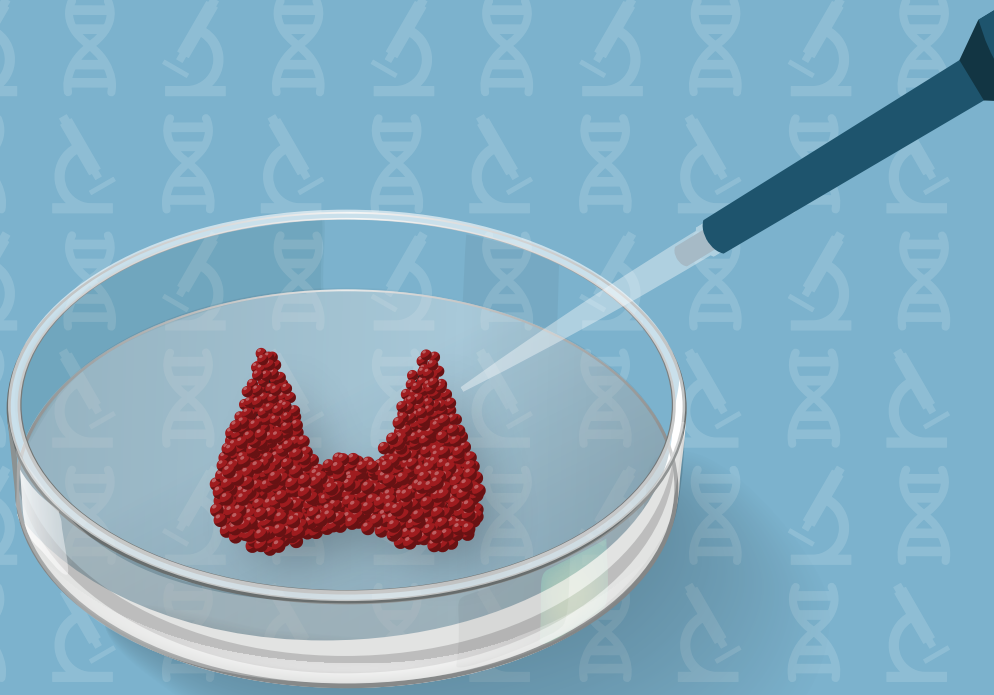
AALTJE ARIËNNE ALIES VAN MULLEM



CLINICAL and MOLECULAR ASPECTS of NUCLEAR THYROID HORMONE ACTION

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CLINICAL AND MOLECULAR ASPECTS OF NUCLEAR THYROID HORMONE ACTION

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CHAPTER 1

General introduction and outline of the thesis

INTRODUCTION

Thyroid hormone production

Thyroid hormone (TH) is synthesized in the thyroid gland [1]. The first step in thyroid hormone production is transport of iodide from the bloodstream into the thyroid follicles trough the sodium iodide symporter (NIS) [1, 2]. The next step is the incorporation of iodide into the tyrosyl residues of thyroglobulin (Tg) by hydrogen peroxide (H2O2) and thyroid peroxidase (TPO) [1]. TPO is also involved in the phenolic coupling of the iodotyrosyl residues to produce thyroxine (T4) [1]. Secretion of the stored TH goes by endocytosis from the apical surface of the thyroid follicular cells [3]. The thyroid gland mainly produces T4, but also a small amount of T3 (90 vs 2 nM) [1, 3, 4]. T4 is the precursor of 3,5,3'-triiodothyronine (T3), the biological active hormone [2, 5]. T4 and to a lesser extend also T3 are bound to carrier proteins such as thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin causing similar free hormone levels [1, 2].

HPT axis

TH production is under negative regulation of the hypothalamus-pituitary-thyroid (HPT) axis [1, 3]. The hypothalamus produces thyrotropin releasing hormone (TRH) which stimulates thyroid stimulating hormone (TSH) production in the pituitary. TSH stimulates production of TH by the thyroid gland by the binding to its TSH receptor (TSHr) [3].

Consequences of abnormal thyroid hormone levels

TH is important for the human body and plays a crucial role in development, differentiation and metabolism [3, 6]. This is illustrated by the consequences of untreated congenital hypothyroidism (CH), which leads to cretinism with brain damage and dwarfism [3, 7-10]. CH also leads to constipation, lethargy and feeding difficulties [9]. CH is detected by 1 in 3000-4000 live births [11]. Approximately 15% of the patients with CH suffer from an autosomal recessive genetic defect in the thyroid hormonogenesis, one of the candidate genes is NIS [1, 11, 12].

Thyroid hormone transport

It has long been assumed that TH crosses the plasma membrane by passive diffusion, because TH are lipophilic and the plasma membrane consist of lipids [13]. However, studies in rat hepatocytes showed that TH is transported over the plasma membrane instead by diffusion by a saturable and energy dependent uptake process [13, 14]. Multiple TH transporters have nowadays been recognized in several cell types and tissues.

Monocarboxylate transporter 8 (MCT8), also known as SLC16A2, is a highly specific TH transporter [15, 16]. MCT8 is expressed in brain, kidney, liver and heart [15, 17]. MCT10 (or SLC16A10), expressed in intestine, kidney, liver, muscle, and placenta, is another TH transporter from the same family that in addition to TH, is also capable of aromatic amino acid transport [16, 18-21]. Another known TH transporter is Na+/taurocholate-cotransporting polypeptide (NTCP), also known as SLC10A1, which is expressed in hepatocytes [13]. Furthermore, there are several organic anion transporting polypeptides (OATPs) and L amino acid transporters capable of TH transport [13, 22, 23]. Besides influx, some of the TH transporters can also facilitate TH efflux [13].

Consequences mutations MCT8

Inactivating mutations in MCT8 lead to the Allan-Herndon-Dudley syndrome (AHDS) [17, 24-26], which was first described in 1944 in patients with X-linked mental retardation [17]. The clinical phenotype of the AHDS exists of cognitive impairment, hypotonia, muscular hypoplasia and developmental retardation [17, 26]. These patients have a high free and total T3, low free and total T4 and normal to mild elevated TSH [17]. Female carriers of MCT8 mutations have no clear phenotype [17]. No cure is (yet) available, but several treatment options are currently under investigation, such as treatment with thyroid hormone analogues as DITPA or Triac (TA3) as well as gene therapy [27-32]. Previous research did not show a beneficial effect of LT4 suppletion on the neurocognitive phenotype [33, 34].

Thyroid hormone metabolism

Three deiodinating enzymes (D1-3) have been identified which catalyze the activation of T4 to T3 or the inactivation of T4 to 3,3',5'-triiodothyronine (reverse T3, rT3) and of T3 to 3,3'-diiodothyronine (3,3'-T2) [2, 35]. Deoidinases, which are selenoproteins, maintain TH homeostasis at cellular level [2, 36].

D1 can either activate or deactivate T4 [2]. D1 is expressed in liver, kidney and thyroid and is important for serum T3 production as well as for clearance of serum rT3 [2, 37]. D2 produces intracellular T3 in vital organs such as brain, pituitary, retina, brown fat, skeletal muscle but also contributes importantly to serum T3 production [2, 38]. D1 and D2 activate T4 by removal of a single outer ring iodine (5') group [1, 2]. D3 decreases the local T3 concentration and inactivates T4 by removing an iodine from the inner ring [2, 38]. D3 is mainly expressed in fetal tissues but also in retina, CNS and pituitary [36, 38, 39]. See figure 1 for a schematic overview of TH metabolism.

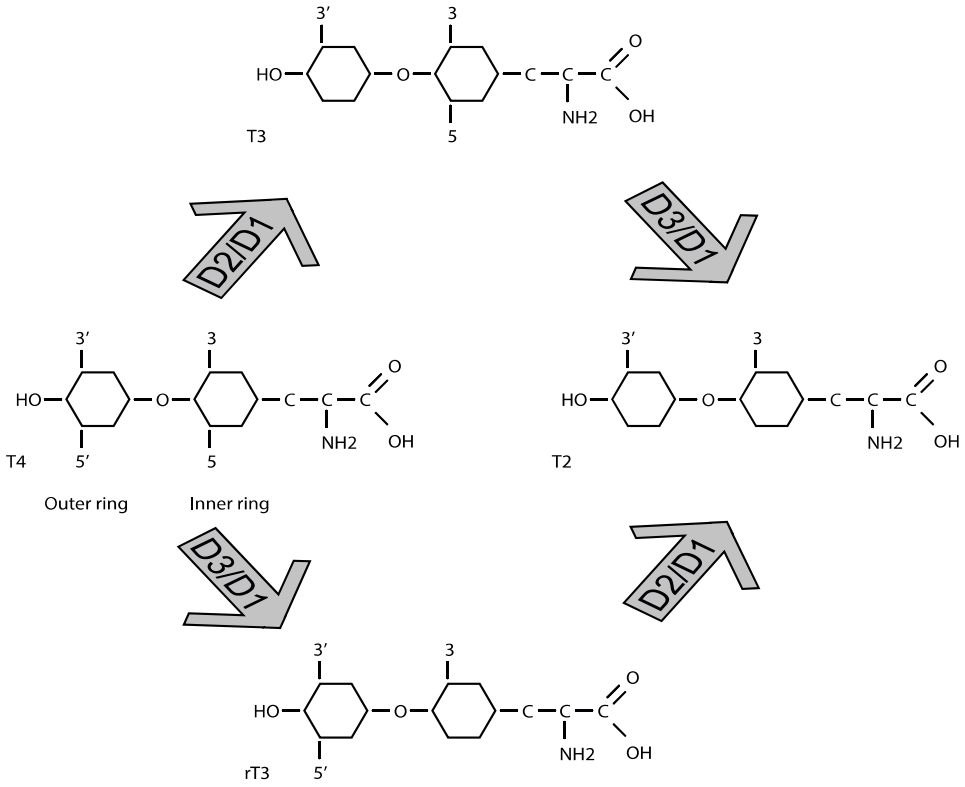

FIGURE 1. Schematic overview of TH metabolism.

Thyroid hormone action

Thyroid hormone receptors (TRs) are family members of the nuclear hormone receptor superfamily [3, 40]. TRs are encoded by *THRA* and *THRB* on chromosomes 3 and 17 respectively [3, 41]. Multiple isoforms are created by usage of different translation start points or different splice sites [3, 42, 43]. The TRs have a similar organization with an A/B domain, DNA binding domain (DBD), hinge region and a ligand binding domain (LBD) (figure 2).

TRα1 and TRβ1 are both ubiquitously expressed, but its expression varies among tissues [40, 44]. TRα is predominantly expressed in bone, brain, intestine, heart and muscle [38, 44], while TRβ is the main isoform in the liver, kidney and thyroid. TRα1 expression in the heart, which regulates cell differentiation, contractile function, pacemaker activity and conduction is 3 times higher than TRβ1 [38].

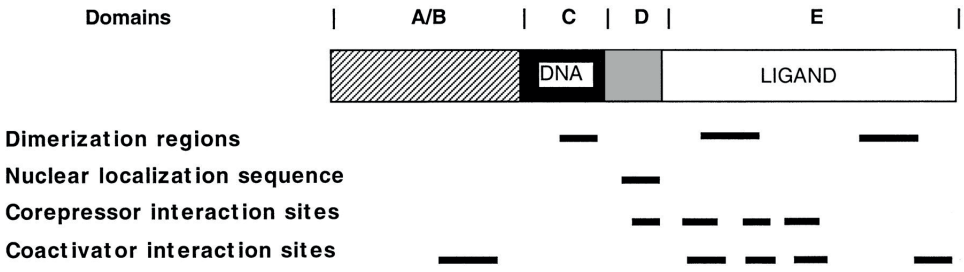

FIGURE 2. Adapted from [3].

TH action is mediated by binding of T3 to its nuclear TR [2, 40]. The TRs regulate gene expression by binding to TH respons elements (TREs) in the promoter region of its target genes [40]. TREs consist of a half-site of AGGTCA in varying orientations [6, 44]. TRs can form monomers, homodimers or heterodimers with retinoid X receptors (RXRs). Unliganded TRs also affect expression of TH target genes by recruiting corepressors, coactivators and histone deacetylase [45-48]. So gene regulation via TRs can either be positive or negative [2]. Besides genomic actions, TH can also mediate non-genomic effects via oxidative phosphorylation and mitochondrial gene transcription [44, 49, 50].

Resistance to thyroid hormone β (RTHβ)

It is known that mutations in *THRB* lead to resistance to thyroid hormone (RTH) for decades [47, 51]. Most mutations are in the LBD of TRβ [47]. The prevalence is estimated at 1:40,000 live births and the inheritance pattern is autosomal dominant [44, 47]. There is no gender prevalence [44]. The disease leads to a decreased response of TH stimulation in TH target organs with predominantly TRβ expression [44]. Patients have a biochemical phenotype of increased free T4 (FT4) and free T3 (FT3) levels with a normal to slightly increased TSH [44, 47, 52]. The mutated receptor has a dominant negative effect on the WT receptor. The clinical phenotype is variable and includes goiter, delayed bone age, developmental delay, hyperactive behavior, raised energy expenditure, learning disabilities and sinus tachycardia [44, 47, 53]. So far, more than hundred mutations in more than 1000 patients have been described with most of the mutations being organized around three so-called hotspots in the LBD and the hinge region [44, 47].

Mouse models

Multiple mouse models have been generated to study the consequences of mutations in *THRA* and *THRB*. The mouse models for RTHβ mutations show a very similar phenotype as patients with RTHβ [54, 55]. Until 2012, no patients with inactivating mutations in TRα

had been identified. In an attempt to predict their clinical phenotype, different mouse models were generated in which mutations identified in patients with RTHβ were introduced at the corresponding position of TRα. All TRα mutated mice (TRα1-PV, TRα1-R384C, TRα1-L400R, TRα1-P398H) had nearly normal TH function tests [56-60], and most suffer from growth retardation and delayed bone development (TRα1-PV, TRα1-R384C, TRα1-L400R) [38, 56, 58, 60-62]. Interestingly, the growth retardation is overcome in adulthood in TRα1-R384C mice, in which the mutation results in a reduced but not absent affinity for T3, whereas the other mice remain dwarfed [56, 57, 59, 60]. The TRα1-R384C mice also have a psychiatric phenotype with anxiety whereas TRα1-R384C and TRα1-L400R mice suffer from seizures [38].

TABLE 1

Mutation in protein	Affected isoform	Reference
G207E	TRα1 and TRα2	[63]
D211G	TRα1 and TRα2	[64]
A263S	TRα1 and TRα2	[65]
A263V	TRα1 and TRα2	[66, 67]
L274P	TRα1 and TRα2	[66]
N359Y	TRα1 and TRα2	[68]
C380fs387X	TRα1	[65]
A382fs388X	TRα1	[69]
R384C	TRα1	[70]
R384H	TRα1	[65]
C392X	TRα1	[71]
F397fs406X	TRα1	[72, 73]
P398R	TRα1	[71]
E403K	TRα1	[71]
E403X	TRα1	[71, 74]

Overview mutations and effect TRα1 in humans - RTHα

Until now several patients with resistance to thyroid hormone α (RTHα) have been described with a total of 15 mutations [38, 63]. All mutations identified so far are localized in the LBD of TRα [3, 63].

RTHα results in hypothyroid features in patients [38]. Patients with RTHα have disproportionate growth retardation, mild to moderate mental retardation, skeletal

dysplasia, constipation, relative macrocephaly and cardiovascular dysfunction [38, 72]. The patients generally have a normal TSH, high-normal FT3, low-normal FT4, while the FT4/FT3 ratio is low and the T3/rT3 ratio high [38] .

