp, Belgium	2013	Poster
Functional analysis of newly discovered mutations in the thyroid hormone activating type 2 deiodinase. Dutch Endocrine Meeting, Noordwijkerhout, The Netherlands	2013	Oral
Functional analysis of newly discovered mutations in the thyroid hormone activating type 2 deiodinase. Annual Molecular Medicine Day, Rotterdam, The Netherlands	2013	Poster

Patients' mutations in the SECIS element of *DIO2:* influence on SECIS element read through and enzymatic activity. 37 th Annual Meeting of the European Thyroid Association, Leiden, The Netherlands	2013	Oral
MCT8, more than a thyroid hormone transporter? Internal Medicine Science Days, Antwerp, Belgium	2014	Oral
Functional analysis of newly discovered mutations in the thyroid hormone activating type 2 deiodinase International workshop on resistance to thyroid hormone and thyroid hormone action, Madrid, Spain	2014	Oral
Transport of thyroid hormone by members of the LAT family 1-5. 38 th Annual Meeting of the European Thyroid Association, Santiago de Compostela, Spain	2014	Oral

Teaching Activities	**Year**	**Workload**
Vaardigheidsonderwijs first year medical students	2012	5 days
Vaardigheidsonderwijs first year medical students	2013	5 days
Vaardigheidsonderwijs first year medical students	2014	5 days

Supervising internships	**Year**	**Workload**
Simanca Deurloo, University of Leiden, Biomedical Sciences MCT8, more than a thyroid hormone transporter (Bachelor Thesis)	2013	6 months

Awards and prizes

2012: Travel grant award, European Thyroid Association, Pisa, Italy

2013: First poster prize, Science Days of Internal Medicine Erasmus MC, Antwerpen, Belgium

2014: Travel grant award, International workshop on resistance to thyroid hormone, Madrid, Spain

2014: Travel grant award, European Thyroid Association, Santiago de Compostela, Spain

Research support

2013-2014: ZonMW Priorities Medicine Rare Diseases Grant

Goal: To study if the thyroid hormone transporter MCT8 can transport alternative ligands, which can lead to an better understanding of the pathophysiology observed in patients with MCT8 mutations.

International Research Experience

July-September 2013: University of Zurich, Department of Medical Molecular Genetics

Supervisor: Dr. Barbara Kloeckener

Goal: To study if the thyroid hormone transporter MCT8, after expression in Xenopus Oocytes, has the ability to transport alternative ligands.

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Lieve **Wilco**, de laatste persoon op een paper is altijd de belangrijkste en dat is nu ook zeker het geval. Al 15 jaar ben jij mijn vriendje. Wat hebben we veel meegemaakt samen, toch zijn we er altijd sterker uitgekomen. In deze periode heb je me altijd weten op te peppen en geloof me, dat was soms nodig. Ook heb je geleerd dat alleen een luisterend oor soms genoeg is ;). Toch heb je me ook regelmatig uit de brand geholpen, als mijn computer weer eens niet mee wilde werken of door gewoon goede ideeën te spuien. Natuurlijk wilde ik die in eerste instantie meestal niet aanhoren, maar toch heb je vaak een punt. Nu is mijn proefschrift klaar en kijk ik uit om aan ons getrouwde leventje te gaan beginnen. Bedankt voor al je steun. Heel veel liefs en ik hou van je!