Several patients have been treated with LT4 showing a normal response of the hypothalamic-thyroid axis, with a blurred effect on growth and overall height [63]. Patients are reported to have a more energetic feeling and better motor coordination with less constipation [38, 63]. The effect of LT4 treatment seems more clear during childhood than in adults [72].

Thyroid hormone binding proteins

CRYM is found in the inner ear, and mutations in CRYM have been associated with non-syndromic deafness [75]. Furthermore, CRYM is abundantly expressed in the central nervous system; predominantly in the cerebral cortex and in the cytoplasm of neurons [76]. T3 binding to CRYM depends on NADPH and thiol cofactors and it is hypothesized that CRYM may deliver TH to the mitochondria or the nucleus [77-79].

Thyroid hormone analogues

Triac (TA3) is an alternative thyroid hormone metabolite produced in the liver by deamination and decarboxylation of the alanine chain [80]. Compared with T3, TA3 has a higher preference for TRβ1 (3.5 fold) and TRα1 (1.5 fold) [80, 81]. TA3 treatment of RTHβ patients suppressed TSH and led to an increased metabolic rate in obese patients [82, 83]. However, due to the short half-life of TA3, higher therapeutic dosages are needed than of T3 [80, 83, 84].

OUTLINE OF THE THESIS

This thesis focuses on the clinical and molecular aspects of nuclear thyroid hormone action. In **chapter 2 and 3** we describe the phenotype of a girl and her father with a mutation in TRα1, and study the consequences of treatment with LT4. In **chapter 4** we investigate the effects of mutations and deletions of TRα1 and TRβ, as well as the consequences of hypothyroidism on deiodinase activity in cerebellum and liver. In **chapter 5** we analyse the role of MCT8 and MCT10 on biological availability of TH for either D3 or the nuclear TR. In **chapter 6,** we show that affinity labelling does not modify MCT8 and MCT10, but an intracellular binding protein. In **chapter 7** we explore the effect of TR ligands (TA3 and 3,5-T2) on gene expression profiles in a human hepatic cell line. Finally, in **chapter 8** we discuss the findings in this thesis and its implications in view of the literature.

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CHAPTER 2

Clinical phenotype associated with mutation of thyroid hormone receptor alpha 1 (TRα1)

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The action of thyroid hormone, which is essential for normal development and metabolism, is largely mediated by the binding of triiodothyronine (T3) to nuclear receptors (TRs), changing the expression of the genes responsive to thyroid hormone. Different TR isoforms are generated by the genes thyroid hormone receptor alpha (*THRA*) and thyroid hormone receptor beta (*THRB*). Mutations in *THRB* cause resistance to the action of thyroid hormone. This resistance is characterized by elevated serum levels of thyroid hormone, the absence of suppression of thyrotropin, and a variable phenotype [1]. Here we report on two Greek patients (the index patient and her father) who have a *THRA* mutation.

In the first 3 years of life, the index patient had macroglossia, omphalocele, congenital hip dislocation, no hip ossification centers, delayed closure of skull sutures, delayed tooth eruption, delayed motor development, and macrocephaly (with head size 1.65 SD above normal). Serum levels of free thyroxine (T4) were low, levels of T3 were high, and levels of thyrotropin were normal (Figure 1A).

At 6 years of age, she was evaluated for short stature, and bone age was clearly delayed (Figure 1B, and Figure 1 in the Supplementary Appendix). In addition to the finding of abnormal levels of thyroid hormone, laboratory testing revealed serum levels of insulin-like growth factor 1 (IGF-1) in the lower end of the normal range and high levels of cholesterol. The patient had clinically determined hypothyroidism, with dry skin, slow tendon reflexes, slow reactions and drowsiness. Treatment with levothyroxine resulted in initial catch-up growth and decreases in serum levels of thyrotropin and cholesterol. At 8.5 years of age, her height remained 2 SD below normal. Therapy with growth hormone was started and had little effect on growth (Figure 1B). At her most recent examination, performed when the patient was 11 years of age, her bone age was 9 years and her height 1.81 SD below normal. She has mild cognitive deficits (IQ 90).

The clinical characteristics of her father are very similar - including short stature (3.77 SD below normal), levels of free T4 in the lower end of the normal range, high levels of T3, and normal levels of thyrotropin – as are his responses to treatment with levothyroxine (75 µg per day) and his high cholesterol levels, levels of IGF-1 in the lower end of the normal range, suppressed growth hormone stimulation, and mild cognitive deficits (IQ 85). Both father and daughter have constipation, having stools every 2 to 4 days when not receiving levothyroxine and every day when receiving it.

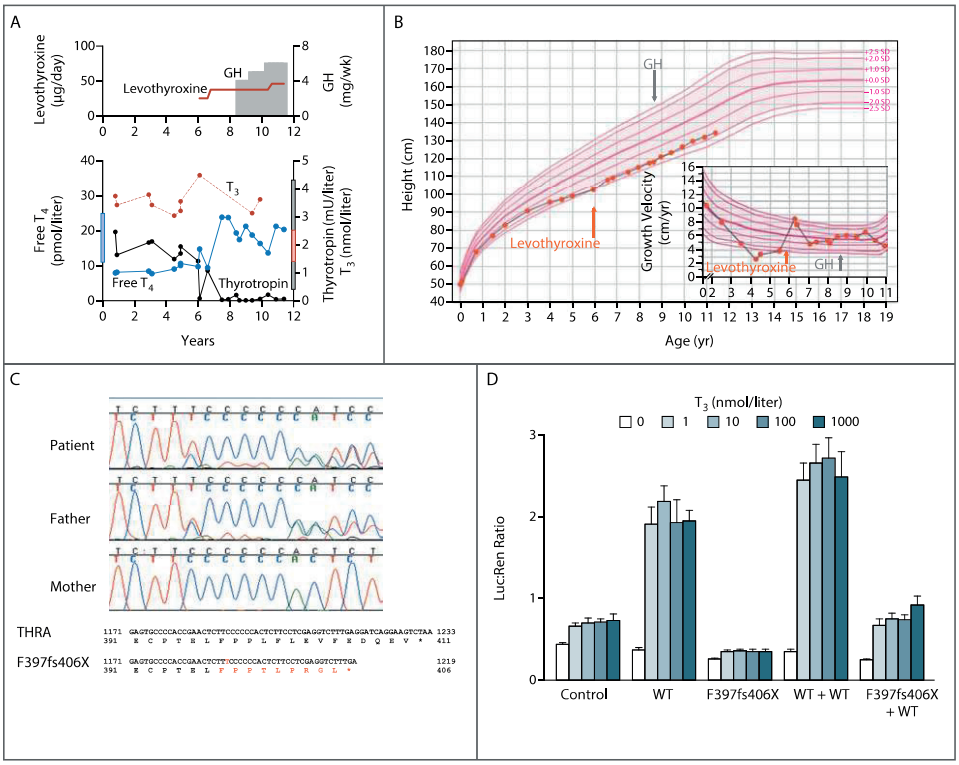

FIGURE 1. Clinical phenotype of the index patient and molecular analysis of *THRA*. Panel A shows the changes in serum levels of free thyroxine (T4), triiodothyronine (T3), and thyrotropin in the index patient from birth through 11 years of age (shaded areas in the vertical axis indicate reference ranges). Also shown are the doses of levothyroxine and growth hormone (GH) the patient received since the age of 6 years and 8 years, respectively. Panel B shows changes in height and in growth velocity (insert). The growth curve of the index patient (dots) is compared with the normal range in the Greek population. In Panel C, sequence profiles for part of exon 9 in *THRA* show the insertion of thymine (T) at codon 397 in the index patient and her father but not her mother. The mutation results in a frameshift, with an early stop at codon 406 (F397fs406X) instead of the natural stop at codon 411. In Panel D, functional tests of wild-type (WT) TRα1 and the F397fs406X mutant show that the latter does not respond to activation by T3. When cotransfected at equal amounts, the mutant exerts a strong dominant-negative effect over the wild type. The expression of firefly luciferase (Luc) is under the control of a nuclear-receptor (TR)–dependent promoter, and the expression of renilla luciferase (Ren) is under the control of a TR-independent promoter. Firefly luciferase activity was normalized to renilla luciferase activity to adjust for transfection efficiency (for further details, see the Supplementary Appendix). To convert the values for free T4 to nanograms per deciliter, divide by 12.87. To convert the values for T3 to nanograms per deciliter, divide by 0.0154. T bars indicate standard deviations.

Given the symptoms of hypothyroidism and delayed bone development – despite high levels of T3 – we hypothesized that there was a defect in the functioning of TRα1. Genomic sequencing revealed that father and daughter were heterozygous for the insertion of thymine at codon 397 in *THRA*, which resulted in a frameshift and an early stop at codon 406 (F397fs406X) (Figure 1C). This mutation was detected neither in more than 300 white controls nor in public databases. Mutations in *MCT8*, *MCT10*, *DIO1*, *DIO2*, *DIO3*, and *THRB* in the patient and her father were excluded by sequence analysis.

The analysis of cells cotransfected with a TR-dependent promoter-reporter construct showed marked T3 stimulation of wild-type TRα1, but no effect on the mutant receptor could be detected (Figure 1D). Furthermore, the mutant had a strong dominant negative effect on wild-type TRα1.

The delayed bone development in the index patient and her father is very similar to that recently reported patient by Bochukova et al. in another patient with a similar TRα1 mutant [2], which suggests that TRα1 plays a major role in bone development [3]. In addition, mice with a similar TRα1 mutant showed reduced endochondral and intra-membranous ossification, severe postnatal growth retardation, and delayed closure of skull sutures [4]. The transient delay in motor development and the mild cognitive deficits in our patients are consistent with the important role of TRα1 in brain development [5].

In conclusion, our findings and those of Bochukova et al. [2] indicate that mutant TRα1 is associated with abnormal levels of thyroid hormone but normal levels of thyrotropin as well as growth retardation, and mildly delayed motor and cognitive development (Table 2 in the Supplementary Appendix).

ACKNOWLEDGMENTS

We thank Drs. Edith Friesema and Monique Kester for sequence analyses of the transporter and deiodinase genes. This work was supported by ZonMw VENI Grant 91696017 (RPP) and an Erasmus MC Fellowship (RPP).

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SUPPLEMENTAL MATERIAL

Methods

Mutational analysis

Genomic DNA was extracted from blood using standard procedures. The coding sequence of all exons (2-10) of *THRA* was analyzed using the primers presented in Supplemental Table 1. PCR products were sequenced on an automated ABI PRISM® 3100 genetic analyzer sequencer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) or by Baseclear (Leiden, The Netherlands). Restriction enzyme XmnI (New England Biolabs, Ipswich, MA) was used for additional conformation of the mutation. Incubation with XmnI will not affect the PCR product of wild-type exon 9 (492 bp), but will generate two fragments (304 and 189 bp) from the mutated exon.

SUPPLEMENTAL TABLE 1. Primers used for PCR amplification and sequencing of *THRA*

Exon	Forward primer (5'-3')	Reverse primer (5'-3')
2	CCTTCCTACCGTGACACCTTC	CTGCTTAGGAGTTGGCATGA
3	ATGAGAAAGGGGCTACTCGAAG	CACATCCAGGTCCAGAGGAG
4	AGGGATGGGGAAAAGTGTG	GTAATATGGGGGCTCAGGTG
5	GTTGGTTCAGGAAGGGGAAG	GGTACCCTGGAAGGAAGCTG
6	TTCTCCAACCTGTACTCTAGGAAGA	TCCTGGAGGAGGCAAGACT
7	CTTGGAGCTCCCCCTGGT	TCCTTGTCCAGAGAACCTCAG
8	GGCTCCCGTAGGACACTCTA	ATTCAGGAGGGAGTTGAGCA
9	TCCCCTCTAGTCCTTTCTTCC	TGTGTGTGTGGGAGCTGAAT
10	CCAGAGGCTCATCTTGGAAT	AGAGGCCTGGGAGAAGGTAT

Functional analysis the TRα1 mutant

hTRα1 cDNA (SC307938) was obtained from OriGene Technologies (Rockville, MD), and subcloned in the pcDNA3 expression vector. The patient's mutation (c.1190-1191insT) was introduced in TRα1 using the QuickChange II Mutagenesis kit (Agilent Technologies, Amstelveen, The Netherlands) and confirmed by sequencing.

JEG3 cells and HepG2 cells were cultured in 96-well plates containing DMEM/F12 with 9% heat-inactivated fetal bovine serum and 100 nM sodium selenate. A construct containing a TRE-dependent firefly luciferase reporter and a control renilla luciferase reporter (pdV-L1) was used1. Cells were transfected for 48 h with 15 ng reporter plus 15 ng wild-type TRα1, mutant TRα1, and/or empty vector using X-tremeGENE 9

Transfection Reagent (Roche Diagnostics, Almere, The Netherlands). After washing, cells were incubated for 24 h with 1-1000 nM T3. After incubation, luciferase and renilla luciferase signals were analyzed in lyzed cells using the Dual-Glo Luciferase Assay System (Promega, Leiden, The Netherlands). Firefly luciferase activity was normalized to renilla luciferase activity to adjust for transfection efficiency.

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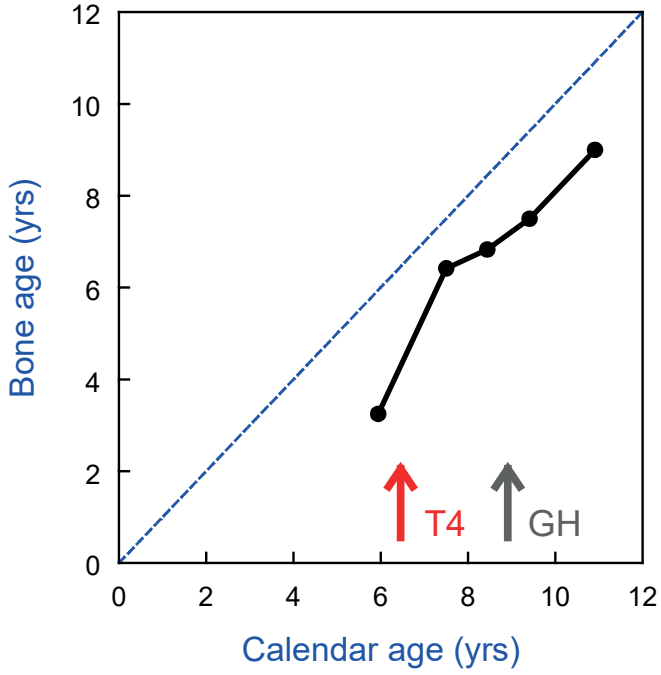
SUPPLEMENTAL TABLE 2. Genotype and phenotype comparison of patients with TRα1 mutations.

	TRα1-F397fs406X	**TRα1-E403X***
Genotype		
Mutation	Frame shift	Missense
Zygosity	Heterozygous	Heterozygous
Phenotype		
Bone development	Delayed	Delayed
Mental development	Mildly affected	Mildly affected
Motor development	Mildly affected	Mildly affected
Constipation	Mild	Severe
FT4	Low-normal	Low-normal
T3	High	High-normal
TSH	Normal	Normal

*Data from Bochukova E, Schoenmakers N, Agostini M, et al. A Mutation in the Thyroid Hormone Receptor Alpha Gene. The New England Journal of Medicine 2011.



SUPPLEMENTAL FIGURE 1. Delayed bone development in the index patient. The graph shows bone age determined by X-ray images of the left hand *versus* calendar age.

CHAPTER 3

Clinical phenotype of a new type of thyroid hormone resistance caused by a mutation of the TRα1 receptor; consequences of LT4 treatment

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ABSTRACT

Context Recently, the first patients with inactivating mutations in TRα1 have been identified. They have low (F)T4, high T3, low rT3 and normal TSH serum levels, in combination with growth retardation, delayed bone development and constipation.

Objective The aim of the current study was to report the effects of levothyroxine (LT4) treatment on the clinical phenotype of two patients (father and daughter) with a heterozygous inactivating mutation in TRα1.

Setting and Participants Both patients were treated with LT4 for the last 5 years. To evaluate the effect of LT4 treatment, LT4 was withdrawn for 35 days and subsequently re-initiated. Data were collected from medical records, by re-analysis of serum collected over the last 6 years, and by a detailed clinical evaluation.

Results Treatment with LT4 resulted in a suppression of serum TSH, normalization of serum (F)T4 and rT3, whereas T3 levels remained elevated in both patients. In addition, there was a normalization of the dyslipidemia, as well as a response in serum IGF1, SHBG and creatine kinase in the index patient. All these parameters returned to pre-treatment values when LT4 was briefly stopped. LT4 also resulted in an improvement of certain clinical features, such as constipation and nerve conductance. However, cognitive and fine motor skill defects remained.

Conclusion This study reports the consequences of LT4 treatment over a prolonged period of time in two of the first patients with a heterozygous mutation in TRα1. LT4 therapy leads to an improvement of certain but not all features of the clinical phenotype.

INTRODUCTION

The importance of thyroid hormone (TH) for normal development is illustrated by the severe consequences of untreated congenital hypothyroidism, which results in growth failure and permanent mental retardation. The production of TH by the thyroid gland is regulated by the hypothalamus-pituitary-thyroid axis (HPT-axis), in which pituitary TSH stimulates the thyroid to produce TH [1]. T4 represents the majority of TH secreted by the thyroid, whereas the biological activity of TH is largely mediated by binding of the active hormone T3 to its nuclear T3 receptor (TR). TRs function as ligand-dependent transcription factors, which regulate target gene expression by binding to T3 response elements (TREs) in the promoter region [2, 3].

Different TR isoforms are generated from the *THRA* and *THRB* genes by alternative splicing and different promoter usage, with TRα1, TRβ1 and TRβ2 as highly homologous T3 binding isoforms [3]. TRα1 and TRβ1 are widely expressed, and their expression is spatio-temporally regulated. TRα1 is preferentially expressed in brain, bone and heart, whereas TRβ1 is considered the major isoform in liver, kidney and thyroid [3, 4]. TRβ2 has a more restricted expression pattern regulating neurosensory development as well as the HPT-axis [5, 6].

Heterozygous mutations in the ligand-binding domain (LBD) of *THRB*, leading to impaired hormone binding and/or transcriptional activity of the receptor result in resistance to TH (RTH). RTH is a syndrome characterized by elevated serum TH levels and a non-suppressed TSH, and a variable phenotype including goiter, tachycardia and raised energy expenditure [7, 8].

Ever since its characterization in 1987, investigators have searched for patients with mutations in TRα1, which had not been identified until recently. The phenotype of the first patients with inactivating mutations in TRα1 includes abnormal thyroid function tests (low free T4 (FT4), high T3, but normal TSH levels), growth retardation, delayed bone development and constipation [9, 10]. In the current study we describe the consequences of treatment with levothyroxine (LT4) for different thyroid related phenotypes in two of these patients.

PATIENTS AND METHODS

Informed consent was obtained from the index patient and her parents. The index patient is a 12-year old girl from Greece [10]. The patient and her father have a heterozygous single nucleotide insertion in exon 9 of *THRA*, resulting in a frame shift and

alteration of the C-terminal domain of TRα1 (F397fs406X). As previously described, the clinical phenotype associated with this mutation includes growth retardation, delayed bone development, mildly delayed motor and cognitive development [10]. Because of hypothyroid symptoms, LT4 treatment was started at 6 years of age in the index patient. The initial LT4 dose was 1.15 µg/kg/day, corresponding to 25 µg LT4 per day. During follow up, the LT4 dose was adjusted based on serum FT4 levels to 37.5-45 µg per day. LT4 therapy resulted in a transient increase in growth [10]. Because of evidence of GH deficiency, GH therapy was started at 8.5 years of age. The index patient received a fixed dose of 0.15 mg/kg body weight/week, corresponding initially to 4 mg GH per week. At that age brain MRI was normal and there was no hearing defect. The father received LT4 treatment from 42 years of age in a daily dose of 50-75 µg. Before LT4 treatment, a TRH test with 200 µg TRH was performed in both patients.

In order to evaluate the effect of LT4 treatment, LT4 was stopped for 35 days at 11 years of age in the girl and at 47 years of age in the father, and a detailed clinical analysis was performed. Seven months after LT4 therapy was re-initiated, clinical analysis was repeated.

Neurological function was evaluated by: a questionnaire on neurological symptoms; assessment of the mental status by the mini-mental state examination (MMSE); a Raven test for IQ; evaluation of the visual-spatial orientation with GFSSFI cards; intelligence with the Weschler Abbreviated Scale of Intelligence; neurological examination, which included Phalen manoeuvre (forced complete flexion of the wrist), grading of muscle strength by the Medical Research Council (MRC) scale, assessment of tendon reflexes and sensory testing of pinprick, joint position and vibration in feet and hands. In addition, a neurophysiological profile was assessed, which consisted of the following parameters: 1) motor conduction of median, ulnar and fibular nerves with measurements of distal motor latency, motor conduction velocity and amplitude of compound muscle action potential; and 2) sensory conduction of median, ulnar and sural nerves with measurements of distal sensory latency, sensory conduction velocity and amplitude of sensory action potential. The neurophysiological examination was performed by employing standard methods, using surface electrodes and maintaining the limb's temperature between 32 and 34 C. Dual-energy X-ray absorptiometry (DEXA) was used to measure bone mineral density (BMD) in the lumbar spine and femoral head.

Thyroid function tests

In the last 6 years, serum samples of both patients had been collected before, during, and after treatment with LT4 and/or GH, and stored at -20˚C until analysis. In all samples we measured serum TSH, FT4, T4 and T3 levels by the Vitros® ECiQ (Ortho-Clinical-

Diagnostics, Amersham, United Kingdom); rT3 was measured by a commercial available radioimmunoassay (Zentech, Angleur, Belgium), and SHBG with the Immulite® 2000XPi (Siemens, Breda, The Netherlands). All other measurements (total cholesterol, LDL cholesterol, HDL cholesterol, creatinine kinase, hemoglobin, erythropoietin, GH, IGF1 and prolactin) were obtained during regular clinical follow-up.

Functional analysis of TRα1- F397fs406X mutation

To understand the *in vivo* effects of LT4 treatment, the possible dominant negative effect of mutant TRα1 on wild-type (WT) TRβ1 was studied *in vitro*, and if this could be overcome by high T3 concentrations. These *in vitro* studies were performed as previously described [10]. In brief, HepG2 cells cultured in 96-well plates were co-transfected with 15 ng TRE-luciferase construct, 15 ng of TK-renilla (Promega, Leiden, The Netherlands), and 10 ng of WT TRα1, mutant TRα1- F397fs406X, or 5 ng WT TRβ1 [10, 11]. As we described previously, mutant TRα1 has a dominant negative effect over WT TRα1 when transfected in a 1:1 ratio. In the current study, we analyzed the effect of co-transfection of mutant TRα1 on the transcriptional activity of WT TRβ1. Cells co-transfected with WT TRα1 and WT TRβ1 in equal ratios were used as a control. After washing and incubation for 24 h with 0-1000 nM T3, luciferase and renilla values were determined by a luminometer (Topcount® NXTTM, Packard instrument company, Meriden, CT, USA).

RESULTS

Serum thyroid function tests and consequences of treatment with LT4

Serum samples had been collected from the index patient and her father during the different periods off and on LT4 and/or GH therapy. In these samples, an extensive thyroid function profile was determined. Results for the index patient are presented in Figure 1. Before treatment, FT4 and T4 levels were low-normal, T3 was increased and rT3 was decreased, while serum TSH was normal. Treatment with LT4 was started at the age of 6 years. This resulted in a suppression of TSH, an elevation of FT4 and T4, and a normalization of rT3, while serum T3 increased slightly. Additional treatment with GH was started at the age of 8.5 years. This was accompanied by a decrease in FT4, T4, T3, and rT3 levels, which may also be due to a relative decrease in the dose of LT4 per kg body weight. The T3/rT3 ratio and T3/T4 ratio were highly elevated before treatment (Figure 1); they were decreased by LT4 treatment but not affected by GH therapy. The rT3/T4 ratio was in the normal range and did not change during treatment. TSH remained suppressed until LT4 was temporarily stopped at the age of 11 years. This resulted in a steep increase in TSH levels, as well as a marked decrease in serum T4, FT4

and rT3 to low-normal levels and of T3 levels to the upper limit of the normal range. Re-initiation of LT4 therapy resulted in a suppression of serum TSH, a normalization of FT4 and rT3, while T3 levels increased.

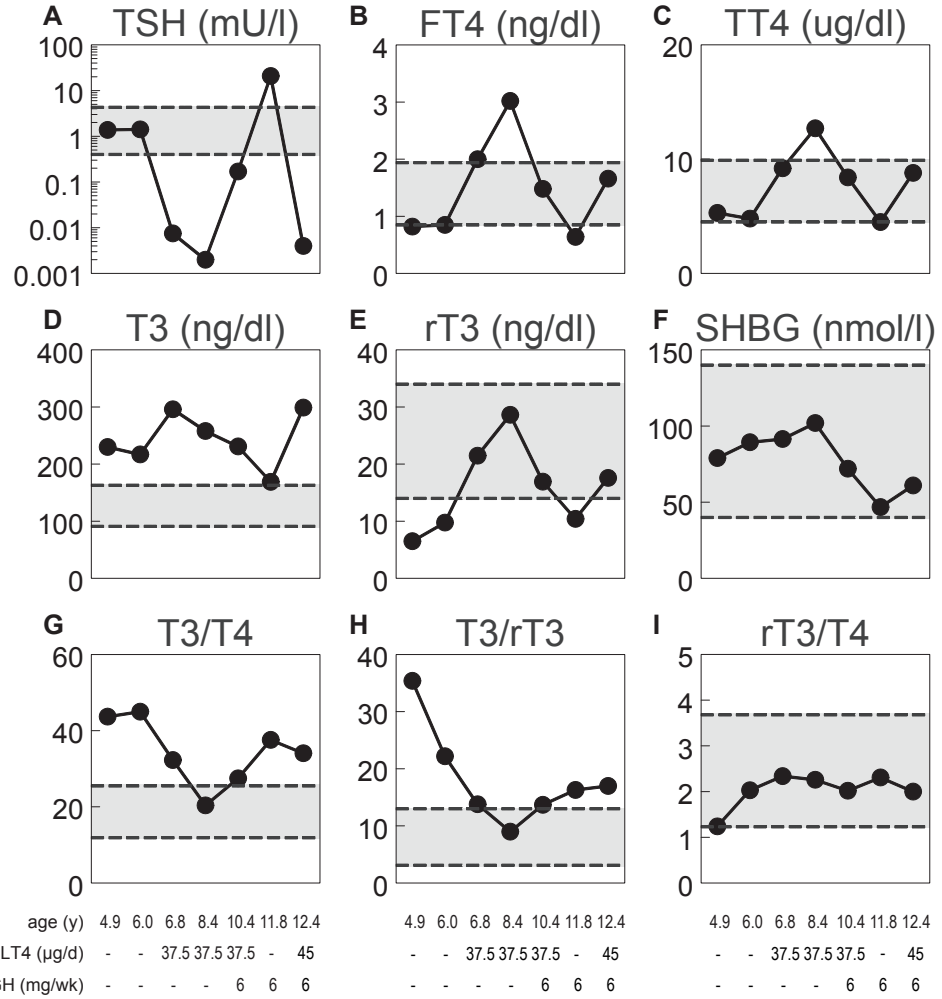

FIGURE 1. Serum thyroid function tests and SHBG levels in samples that were collected from the index patient under different treatment modalities (on and off LT4 and/or GH therapy) at different time points. **A**: TSH, **B**: FT4, **C**: TT4, **D**: T3, **E**: rT3, **F**: SHBG, **G**: T3/T4 ratio, **H**: T3/rT3 ratio, **I**: rT3/T4 ratio. Horizontal lines represent the different reference ranges.

Also in the father, FT4 and T4 levels were low-normal, T3 was increased and rT3 was decreased in combination with a normal TSH before treatment (Figure 2). LT4 treatment resulted in a suppression of serum TSH and a normalization of serum (F)T4 levels, whereas serum T3 remained elevated. Serum rT3 increased, but remained in the low-normal range. TSH levels normalized when LT4 was temporarily stopped. LT4 withdrawal resulted in low serum (F)T4 and rT3 levels, whereas T3 decreased to the upper limit of the normal range (Figure 2). When LT4 was restarted, TSH and iodothyronine levels returned to their previous treatment values. The elevated T3/rT3 and T3/T4 ratios also decreased by LT4 treatment, whereas the normal rT3/T4 ratio did not change during treatment. Thus, the effects of LT4 therapy on the different iodothyronine levels and their ratios showed a similar pattern in the father and index patient.

The negative feedback of TH on TSH secretion is predominantly regulated by serum FT4 [12, 13]. There was a clear, log-linear negative relationship between TSH and FT4 in both patients (Figure 3A,B), suggesting that the negative feedback of FT4 on TSH secretion is intact. Before LT4 therapy, pituitary function was additionally tested with a TRH test, which showed a sub-normal TSH response but normal prolactin response in both patients (Figure 3C,D).

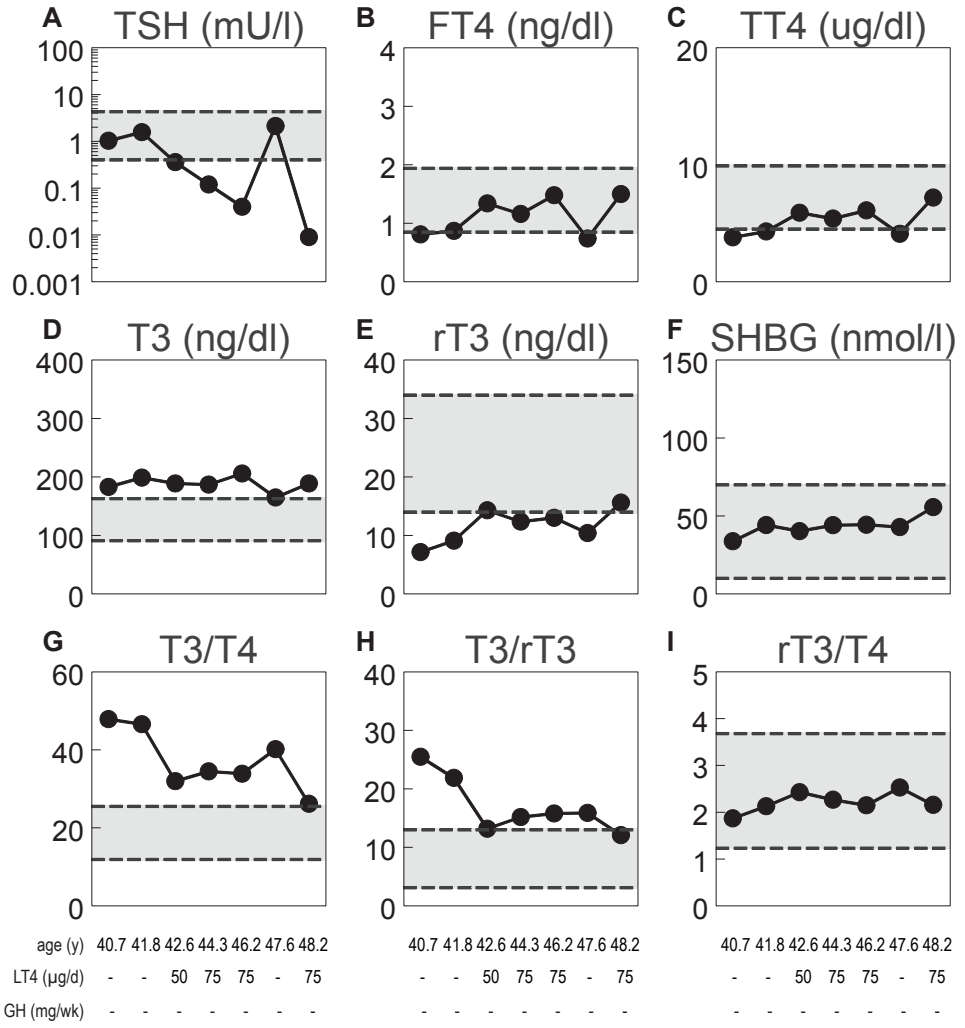

FIGURE 2. Serum thyroid function tests and SHBG levels in samples that were collected from the father on and off LT4 therapy at different time points. **A**: TSH, **B**: FT4, **C**: TT4, **D**: T3, **E**: rT3, **F**: SHBG, **G**: T3/T4 ratio, **H**: T3/rT3 ratio, **I**: rT3/T4 ratio. Horizontal lines represent the different reference ranges.

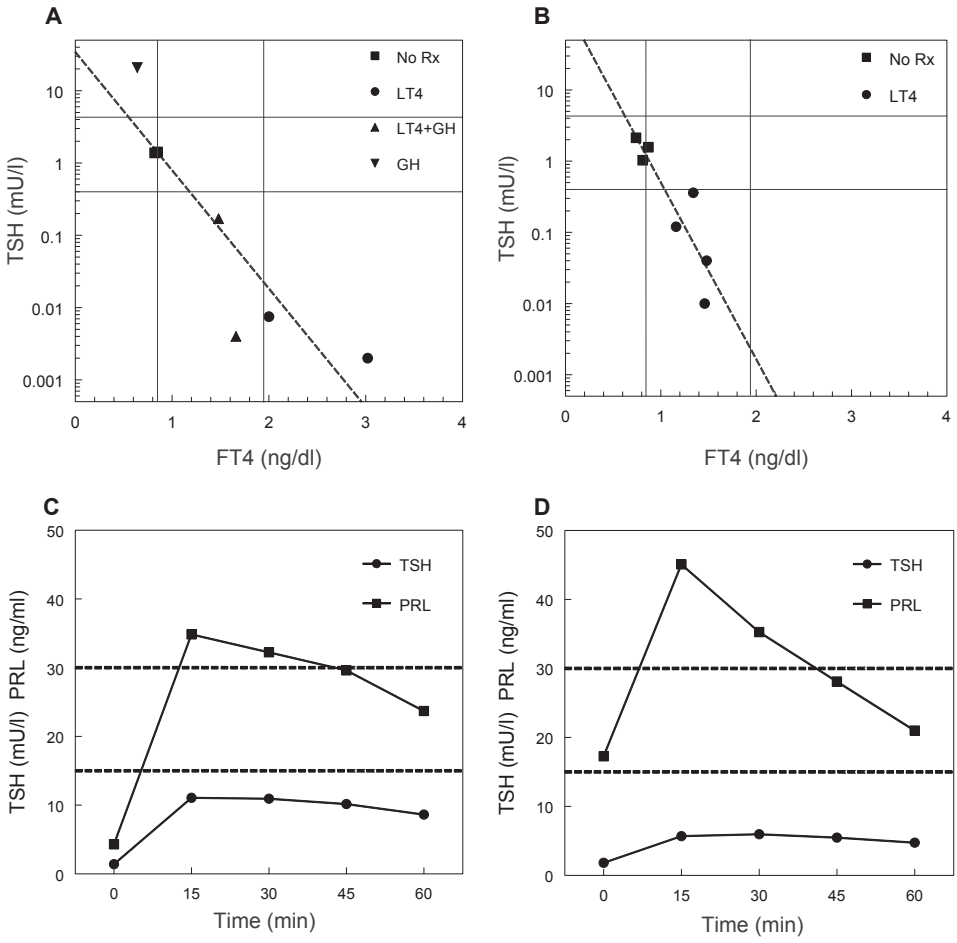

FIGURE 3. Serum TSH plotted as a function of serum FT4 levels, in the index patient (**A**) and the father (**B**). The different symbols represent the different treatment modalities (on and off LT4 and/or GH therapy) of the patients when serum thyroid function tests were determined. The regression line is based on all data points. Reference ranges are indicated by the horizontal and vertical lines. Serum TSH and PRL levels after TRH stimulation in the index patient (**C**) and father (**D**).

Serum markers reflecting thyroid state and consequences of treatment with LT4

Total and LDL cholesterol levels, which are increased in hypothyroid patients, were clearly elevated in both patients despite the elevated serum T3 levels (Figure 4). In the index patient, LT4 treatment resulted in a normalization of the elevated total and LDL cholesterol levels (Figure 4A). After 35 days of LT4 withdrawal, both total and LDL cholesterol returned to their elevated pre-treatment values. Re-initiation of LT4 resulted in a normalization of the serum cholesterol levels. There was a significant negative correlation of total and LDL cholesterol with serum FT4 levels (Figure 4B), but not with serum T3 levels (data not shown). The relationship between FT4 and cholesterol levels in the father could not be studied, because he is currently treated with rosuvastatin for his dyslipidemia.

Serum IGF1 levels, which are known to be influenced by thyroid function, were low to low-normal in both patients. IGF1 was increased by LT4 treatment in the index patient but not in the father (Figure 4C,D). GH stimulation tests were performed during LT4 therapy, showing a subnormal response to both clonidine and L-DOPA in the index patient, and a blunted response to clonidine in the father (data not shown). IGF1 levels were normalized in the index patient by treatment with LT4 plus GH. Her IGF1 levels decreased in response to LT4 withdrawal, and increased again after LT4 continuation (Figure 4C). In the father, no clear changes in IGF1 levels were observed during temporary LT4 withdrawal (Figure 4D).

In the index patient, serum SHBG levels were in the normal range and roughly followed serum FT4 levels in the different treatment periods (Figure 1). Also in the father, serum SHBG was normal but it showed little response to LT4 treatment (Figure 2). Serum creatine kinase was clearly responsive to LT4 treatment in the index patient (on-off-on LT4: 149-196-121 U/L [reference range <190 U/L]) but this was less clear in the father (not shown).

Other laboratory findings were a normocytic anemia with low red blood cell count and low erythropoietin levels in the index patient. Her hemoglobin did not respond to LT4 treatment (11.5 and 10.6 g/dL before and on LT4, respectively). The father had a similar normocytic anemia which normalized during LT4 treatment (hemoglobin 10 and 14.2 g/dL before and one year on LT4, respectively).

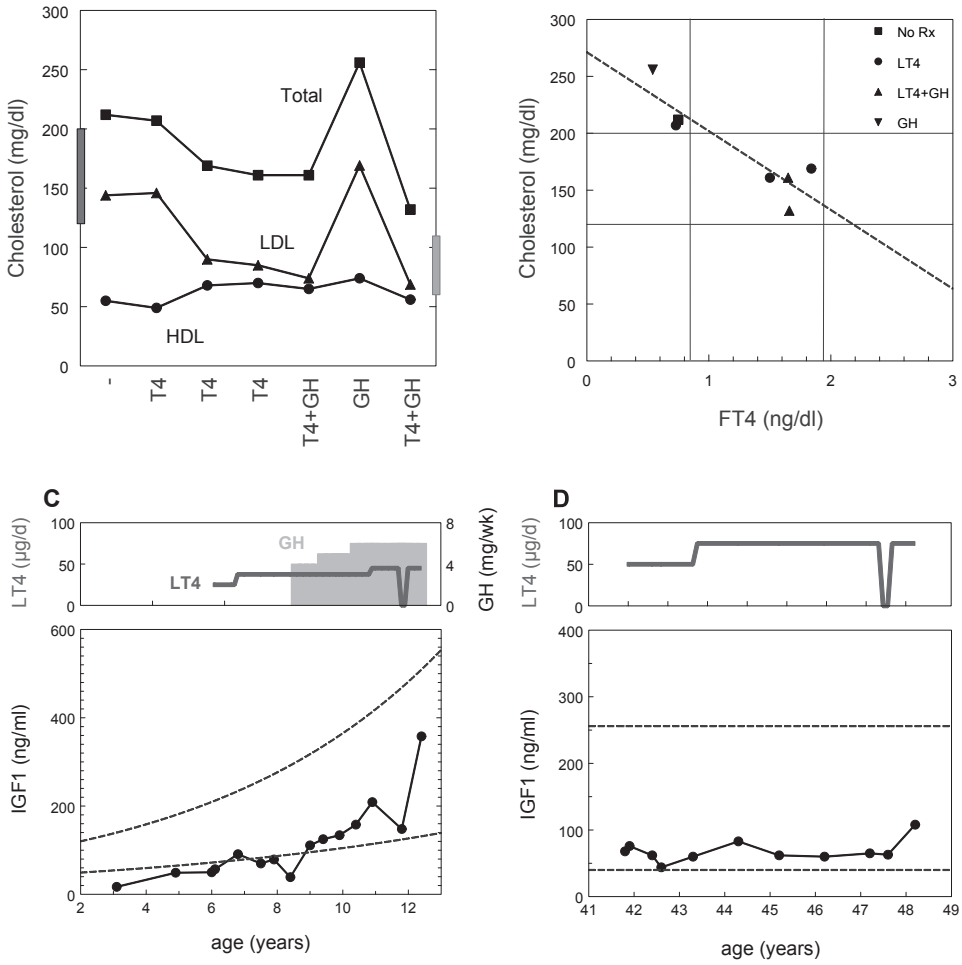

FIGURE 4. Serum total cholesterol, LDL cholesterol and HDL cholesterol values in relation to different treatment modalities (on and off LT4 and/or GH therapy) in the index patient. The reference range of total cholesterol is indicated with the shaded area in the left vertical axis, while the reference range of LDL cholesterol is indicated with the shaded area in the right vertical axis. The reference range for HDL cholesterol is 30-65 mg/dl (**A**). Serum total cholesterol plotted as a function of serum FT4 during different treatment modalities in the index patient (**B**). Serum IGF1 levels determined over the years in the index patient (**C**) and the father (**D**), in relation to T4 dose (µg/day) and/or GH dose (mg/week). Stippled lines indicate IGF1 reference ranges

Additional description of the clinical phenotype, and consequences of LT4 treatment

To study the effect of LT4 treatment on other aspects of the clinical phenotype, both patients underwent a detailed clinical examination 35 days after LT4 withdrawal and 7 months after LT4 treatment was re-initiated. Off LT4, the index patient had a heart rate of 88±3.2 bpm (mean±SEM, n=7 measurement on 2 separate days), a normal blood pressure (106±2.4 over 56±2.6 mm Hg) and normal body temperature (36.2±0.2 C) during admission. Electrocardiography was normal, both in the index patient and her father, with a normal duration of the QRS complex (103 ms and 110 ms, respectively), as well as a normal corrected QT interval (381 ms and 414 ms, respectively). Furthermore, echocardiography showed no major clinical abnormalities. At 12 years of age, she was pubertal (breast development Tanner stage II), she was pale, had a small soft goiter and slow deep tendon reflexes. Her height was 137 cm and her weight was 40 kg, corresponding to a high-normal BMI of 21. She did not report more drowsiness and did not get tired more rapidly, but as we described previously, she did report more constipation after the LT4 was stopped (stool frequency off LT4 every 2-3 days, while it was every day on LT4) [10].

Evaluation by a paediatric neurologist at the age of 11 years when the patient was off LT4 showed that she had mild coarse features and difficulties in running tests requiring coordination. She scored 19 out of 30 points on an MMSE test (<23 points indicates mild cognitive impairment). She was delayed in orientation, attention, calculations and language. Her mental age based on drawing of a human figure corresponded to a child at the age of 7 years. Her visual-motor coordination corresponded to a girl of 9 years. Her general behaviour was characterized by slowness but also by significant improvement of her performance after reinforcement. Memory evaluation by a neuropsychologist revealed that she had mild cognitive deficits especially to code new materials acoustically and verbally, and mild problems concerning attention and working memory. Her IQ was 90 but her functional ability was normal. Her hearing, evaluated by an ear-nose-throat physician, was normal. In conclusion, she had a 3-5 year delay in higher mental functions, corresponding to the age of 7-9.

Seven months after the re-initiation of LT4 treatment, she was re-admitted for clinical evaluation. Her heart rate had slightly increased to 94±2.0 bpm (n=7) and the blood pressure was 99±0.6 over 55±1.3 mmHg. Her body temperature was still 36.2±0.1 C. She reported that the stool frequency had normalized. Breast development had progressed to Tanner stage III. Her neurological and developmental assessment had not improved significantly. She was still behind in orientation, attention, calculations and language,

and she had the same score with the MMSE and IQ test as in the period off LT4. The only difference was that she was much more energetic and that her defecation pattern had improved.

The father was clearly overweight, with a BMI of 36. Also in the father, the heart rate increased after the re-initiation of LT4 treatment (from 75 to 90 bpm), whereas blood pressure (125/70 mm Hg) and body temperature (36.2 C) were unaffected. Neuropsychological evaluation resulted in the same IQ on and off LT4 (IQ=85). The father has no cognitive deficits but off LT4 treatment a mild delay in recall of new materials and processing speed was noted compared with his functioning on LT4 treatment. It should be noted that the father has acquired hearing loss, which is most likely due to otosclerosis (confirmed by CT scan). Since the father used a hearing device during the neurocognitive evaluation, it is unlikely that the hearing deficit influenced the results of the evaluation.

Neurophysiological analysis of the index patient showed mildly affected distal motor latency prolongation and borderline sensory conduction velocity in the median nerve, findings which suggest, in association with the normal measurements in all other nerves, a subclinical carpal tunnel syndrome (Table 1). The patient's father had typical bilateral sensory symptoms of carpal tunnel syndrome, experienced severe difficulty making fine hand movements and had a positive Phalen manoeuvre. Clinical examination revealed atrophy of the thenar muscles and slow relaxation of Achilles tendon reflex bilaterally. The findings of the nerve conduction studies supported the diagnosis of a severe bilateral carpal tunnel syndrome in the father (Table 1), whereas measurements in the ulnar nerve were normal in both patients (data not shown). A follow-up neurophysiological examination after LT4 re-initiation showed a normalization of the motor conduction of the median nerve in the index patient, as well as a slightly improved (but not normal) motor conduction in the father (Table 1).

TABLE 1. Electrophysiological measurements of the median nerve in the index patient and her father

		Index patient		Father		Reference range
		Off LT4	On LT4	Off LT4	On LT4	
Motor conduction study	distal motor latency (ms)	**4.1**	3.4	**11.8**	**10**	<3.5 (children) <4.1 (adults)
	amplitude of compound muscle action potential (mV)	6.2	9.4	**2.3**	**4.5**	>5
	motor conduction velocity wrist to elbow (m/s)	65	59	51	53	>50
Sensory conduction study	distal sensory latency (ms)	2.4	2.7	**absent**	**absent**	<2.8
	amplitude of sensory action potential (mV)	21	15			>7
	sensory conduction velocity wrist-2nd finger (m/s)	51	55			>50

The neurophysiological examination was performed by employing standard methods, using surface electrodes and maintaining the limb's temperature between 32 and 34 C. **Underlined** data indicate abnormality.

In the index patient, BMD was 0.911 g/cm2 (Z-score = +0.3 SD, T-score = -0.3 SD) in the lumbar spine and 0.881 g/cm^2 (Z-score = +0.3 SD, T-score = -1,5 SD) in the femoral head. In addition, the father had a normal BMD of 1.110 g/cm^2 (Z-score = +0.5 SD, T-score = +0.2 SD) in the lumbar spine and 1.209 g/cm^2 (Z-score = +1.5 SD, T-score = +1.2 SD) in the femoral head.

Functional analysis of TRα1- F397fs406X mutation

As described previously, the TRα1- F397fs406X mutant showed a complete lack of T3 activation and a dominant-negative effect towards WT TRα1, which could not be overcome by high concentrations of T3 [10]. However, a clear beneficial effect of LT4 treatment was observed on clinical characteristics such as dyslipidemia and constipation. Since the effects of TH on cholesterol metabolism seem to be mediated predominantly via TRβ [14], we evaluated if the TRα1- F397fs406X mutant had a dominant negative effect on TRβ1 as well. Co-transfection studies revealed a dominant-negative effect of mutant TRα1 on WT TRβ1 in the presence of low concentrations of T3, but in contrast to the dominant-negative effect on WT TRα1, the dominant-negative effect on WT TRβ1 could be partially overcome by higher concentrations T3 (10-1000 nM) (Figure 5).

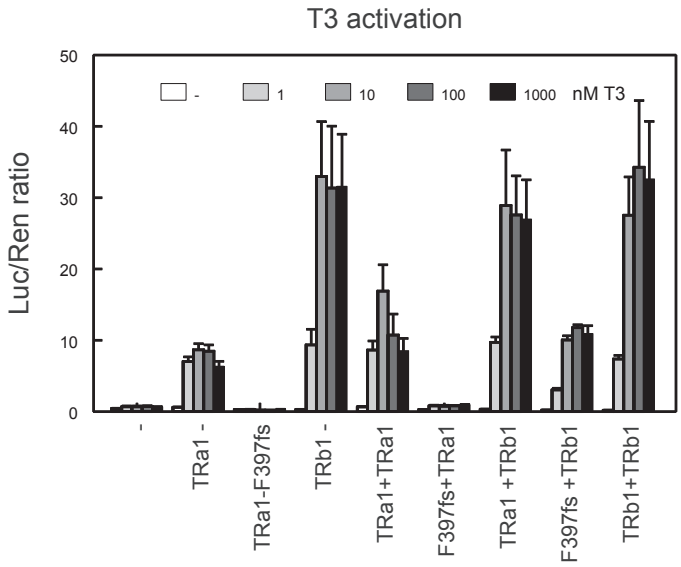

FIGURE 5. Functional analysis of WT TRα1, WT TRβ1 or TRα1-F397fs406X mutant alone or in combination. The nuclear receptors were co-transfected in cells with a TRE-luciferase reporter construct and TK-renilla, and cells were incubated for 24 h with increasing concentrations of T3 (0-1000 nM). As we described previously, mutant TRα1 has a dominant negative effect over WT TRα1 when transfected in a 1:1 ratio (F397fs+TRa1) [10]. In the current study, we analyzed the effect of co-transfection of mutant TRα1 on WT TRβ1 (F397fs+TRb1). Cells co-transfected with double amounts of WT TRα1 (TRa1+TRa1), double amounts of WT TRβ1 (TRb1+TRb1), or a combination of both receptors (TRa1+TRb1) were used as controls.

DISCUSSION

In the current study we investigate the consequences of LT4 treatment in two of the first patients (father and daughter) with a dominant-negative mutation in the C-terminal domain of TRα1 [10]. We demonstrate an effect of LT4 treatment on different serum markers reflecting tissue thyroid state (especially total and LDL cholesterol levels) and certain features associated with hypothyroidism, but not on cognitive performance or fine motor skills. *In vitro* analysis showed that mutant TRα1 had a dominant negative effect on WT TRβ1, which could be partially overcome by high doses of T3. For a comparison of the clinical features of patients with inactivating mutations in TRα1 and TRβ, the reader is referred to recent reviews [7-10, 15, 16].

The altered thyroid function tests in all three patients with TRα1 mutations that have been described so far is remarkable considering the primary importance of the TRβ2 isoform

in the HPT-axis [3, 9, 10]. The current study demonstrates that the negative feedback of FT4 on TSH secretion is intact in both TRα1- F397fs406X patients, which is in line with a predominant role for TRβ2 in the HPT-axis [17]. Also the marked suppression of serum TSH by relatively small increases in serum FT4 argues for a normal feedback action and thus for a minor role of TRα1 therein. A decreased TSH response was seen after TRH testing despite normal basal levels of TSH. This suggests a decreased sensitivity of the thyrotrophs to TRH, which may be caused by the elevated serum T3 levels. Despite the normal relationship between TSH and FT4, both patients have clearly disturbed serum T4, T3 and rT3 levels, and ratios thereof, suggesting altered peripheral TH metabolism by the deiodinases D1-3 [12, 18-20]. As a result of the low (F)T4 and rT3 levels, the rT3/T4 ratio is in the (low) normal range in both patients.

Interestingly, TRα1PV mutant mice, with a very similar frame-shift mutation in TRα1 as in our patients, have elevated levels of T3 in combination with a normal TSH as well [21, 22]. These mice have markedly increased mRNA and activity levels of liver and kidney D1 [21, 22]. Increased activity of D1, which plays a key-role in the production of serum T3 from T4 and in the degradation of the metabolite rT3 [18, 23], will contribute to the elevated T3/T4 and T3/rT3 ratios as observed in both patients. Since D1 is a T3-responsive gene, the increased D1 expression in these animals may be the cause as well as the consequence of the elevated T3 levels. However, since D1 activity is not different between TRα1PV mutant and WT mice under hypothyroid conditions, it is more likely that the elevated D1 in TRα1PV mutant mice is the result rather than the cause of the elevated serum T3 levels [22]. In contrast to TRα1PV mutant mice, TRα1-/- mice have normal liver D1 activity [24]. Cortex D2 activity, which plays an important role in local T3 production in the brain, is not different between WT and TRα1PV mutant mice under euthyroid conditions [22]. In addition to an increased D1 activity, a decreased degradation of TH by D3 could also contribute to the high T3 and low rT3 levels observed in patients with TRα1 mutations. It has recently been shown that TRα1 mediates the up-regulation of D3 by T3, and that TRα1-/- mice display an impaired regulation of D3, resulting in a reduced clearance rate of T4 and in particular T3 [24, 25]. This may contribute to the alterations in serum T3 and rT3 levels observed in our patients. Although TRα1PV mutant mice have normal cortex D3 activity under euthyroid conditions, they completely lack T3-induced D3 expression [22]. This results in a decreased T3 clearance when T3 levels are high [22]. Whether the changes in iodothyronine levels in patients with TRα1 mutations are due to an increased D1 activity, a decreased D3 activity, or combination of both remains to be determined in future studies.

GH treatment was associated with a decrease in T4 and rT3 levels. This could be due to the stimulatory effect of GH treatment on D1 activity [26]. Nevertheless, the observation

that the T3/T4 ratio was not affected by GH treatment argues against this hypothesis. Also a relative decrease in the dose of LT4 per kg body weight with increasing age may have contributed to the decrease in serum FT4 and T3.

Cessation and re-initiation of therapy provided the opportunity to study the direct effects of LT4 treatment on different markers reflecting tissue thyroid status. Different serum parameters, known to be altered during hyper- and hypothyroidism, were measured. Both patients suffered from clear dyslipidemia, which is remarkable in view of the high T3 levels. It has been shown in rodents that TRβ is necessary for the stimulatory effects of T3 on cholesterol metabolism [14], and TRβ selective agonists have lipid-lowering effects in humans [27]. However, the dyslipidemia in both patients may suggest an involvement of TRα1 in lipid metabolism as well. Our *in vitro* data, demonstrating a dominant negative effect of mutant TRα1 on TRβ1 in transfected cells, suggest that at least part of the dyslipidemia may be caused by dominant-negative effects of mutant TRα1 on hepatic TRβ1 function. Whereas we previously showed that the dominant-negative effect of mutant TRα1 on WT TRα1 was resistant to high levels of T3 [10], the current study suggests that high doses of T3 can partially overcome the dominant-negative effects on TRβ1 *in vitro*. Although it is presently unknown to what extent TRα1 and TRβ1 are expressed in the same human liver cells, this mechanism may very well contribute to the beneficial effects of LT4 treatment on the dyslipidemia in the index patient. However, other causes of the dyslipidemia unrelated to the TRα1 mutation cannot be excluded.

Other serum markers of thyroid state also showed a response to LT4 therapy. Serum IGF1 levels have been shown to be regulated by TH via direct effects as well as via effects on GH secretion [28]. Without a change in GH treatment, cessation of LT4 therapy resulted in a significant drop in serum IGF1 levels in the index patient. This is in agreement with findings by Bochukova and co-workers, who reported an increase in IGF1 levels after LT4 treatment of their patient [9]. In the father, serum IGF1 did not respond to LT4 treatment.

Serum SHBG, of which the hepatic synthesis is stimulated by TH [29], was normal despite the elevated serum T3 levels. This suggests that the liver may be partially resistant to the high T3 levels. In contrast, the patient described by Bochukova *et al*. had high SHBG levels, independent of LT4 treatment [9]. On LT4 treatment, CK levels were normal in both of our patients. SHBG levels decreased and CK levels increased in the index patient when LT4 was stopped. The increase in CK levels after LT4 cessation might suggest a relatively low thyroid state in skeletal muscle, since serum CK levels increase in hypothyroidism [30, 31]. Together, these markers suggest tissue-specific responsiveness to LT4 in patients with TRα1 mutations.

Some clinical parameters also responded to LT4 treatment. The effect on constipation, which clearly improved after LT4 treatment in both patients [10], was most evident and is in agreement with the findings by Bochukova et al [9]. In addition, the index patient was more energetic on LT4 therapy, and both patients showed improved motor conductance of the median nerve. The father had severe carpal tunnel syndrome as well, which did not respond to LT4 treatment, possibly because of the chronic nature of the lesion and the secondary degeneration of the nerve fibres. Interestingly, similar findings of carpal tunnel syndrome have also been described in acquired hypothyroidism, predominantly in adults [32]. In contrast to the bradycardia in the patient of Bochukova *et al.*, our patients had a normal heart rate which appeared to increase on LT4 therapy [9]. However, based on the high serum T3 levels a higher heart rate would have been expected [9, 10]. This relative bradycardia is in agreement with findings in TRα$^{0/0}$ mice and TRα1-R384C mice [25, 33].

With regard to other features of the clinical phenotype, there was no response to LT4 treatment. In agreement with Bochukova et al., blood pressure did not respond to LT4 treatment [9], nor did body temperature. TH therapy did not improve the mild cognitive defects of both patients either, nor did it result in a significant improvement of the developmental assessment of the index patient. This lack of effect of LT4 treatment on cognition might be due to the fact that TRα1 is the principal receptor expressed in brain [34-36], and that the dominant-negative effect of mutant TRα1 on WT TRα1 is not overcome by high levels of T3 [10]. At present, the index patient is still 3-5 years behind with regard to higher mental functions. This may be irreversible, given the importance of TRα1 in early development, since TRα1 is already expressed in brain at week 10 of gestation. Interestingly, TRα1-R384C mice have persistent locomotor deficiencies that can be prevented by early post-natal treatment with TH [37], but TH treatment in adulthood does not improve these locomotor deficiencies. However, in contrast to the TRα1-F397fs406X mutation, which results in a complete lack of T3 activation even at very high doses of T3, the loss of function of TRα1-R384C is overcome by higher T3 concentrations [37]. This suggests that patients with milder loss of function mutations in TRα1 may benefit from early LT4 therapy.

Both patients had a normocytic anemia, which is frequently associated with hypothyroidism [38, 39]. This is in line with studies in rodents, since TRα$^{-/-}$ mice have compromised fetal and adult erythropoiesis [40]. Serum levels of erythropoietin were low in both patients, and in the index patient the anemia did not improve after LT4 therapy, while the anemia normalized in the father.

Both patients had a normal BMD compared to age-matched controls. These findings are in contrast to TRα$^{-/-}$ mice, which have osteosclerosis with increased trabecular bone mass at adult age. Moreover, heterozygous mutant TRα1 mice have an even more severe phenotype of increased bone mass [41].

The addition of PTU to the LT4 therapy of both patients may normalize serum T3 levels, by blocking D1 activity. This could be beneficial by reducing thyrotoxicosis in cells that predominantly express TRβ. However, since we did not see clear clinical features of hyperthyroidism in these patients, and since we even observed beneficial effects of LT4 therapy on the dyslipidemia, which is predominantly mediated via TRβ, we have refrained from treating our patients with PTU.

In conclusion, this report studies the consequences of LT4 treatment in two patients with a heterozygous mutation in TRα1, resulting in a new type of reduced sensitivity to TH. Treatment with LT4 leads to an improvement of certain features of the phenotype, but cognitive and fine motor skill defects remained. The identification of additional patients will provide more insights into the exact mechanisms involved and the possible beneficial effects of LT4 treatment.

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CHAPTER 4

Opposite regulation of the thyroid hormone-degrading type 3 deiodinase in brain and peripheral tissues by thyroid hormone and thyroid hormone receptor subtypes

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MANUSCRIPT IN PREPARATION

ABSTRACT

Patients with mutations in the T3 receptor TRα1 show developmental abnormalities as well as relatively low serum T4 and rT3 levels, high T3, and normal TSH levels. We hypothesized that these altered thyroid function tests result from defective T3-dependent expression of the type 3 deiodinase (DIO3), the enzyme that catalyses the degradation of T3 and production of rT3 out of T4. This hypothesis was tested by studying the effects of deletion or mutations of TRα1 and TRβ, as well as the consequences of hypothyroidism on deiodinase activity in cerebellum and liver. Mice heterozygous for the dominant-negative TRα1-PV mutation showed decreased Dio3 activity in cerebellum as well as liver. Mice made hypothyroid by genetic and pharmacological methods showed the expected decrease in cerebellum Dio3 activity but surprisingly also an increased hepatic Dio3 activity. The hypothyroidism-induced decrease in cerebellum Dio3 activity was largely prevented in TRα1-/- mice but not in TRβ1-/- mice. The negative control of liver Dio3 by TH was still observed in TRα-/- mice and to some extent also in TRβ-/- mice. These results suggest opposite regulation of Dio3 expression by T3 and T3 receptor subtypes in mouse brain and liver.

INTRODUCTION

Thyroid hormone (TH) is essential for normal development and metabolic control of tissues [1-3]. It has become increasingly clear that these processes are controlled by paracrine and autocrine mechanisms regulating TH bioactivity at the tissue level. Important components of these regulatory mechanisms are TH transporters, deiodinases which catalyse the activation or inactivation of TH, and TH receptors which mediate tissue and receptor subtype-specific TH actions.

Peripheral TH metabolism is predominantly mediated by 3 iodothyronine deiodinases (DIO1-DIO3) [4]. These enzymes catalyse the outer ring deiodination (ORD) of the prohormone T4 to the bioactive T3 (DIO1, DIO2) and/or the inner ring deiodination (IRD) of T4 and T3 to the receptor-inactive metabolites rT3 and 3,3'-T2 (DIO1, DIO3). DIO1 is importantly expressed in liver and kidney, where it is a major site for serum T3 production and clearance of rT3. DIO2 is expressed at high levels in brain, pituitary and brown adipose tissue (BAT), where it is responsible for the local conversion of T4 to T3. DIO3 is highly expressed in the adult brain, even more so in fetal brain, as well as other fetal tissues. It has an important function in local TH regulation by mediating the degradation of both T4 and T3. It is generally accepted that DIO1 and DIO3 expression are up-regulated by TH, whereas DIO2 activity is down-regulated by TH.

There are two genes coding for the T3 receptors, *THRA* and *THRB*. Multiple receptor isoforms are generated from both genes, with TRα1, TRβ1 and TRβ2 as the most important hormone-binding subtypes. T3 receptors are widely expressed, with TRα1 being predominant in brain, bone and heart, TRβ1 in liver, kidney and thyroid, and TRβ2 in hypothalamus, pituitary and neurosensory organs [5, 6]. An extensive literature exists about patients with resistance to thyroid hormone (RTHβ) caused by mutations in TRβ1/2, but only a limited number of patients have been reported with RTHα due to mutations in TRα1 with the first as recently as 2012 [7-9]. Patients with RTHα show markedly delayed bone development, various degrees of motor and cognitive disabilities, and mild to severe constipation. In addition, they have a peculiar combination of thyroid function tests, consisting of relatively low serum T4, relatively high T3, low rT3, and normal TSH levels. It has been demonstrated that the up-regulation of DIO3 in brain by T3 is mediated specifically by TRα1 [10]. Therefore, we hypothesized that the high T3 and low rT3 levels in serum of RTHα patients are largely due to impaired expression of DIO3.

To test this hypothesis, we studied the regulation of deiodinase expression in general in different tissues of mice with a mutation or deletion of TRα1 or TRβ, as well as in mice

made hypothyroid by genetic and pharmacological approaches. Surprisingly, our results indicate that, in contrast to the positive regulation of Dio3 in brain, Dio3 expression in liver is under negative control of TH.

MATERIALS AND METHODS

Animal studies

TRα1-PV mice, TRα1 knockout (KO) mice, TRβ heterozygous and homozygous KO mice, Pax8 KO mice, Trhr1 KO mice and TRα1/Pax8 double knockout mice (DKO) [11-15] were obtained as previously described. Thyroid state of mice was manipulated as described previously [16]. Wild-type mice were made hypothyroid by treatment with methimazole (MMI) in the drinking water, Trhr1 KO mice were rendered euthyroid by treatment with T4 and T3 in the drinking water, and TRβ heterozygous and homozygous KO mice made hypothyroid by treatment with MMI in the drinking water and euthyroidism restored by supplementation with T3. Both male and female mice were used in the experiments. Handling was according the guidelines and with approval of the Animal Welfare Committee of the Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz, Germany, and the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health, Bethesda, MD, USA (TRα1-PV mice).

Measurement of tissue deiodinase activities and serum T4 and T3 levels

Tissue deiodinase activities and serum T3 and T4 levels were measured as previously described [12, 17, 18]. In short, deiodinase activity was measured by monitoring the conversion of radioactive outer ring labeled T4, T3 or rT3 into their metabolites. D1 activity was illustrated by ORD of rT3, D2 activity by ORD of T4 and D3 activity by IRD of T3. Tissues were homogenized in 10 volumes of PED10 buffer (0.1 M sodium phosphate (pH 7.2), 2 mM EDTA, 10 mM DTT) and protein levels determined with the Bio-RAD protein assay according to the manufacturer's instructions. Liver and kidney homogenates were incubated at 37 ˚C in PED10 buffer with 100 nM ($4x10^5$ cpm) ^{125}I-rT3 for 30 minutes to determine D1 activity or 1 nM ($2x10^5$ cpm) ^{125}I-T3 for 120 minutes determine D3 activity. Cerebellum homogenates were incubated at 37 ˚C in PED10 buffer for 120 minutes with 1 nM ($2x10^5$ cpm) ^{125}I-T4 to determined D2 activity, in the presence of 0.5 μM T3 to block D3 activity, or for 60 minutes with 1 nM ($2x10^5$ cpm) ^{125}I-T3 to determine D3 activity. All incubations were performed in duplicate. For blanks, incubations were carried out with PED10 only. After the incubations the reaction was stopped by adding 0.1ml ice cold ethanol. Samples were centrifuged, the supernatant diluted 1:1 with 0.02 M ammonium

acetate and analyzed by HPLC or UPLC (Waters). Radioactivity was measured by a Radiomatic A-500 flow scintillation detector (Packerd). The activity was corrected for the activity measured in the blanks and protein content.

Serum T4 and T3 concentrations were determined by RIA as described [12].

Statistical analysis

Values are presented as means ± SEM. Statistical differences were calculated using Student's t-test. $P<0.05$ was considered significant.

RESULTS

To test the hypothesis that the high T3 and low rT3 levels in serum of RTHα patients are largely due to impaired expression of DIO3, we first investigated tissue Dio3 expression using heterozygous TRα1-PV mutant mice, because these mice have a similar mutation (T394fs406X) as the mutation (F397fs406X) we described previously in RTHα patients [8, 11]. On P15, liver Dio1 activity was significantly increased, whereas liver Dio3 activity was significantly decreased in TRα1-PV vs. WT mice (Figure 1). Similarly, Dio3 activities in cerebellum (Figure 1) and retina (Figure S1) were also lower in TRα1-PV than in WT mice. Earlier in development (P5), when T3 levels are much lower [19] there was no difference in Dio1 or Dio3 activities (data not shown).

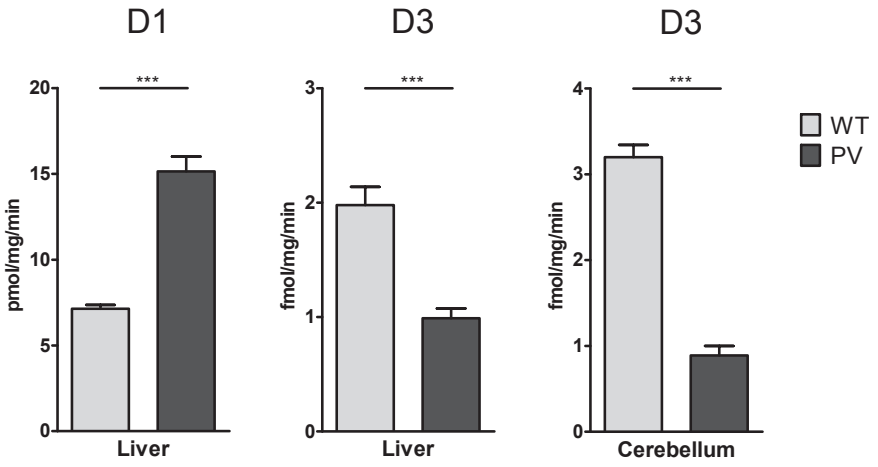

FIGURE 1. Deiodinase activity in cerebellum of P15 WT and TRα1-PV mice. Liver Dio1 activity was significantly higher in liver of TRα1-PV, whereas Dio3 activity was significantly decreased in liver and cerebellum of TRα1-PV mice. Data presented as mean±SEM. ***$p<0.001$

As the low Dio3 activity in P15 TRα1-PV mice could theoretically be attributed to a low TRα signaling as well as a high TRβ signaling, we next investigated the effects of hypothyroidism on liver Dio1 and Dio3 activity. For this purpose we used 1) mice treated with the TH synthesis inhibitor methimazole (MMI) [16], 2) Trhr-1-/- mice which have central hypothyroidism because of the deletion of the TRH receptor 1, with a roughly 50% reduction in serum T4 and T3 [16], and 3) Pax8-/- mice which have no thyroid and thus have extremely low TH levels [12]. In all hypothyroid mice, liver Dio1 was substantially decreased, compared with the positive control of hepatic Dio1 expression by T3 in WT mice (Figure 2A-C) [4, 20, 21]. More remarkably, liver Dio3 activity was significantly increased in all hypothyroid animals, suggesting that hepatic Dio3 expression is under negative control of TH. This is further supported by the normalization of both liver Dio1 and Dio3 activities after treatment of Trhr1-/- mice with TH (Figure 2B). Similar findings regarding regulation of Dio1 and Dio3 in Pax8-/- mice were obtained by analysis of kidney deiodinase activities (Figure S2). Therefore, Dio3 expression in both liver and kidney undergoes negative regulation by TH. This is in sharp contrast to the well-established positive regulation of Dio3 expression by TH in the brain (Figure 2D). As expected, cerebellum Dio2 activity was increased in Pax8 KO vs. WT mice (Figure 2D) due to the absence of T3-dependent downregulation of Dio2 under the hypothyroid conditions.

To further delineate the role of TRα1 and TRβ1 in the TH-dependent regulation of Dio3 expression in liver and cerebellum, we subsequently studied the effects of Pax8 deletion in WT and TRα1 KO mice at P21. Compared with WT mice, TRα1 KO mice have mildly decreased T4 and mildly increased T3 levels (Table 1). As expected, Pax8 deletion resulted in a complete loss of serum TH in both the WT and the TRα1 KO (Table 1).

In mice with an intact thyroid, deletion of TRα1 did not affect liver Dio1 activity (Figure 3A). Moreover, congenital hypothyroidism due to Pax8 deletion resulted in the same dramatic decrease in liver Dio1 expression in TRα1 KO as in TR WT mice. These results suggest that the TH-dependent regulation of liver Dio1 is not mediated by TRα1. In mice with an intact thyroid, TRα1 deletion resulted in a modest increase in liver Dio3 activity. Furthermore, Pax8 deletion induced a similar increase in liver Dio3 activity in TRα1 KO mice as in TR WT mice (Figure 3A). Similar findings were obtained in kidney (Figure S3), although the blunted Dio3 response to hypothyroidism in the DKO suggests some residual function in Dio3 regulation for TRα1 in the kidney. These results suggest that TRα1 also does not play a prominent role in the regulation of liver and kidney Dio3 by thyroid state.

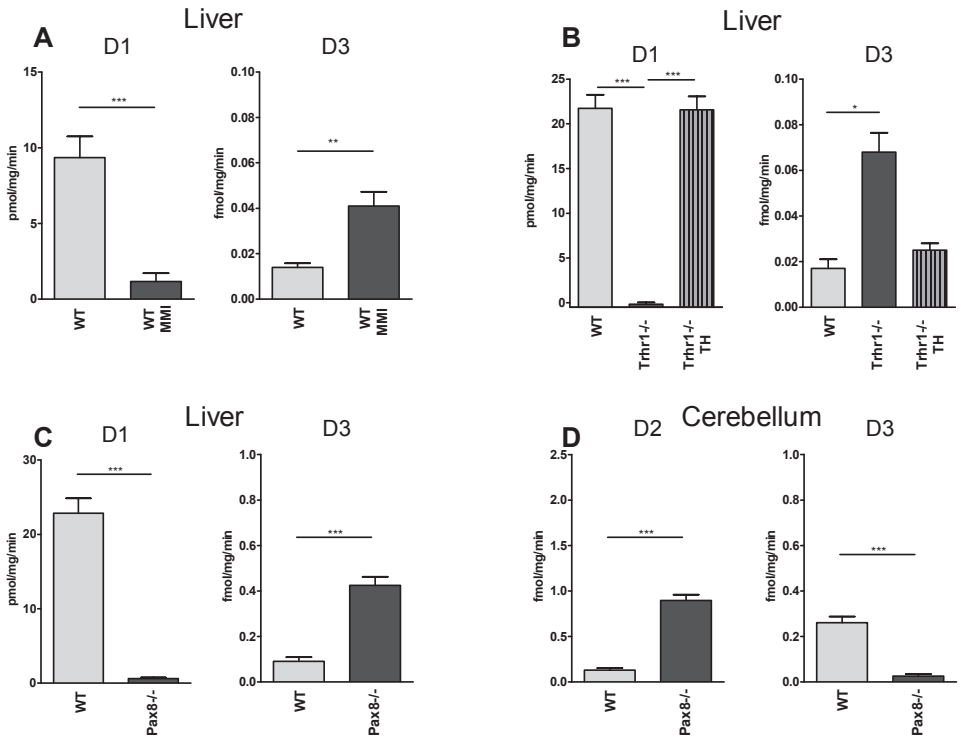

FIGURE 2. Deiodinase activity in liver and cerebellum of mice rendered hypothyroid due to MMI treatment (A), Trhr1 deletion (B) or Pax8 deletion (C and D). Liver Dio1 activity was decreased and liver Dio3 activity increased in all hypothyroid mice compared to WT control mice (A-C), and restored to WT levels when euthyroidism in Trhr1-/- mice was achieved (B). Dio2 activity was increased and Dio3 activity decreased in in hypothyroid Pax8-/- mice. Data presented as mean±SEM. *p<0.05; **p<0.01; ***p<0.001

TABLE 1. Serum TH levels in TRα1 and Pax8 KO mouse models

Genotype	T4 (nmol/l)	T3 (nmol/l)
WT	64.8 (4.29)	0.90 (0.06)
Pax8-/-	0.9 (0.9)	0.24 (0.03)
TRα1-/-	49.9 (3.7)	1.20 (0.09)
TRα1-/- , Pax8-/-	1.3 (0.6)	0.26 (0.04)

Mean (SEM)

In cerebellum, the up-regulation of Dio2 activity in mice with congenital hypothyroidism was not different between TRα1 KO and TR WT mice (Figure 3B). In mice with functional TRα1, cerebellum Dio3 activity was markedly suppressed by congenital hypothyroidism due to Pax8 deletion (Figure 3B). In mice with an intact thyroid, TRα1 deletion was associated with a decreased Dio3 activity which was not further affected by Pax8 deletion. These findings are consistent with the predominant role of TRα1 in the positive regulation of brain Dio3 expression by T3. The lower Dio3 activity in Pax8 KO mice with an intact TRα1 compared to both TRα1 KO strains likely reflects Dio3 repression by unliganded TRα1 under hypothyroid conditions.

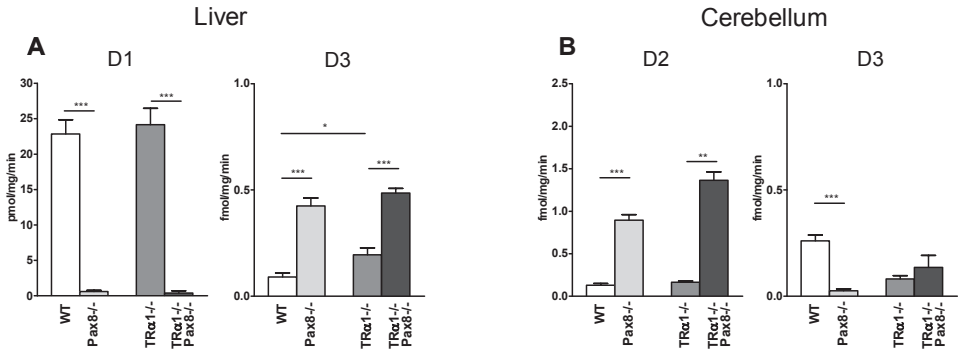

FIGURE 3. Deiodinase activity in liver (A) and cerebellum (B) of TRα1 KO, Pax8 KO and TRα1/Pax8 DKO mice. (A) In liver, downregulation of Dio1 and upregulation of Dio3 activity by hypothyroidism is unaffected by TRα1 deletion. (B) In cerebellum, Dio2 activity is not affected by TRα1 deletion. Dio3 activity is decreased by Pax8 deletion in a WT but not TRα1 KO background indicating that Dio3 expression is insensitive to thyroid state in the absence of TRα1. The lower Dio3 activity in the Pax8 KO compared to the TRα1/Pax8 DKO mice indicates gene repression by the unliganded TRα1. Data presented as mean±SEM. *p<0.05; **p<0.01; ***p<0.001

The role of TRβ1 in the regulation of liver Dio1 and Dio3 was studied by comparing tissue deiodinase activities in TRβ+/- or TRβ-/- mice made hypothyroid by MMI treatment or euthyroid by MMI plus T3 treatment, where TRβ+/- mice behave like WT mice [22]. As expected, serum TH levels were abolished by MMI treatment, whereas serum T3 was restored to WT levels by T3 treatment (Table 2).

T3-treated TRβ+/- mice show high liver Dio1 activities, whereas T3-treated TRβ-/- mice showed very low liver Dio1 activities (Figure 4A). This is in agreement with the involvement of TRβ1 in the regulation of liver Dio1 by T3. Liver Dio1 activity was even further decreased by treatment of TRβ1-/- mice with MMI, suggesting that in the absence of TRβ1, regulation of liver Dio1 by T3 may take place to some extent via TRα1.

Compared with T3-treated TRβ+/- mice, liver Dio3 activity was increased in T3-treated TRβ-/- mice and further increased in MMI-treated TRβ-/- mice (Figure 4A). These findings support the role of TRβ1 in TH-dependent downregulation of liver Dio3 and show the involvement of TRα1 in the absence of TRβ1. Similar findings in kidney show that Dio1 and Dio3 activities depend on TRβ1 in the same way in kidney as in liver (Figure S4). No significant changes in cerebellum Dio2 activities were observed between T3-treated TRβ1+/- mice, T3-treated TRβ1-/- mice, and MMI-treated TRβ-/- mice (Figure 4B). Cerebellum Dio3 activities were also not significantly different between T3-treated TRβ1+/- and T3-treated TRβ1-/- mice, but they were lower in the MMI-treated TRβ1-/- mice, suggesting that TRβ1 is not involved in the regulation of cerebellum Dio3 by T3.

TABLE 2. Serum TH levels in TRβ KO mouse models

Genotype/treatment	T4 (nmol/l)	T3 (nmol/l)
TRβ-/- MMI	1.11 (0.47)	0.17 (0.07)
TRβ-/- MMI + T3	0.20 (0.09)	0.87 (0.09)
TRβ+/- MMI + T3	0.50 (0.17)	1.13 (0.08)

Mean (SEM)

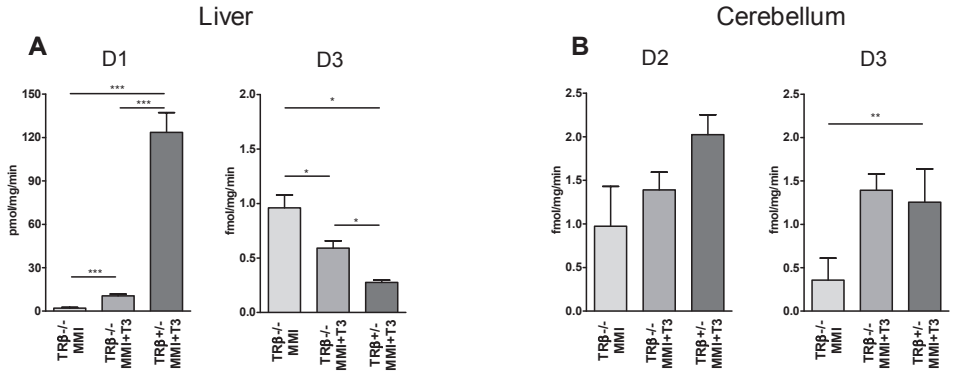

FIGURE 4. Deiodinase activity in liver (A) and cerebellum (B) of hypothyroid heterozygous and homozygous TRβ KO mice treated with or without T3. Mice were rendered hypothyroid by treatment with MMI and euthyroidism was restored by treatment with T3. (A) Dio1 activity was strongly reduced by TRβ deletion and further reduced when TRβ KO mice were hypothyroid. Conversely, Dio3 activity was lowest in euthyroid mice, increased by TRβ depletion and further increased when TRβ KO mice were made hypothyroid. (B) In cerebelllum, D2 activity was not affected by TRβ deletion or thyroid state in TRβ KO mice, whereas the T3-induced increase in Dio3 activity was independent of TRβ. Data presented as mean±SEM. *p<0.05; **p<0.01; ***p<0.001

DISCUSSION

In this study, using different knockout mouse models with manipulation of thyroid state, we show that Dio3 expression is differentially regulated between tissues and by the different T3-receptor isoforms. As expected, cerebellum Dio3 expression is positively regulated by T3, which is mediated by TRα1. Surprisingly, liver Dio3 expression is negatively regulated by T3, which is predominantly mediated via TRβ1.

Patients with TRα1 mutations usually show low serum T4, high T3 and low rT3 levels [7, 8, 23-28]. These changes may be caused by an increased tissue DIO1 expression and/or decreased DIO3 expression. The present study was done to better understand the biochemical profile of RTHα patients, as well as the role of TRα1 in the regulation of tissue Dio3 expression. Using heterozygous TRα1-PV mutant mice with a similar TRα1 mutation as the F397fs406X mutation we identified previously in a family with RTHα [8, 24], we showed that liver Dio1 activity is higher in TRα1-PV mice than in WT mice on P15. This is explained by the increased serum T3 levels stimulating Dio1 expression via regular TRβ1. In contrast, liver Dio3 was significantly decreased in TRα1-PV mice. On P5, no significant differences were detected in liver Dio1, and liver and brain Dio3 activities between TRα1-PV and WT mice. This is in line with a previous study which showed no change in brain Dio3 expression in 2-4 months old TRα1-PV vs. WT mice [29]. These findings indicate that the effects of the TRα1-PV mutation on tissue deiodinase expression is age dependent and only significant around P15, when serum T4 and T3 reach peak levels [19].

Although studies using luciferase reporter assays have shown that the TRα1-F397fs406X mutant exhibits a strong negative effect on TRβ1 activity in vitro [8], it is unlikely that this mechanism is relevant given the low levels of TRα1 expression compared with TRβ1 in liver [30, 31]. Alternatively, the low Dio3 activity could be caused by T3-induced down-regulation of hepatic Dio3 expression mediated by TRβ1.Our hypothesis that liver Dio3 expression is under negative control of TH via TRβ1 was subsequently corroborated in studies using 1) MMI-treated WT mice with acute drug-induced hypothyroidism (Figure 2A), 2) Trhr1 KO mice with mild congenital central hypothyroidism (Figure 2B), and 3) Pax8 KO mice with severe congenital primary hypothyroidism (Figure 2C). The decrease in liver Dio1 we observed in all hypothyroid mice is in agreement with previous studies demonstrating that hepatic Dio1 expression is positively regulated by T3 [4, 21]. The remarkable increase in liver Dio3 in all hypothyroid animals supports our hypothesis that liver Dio3 expression is negatively controlled by TH. This was confirmed by the normalization not only of liver Dio1 but also of Dio3 activity after treatment of Trhr1 KO mice with TH (Figure 2B). Parallel studies in kidney (Figure S2) indicated the same TH-

dependent regulation of Dio1 and Dio3 as in liver. Therefore, Dio3 expression was down-regulated by TH in both liver and kidney, in contrast to the established up-regulation of Dio3 expression by TH in the CNS (Figure 2D) [32].

Subsequent studies focused on the role of the different T3-receptor isoforms in this tissue-specific regulation. Comparison of tissue deiodinase activities in WT, Pax8 KO, TRα1 KO and Pax8/TRα1 DKO mice suggested that basal cerebellum Dio3 activity as well as its response to hypothyroidism were impaired in TRα1 KO mice (Figure 3B). On the other hand, cerebellum Dio3 activity was not different between T3-treated TRβ+/- and TRβ-/- mice, and the hypothyroidism-induced down-regulation of cerebellum Dio3 was not prevented in TRβ-/- mice (Figure 4B). These findings are in agreement with the overriding importance of TRα1 in the regulation of brain Dio3 expression [33]. The hypothyroidism-induced increase in cerebellum Dio2 activity appeared to be even greater in TRα1 KO mice than in TR WT mice (Figure 3B), suggesting that TRα1 does not play an important role in the negative regulation of Dio2 by TH. We did not observe an up-regulation of cerebellum Dio2 activity in MMI-treated TRβ1-/- mice compared with T3-treated TRβ1-/- mice (Figure 4B). Since the post-translational mechanism for the negative regulation of Dio2 occurs by T4-induced inactivation of Dio2 enzyme, and since T4 levels are negligible in both T3-treated and MMI-treated TRβ1 KO mice, these results suggest that the transcriptional regulation of Dio2 expression by T3 is mediated by TRβ1 [34].

Regarding the TH-dependent regulation of liver Dio1, we demonstrated that 1) Dio1 activity in euthyroid animals was not affected by deletion of TRα1 (Figure 3A); 2) congenital hypothyroidism resulted in the same dramatic decrease in Dio1 expression in TRα1 KO as in TR WT mice (Figure 3A); 3) Dio1 activity was dramatically decreased in T3-treated TRβ-/- mice compared with T3-treated TRβ+/- mice (Figure 4A). These findings are in agreement with previous findings regarding the positive regulation of Dio1 by T3, and the predominant expression of TRβ1 compared with TRα1 in liver (and kidney) [31]. However, liver Dio1 activity was further decreased in MMI-treated TRβ-/- mice compared with T3-treated TRβ-/- mice (Figure 4A), suggesting that in the absence of TRβ1 some T3-dependent regulation of Dio1 may be mediated by TRα1, in agreement with previous studies [21].

The most remarkable finding involves the down-regulation of liver and kidney Dio3 by TH, and the involvement of TRα1 and TRβ1 herein. Dio3 activity was equally high in congenitally hypothyroid mice without or with TRα1 deletion (Figure 3A), suggesting that the negative regulation of Dio3 in liver (and kidney) is not mediated by TRα1. This is not surprising in view of its low expression in these tissues. Also the higher liver Dio3

activity in T3-treated TRβ1-/- mice than in T3-treated TRβ+/- mice (Figure 4A) supports the hypothesis that TRβ1 plays an important role in the negative regulation of liver Dio3 by TH. The further increase in liver Dio3 by making TRβ-/- mice hypothyroid using MMI (Figure 4A) may again may point to the involvement of TRα1 in the hypothyroidism-induced increase in liver Dio3 in mice lacking TRβ1 from conception. This is in line with other studies showing that TRα also plays a role in regulation of the HPT-axis in TRβ-/- mice. Liver Dio3 activity was higher in euthyroid TRα1 KO mice than in euthyroid TR WT mice (Figure 3A), the mechanism of which remains to be investigated. Altogether, these data show that cerebellum Dio3 activity is positively regulated by T3 via TRα1, whereas liver and kidney Dio3 expression is negatively regulated by T3, predominantly via TRβ1. Obviously, the contribution of indirect mechanisms that regulate Dio3 expression independent of liver TRα1 and TRβ1 expression cannot be excluded.

The mechanisms behind the differential regulation of Dio3 in brain and peripheral tissue are as yet unclear. The ~1.5 kb 5′ flanking regions of human and mouse DIO3 are highly homologous, including a AGGTCA thyroid hormone response element (TRE) half-site at -1.5 kb and two translation start sites (TLSs). The downstream TLS appears to be used most often, since transcriptional start sites (TSSs) have been localized by RNA 5′ extension analysis at or downstream of the upstream TLS in the mouse Dio3 gene [32, 35]. Furthermore, the 5′ end of most DIO3 sequences cloned as expressed sequence tags (ESTs) from different human and mouse tissues is located downstream of the upstream TLS. Northern blot analysis has indicated that DIO3 is mostly expressed as a ~2.1 kb mRNA in different mouse and human tissues, although much larger Dio3 mRNA species have been detected in mouse brain [36], suggesting the involvement also of even more upstream located TSS(s) that could undergo different modes of regulation. In brain, DIO3 is predominantly expressed in neurons to limit T3 action in these target cells [20, 37]. It is well known that the expression of DIO3 in brain is up-regulated by T3, and Barca-Mayo et al. have demonstrated in mice in vivo and in mouse neuronal N2A cells and pituitary GH3 cells in vitro that this regulation is largely mediated by TRα1, possibly via the TRE half-site at -1.5 kb [10]. Whether this domain is also required for the negative T3-regulation of Dio3 in liver is not known.

The different effects of TRα1 and TRβ1 on Dio3 expression could simply be explained by preferential expression of a single TR in specific tissues with other factors determining the direction of expression. However, these different effects may also be due to intrinsic differences between TRs. Whole transcriptome analyses in mouse neuronal C17.2 cells [38], and HeLa human hepatocarcinoma HepG2 cells [39] that stably overexpress thyroid hormone receptors did find a substantial number of genes that were differentially regulated by TRα1 and TRβ1 in the same cell type. In addition, whole cistrome analysis

in the C17.2 cells also indicated differences in chromatin occupation [38]. To our knowledge, neither this study nor similar studies for TRβ1 in HepG2 cells [40] and in mouse liver [41, 42] have reported functional TR binding sites (TRBSs) in the vicinity of the Dio3 gene. Therefore, we cannot exclude that T3-regulation of Dio3 occurs by an indirect mechanism involving another factor which is controlled by T3/TR, as has been shown for several genes [38, 39].

In conclusion, our results show opposite regulation of DIO3 expression by T3 and T3 receptor subtypes in brain versus liver and kidney. The hypothyroidism-induced increase in hepatic and renal DIO3 expression that we demonstrate in the current study is counter-intuitive as one would expect a reduction in TH catabolism in situations of TH shortage, similar to the adaptation of brain DIO3 expression to changes in thyroid state. It also casts a different light on previous studies where induction of DIO3 activity in peripheral tissues was interpreted to *be* causal for the generation of low serum T3 levels in various pathophysiological conditions [43]. It has been reported among others that hepatic DIO3 expression is increased in severely ill patients [17], in fasting mice [44], in aging mice [45], in rats and mice after partial hepatectomy [46], and in rats treated with sunitinib [47], which are all associated with low serum T3 levels. Further studies are required to resolve the causal relationship between the changes in hepatic and renal DIO3 expression and serum TH levels in these pathophysiological conditions.

ABBREVIATIONS

BAT	Brown adipose tissue
DIO	Deiodinase
DKO	Double knockout
EST	Expressed sequence tags
KO	Knockout
MMI	Methimazole
rT3	3,3′,5′--triiodo-L-thyronine
RTH	Resistance to thyroid hormone
T3	3,5,3′-triiodo-L-thyronine
T4	L-THYROXINE
TH	Thyroid hormone
TLS	Translation start site
TR	Thyroid hormone receptor
TRBS	Thyroid hormone receptor binding site
TRE	Thyroid hormone response element
TRH	Thyrotropin-releasing hormone
TRHR	Thyrotropin-releasing hormone receptor
TSH	Thyroid-stimulating hormone
TSS	Transcription start site
WT	Wild type

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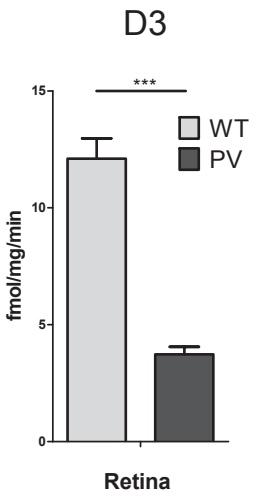

FIGURE S1. Dio3 activity in Retina of P15 WT and TRα1-PV mice. Dio3 activity was significantly decreased in retina of TRα1-PV mice. Data presented as mean±SEM. ***p<0.001

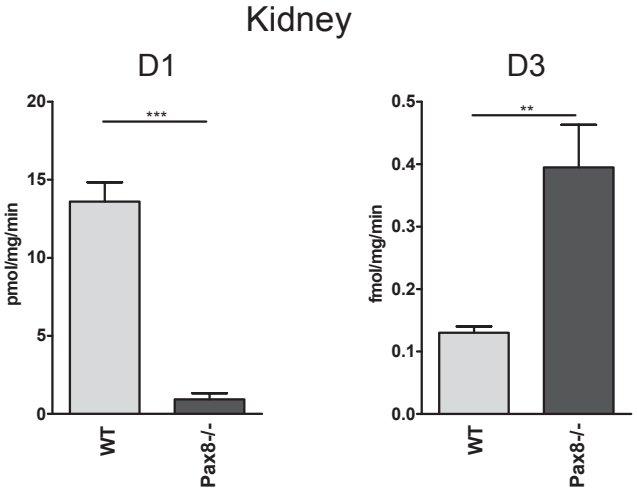

FIGURE S2. Deiodinase activity in kidney of Pax8 KO mice. Dio1 and Dio3 were significantly reduced and increased respectively in kidney of Pax8 KO mice. Data presented as mean±SEM. **p<0.01; ***p<0.001

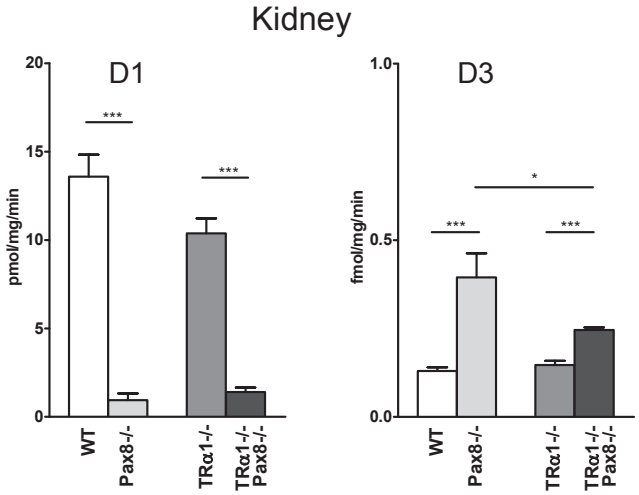

FIGURE S3. Deiodinase activity in kidney of TRα1 KO, Pax8 KO and TRα1/Pax8 DKO mice. Hypothyroidism induced downregulation of kidney Dio1 activity is unaffected and upregulation of Dio3 activity only partially reduced by TRα1 deletion. Data presented as mean±SEM. *p<0.05; ***p<0.001

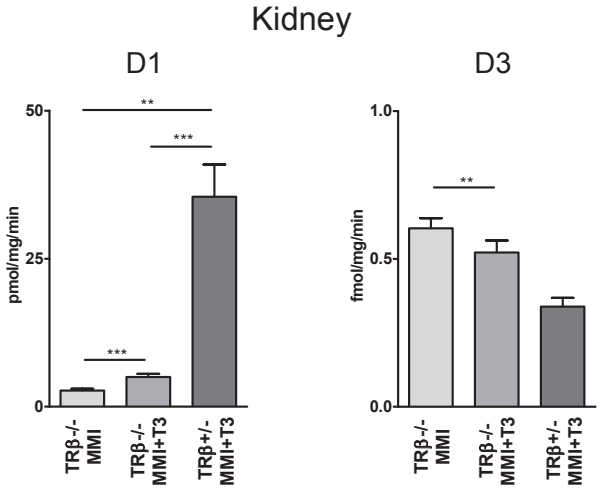

FIGURE S4. Deiodinase activity in kidney of hypothyroid heterozygous and homozygous TRβ KO mice treated with or without T3. Mice were rendered hypothyroid by treatment with MMI and euthyroidism was restored by treatment with T3. Comparable to liver, Dio1 activity in kidney was strongly reduced by TRβ deletion and further reduced when TRβ KO mice were hypothyroid. Dio3 activity was lowest under euthyroid conditions, increased by TRβ depletion and further increased in hypothyroid TRβ KO mice. Data presented as mean±SEM. **p<0.01; ***p<0.001

CHAPTER 5

Effects of thyroid hormone transporters MCT8 and MCT10 on nuclear activity of T3

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ABSTRACT

Transport of thyroid hormone (TH) across the plasma membrane is necessary for the genomic action of T3 mediated by its nuclear T3 receptor. MCT8 and MCT10 have been identified as important TH transporters. Mutations in MCT8 result in severe psychomotor retardation. In addition to TH transport into the cell, MCT8 and MCT10 also facilitate TH efflux from cells. Therefore, the aim of this study was to examine if MCT8 and MCT10 increase the availability of T3 for its nuclear receptor rather than generate a rapid equilibrium between cellular and serum T3.

T3 action was investigated in JEG3 cells co-transfected with TRβ1 and a T3 response element-driven luciferase construct, and T3 metabolism was analyzed in cells transfected with type 3 deiodinase (D3). In addition, cells were transfected with MCT8 or MCT10 and/or the cytoplasmic T3-binding protein mu-crystallin (CRYM). Luciferase signal was markedly stimulated by incubating cells for 24 h with 1 nM T3, but this response was not augmented by MCT8 or MCT10 expression. Limiting the time of T3 exposure to 1-6 hours and co-transfection with CRYM allowed for a modest increase in luciferase response to T3. In contrast, T3 metabolism by D3 was potently stimulated by MCT8 or MCT10 expression, but it was not affected by expression of CRYM.

These results suggest that MCT8 and MCT10 by virtue of their bidirectional T3 transport have less effect on steady-state nuclear T3 levels than on T3 levels at the cell periphery where D3 is located. CRYM alters the dynamics of cellular TH transport but its exact function in the cellular distribution of TH remains to be determined.

INTRODUCTION

Lack of thyroid hormone (TH) in early life leads to severe neurological dysfunction [1-3]. TH regulates gene expression via binding of the biological active hormone T3 to its nuclear receptor (TR), which is bound to a T3 response element (TRE) in the promotor region of target genes. To mediate its effects, TH must first be transported across the plasma membrane by specific transporters. Several TH transporters from different families have been identified, such as organic anion transporting polypeptides (OATP), bile acid transporters, L-type amino acid transporters (LAT) and monocarboxylate transporters (MCT) [4]. Two highly homologous monocarboxylate transporters, MCT8 and MCT10, are so far the only identified transporters with a high activity and specificity for TH [5-8].

MCT8 is expressed in neurons and at the blood-brain barrier [9-15]. Other expression sites are the heart, placenta, kidney, liver and the adrenal glands [14, 16]. Inactivating mutations in MCT8 result in the X-linked Allan-Herndon-Dudley syndrome (AHDS) characterized by severe psychomotor retardation and abnormal serum TH levels (high serum T3, low T4 and normal TSH levels) [17-20]. Patients with AHDS also suffer from hypotonia, muscular hypoplasia, and a delayed development and myelination [19, 21]. The complete pathogenesis of AHDS is not yet completely understood, but it is hypothesized that AHDS is due to a hypothyroid state of the MCT8-expressing neurons as a result of the inactivating mutations in MCT8 [11, 12, 21-23]. In contrast to humans, MCT8 KO mice do not show a neurological phenotype, although they have very similar abnormal serum TH levels as AHDS patients and low T3 levels in the brain [14, 24, 25]. The absence of brain abnormalities in MCT8 KO mice may be explained by the more abundant expression of OATP1C1 at the blood-brain barrier in mice than in humans [10, 26].

MCT10, the highly homologous family member of MCT8, is expressed in multiple tissues, including intestine, kidney, liver, muscle, and placenta [16, 22, 27]. There is little expression of MCT10 in the brain [28]. In contrast to MCT8, no patients with mutations in MCT10 have been identified so far. Apart from TH, MCT10 is also capable of transporting amino acids [7].

MCT8 and MCT10 facilitate not only the cellular uptake but also the efflux of TH from cells. It is therefore unclear if MCT8/MCT10 transported T3 is preferentially directed towards the nucleus, thereby increasing the availability of TH for its nuclear receptors, or that it results in a rapid equilibrium near the plasma membrane. We therefore investigated if the presence of MCT8 and MCT10 has an effect on directing T3 to the nucleus, thereby

increasing the availability of T3 for its nuclear receptors. This was done by analyzing the effects of the overexpression of MCT8 or MCT10 on the TRβ1-mediated stimulation of a TRE-driven luciferase reporter by T3 in JEG3 human choriocarcinoma cells, which show low endogenous expression of TH transporters [29]. The findings were compared with the effects of MCT8 and MCT10 on the metabolism of T3 by type 3 deiodinase (D3) located in the plasma membrane [30]. In addition, we studied the effects of the cytoplasmic TH-binding protein μ-crystallin (CRYM) on the availability of T3 for binding to its receptor in the cell nucleus and its metabolism by D3 at the cell periphery.

MATERIAL AND METHODS

Materials

Unlabeled T3 was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands (NL)). Na125I was obtained from Perkin Elmer (Groningen, NL). [3'-125I]T3 was prepared as previously described [31].

Plasmids

Human MCT8 [8], MCT10 [7], CRYM [7], and D3 [8] plasmids were obtained as previously described. hTRβ1 was cloned into the pcDNA3.1 expression vector by Dr. W.M. van der Deure (Erasmus MC, Rotterdam, NL). N-terminal Flag-tagged hTRβ1 with an optimized Kozak translation start site was also constructed in pcDNA3. The pdV-L1 construct containing a T3 responsive firefly luciferase (LUC) reporter and a control renilla luciferase (REN) reporter was kindly provided by Dr. W.S. Simonides (VU Medical Center, Amsterdam, NL) [32]. Both LUC and REN genes are driven by the SERCA1 minimal promoter, and two T3 response elements (TREs) are inserted in front of the minimal promoter of the LUC gene.

Cell culture

JEG3 cells were cultured at 37 C and 5% CO2 in DMEM/F12 medium (Invitrogen, Bleiswijk, NL) supplemented with 9% heat-inactivated fetal bovine serum (FBS) and 100 nM Na2SeO3 (Sigma-Aldrich).

[^{125}I]T3 transport

JEG3 cells were cultured in 24 or 96-well plates and transiently transfected with various combinations of pdV-L1, hTRβ1 or Flag-hTRβ1, hMCT8 or hMCT10, hCRYM, and/or hD3, and the total amount of plasmid was brought to 60-75 ng (96 wells) or 200 ng (24 wells) with empty pcDNA3. Transfection was performed according to the

manufacturer's protocol using FuGene6 or X-tremeGENE9 (Roche, Almere, NL). In some experiments, cells were washed and incubated for 24 hours with 500 µl DMEM/F12 containing charcoal-stripped FBS prior to transfection. Two days after transfection, cells were washed twice with 200 µl (96 wells) or 500 µl (24 wells) DMEM/F12 + 0.1%BSA and incubated for different time periods at 37 C and 5% CO2 with 100 µl (96 wells) or 500 µl (24 wells) DMEM/F12 + 0.1%BSA with 1 nM T3 and 10,000 cpm (96 wells) or 50,000 cpm (24 wells) [^{125}I]T3. After incubation, the cells were again washed, lysed with 0.1 M NaOH, and counted for radioactivity.

Intact cell T3 metabolism

JEG3 cells were transfected as described above for the uptake assay in 96 well plates. Incubations were performed for different time periods at 37 C and 5% CO2 with 1 nM T3 and 100,000 cpm [125I]T3 in 100 µl DMEM/F12 + 0.1%BSA. Next, 100 µl medium was added to 100 µl ice-cold ethanol. The mixtures were incubated for 30 minutes on ice, and then centrifuged for 15 minutes at 3500 rpm and 4 C. Finally, 100 µl supernatant was mixed with 100 µl 0.02M ammonium acetate (pH 4.0), and the samples were analyzed by HPLC as previously described [8].

Luciferase activity

Two days after transfection, cells were incubated for the different time periods with 1 nM T3. After washing and replacement with medium without T3, incubations were continued for a total time of 24 hours. LUC and REN activities were determined as previously described [33]. T3 has only a small effect on REN activity compared with its effect on LUC expression. Results are presented as LUC/REN ratio but are not essentially different from the absolute LUC activity.

Statistical tests

All experiments were performed in triplicate and presented as mean ± SEM of n experiments. Statistical differences were calculated using Student's t-test or a 2-way ANOVA followed by Bonferroni post-hoc test, as indicated in the figure legends. $P<0.05$ was considered significant.

RESULTS

The transcriptional activity of T3 was studied using JEG3 cells transfected with human TRβ1 and a construct expressing LUC under control of a TRE-dependent promoter and REN under control of a constitutive promoter. Initial experiments were conducted under steady-state conditions, where cells were incubated for 24 hours without or with 1 nM

T3. As demonstrated before using TRα1 [33], this resulted in a marked stimulation of the LUC/REN ratio. To investigate if MCT8 or MCT10 increase the nuclear availability of T3, the effect of 24-hour incubation with 1 nM T3 was also studied in cells co-transfected with plasmids coding for MCT8 or MCT10. Remarkably, this did not result in any further increase in the T3-stimulated LUC/REN ratio compared with control cells transfected with empty plasmid (Figure 1A).

Cellular uptake of 1 nM [^{125}I]T3 was determined in parallel 24-hour incubations, showing that transfection with MCT8 or MCT10 did not stimulate cellular T3 accumulation under these steady-state conditions (Figure 1C). This is in keeping with previous observations that MCT8 and MCT10 facilitate both cellular T3 influx and efflux. Therefore, expression of MCT8 or MCT10 has little effect on the steady-state cellular T3 concentration in the presence of other, endogenous transporters. It has also been shown previously that expression of the cytoplasmic T3 binding protein μ-crystallin (CRYM) markedly augments the cellular accumulation of T3 induced by MCT8 or MCT10 during short incubations. However, co-transfection with CRYM did not enable MCT8 or MCT10 to stimulate the transcriptional activity (Figure 1B) or cellular uptake (Figure 1D) of T3 in these prolonged incubations.

Little metabolism of T3 was observed during 24-hour incubation with JEG3 cells transfected with empty vector, MCT8 or MCT10 alone or in combination with CRYM (Figure 1E,F). About 25% of added T3 was metabolized in parallel incubations with cells transfected with D3 alone or in combination with CRYM. Co-transfection of MCT8 or MCT10 resulted in a similar, marked stimulation of T3 metabolism, amounting to ~75% and ~90% of added T3 in the absence or presence of CRYM, respectively (Figure 1E,F).

Since expression of MCT8 or MCT10 in the absence or presence of CRYM did not have a significant effect on the steady-state cellular and nuclear accumulation of T3 during prolonged incubations, we decided to test shorter incubation times. Therefore, JEG3 cells transfected with the various constructs were incubated for 0.5-24 hours with 1 nM T3; after replacing the medium with T3 deplete medium the incubation was continued until a total period of 24 hours to allow sufficient synthesis of reporter activity. Figure 2 shows that in the absence of CRYM, expression of MCT8 or MCT10 stimulated the cellular accumulation of T3 only during the first 30 minutes of incubation.

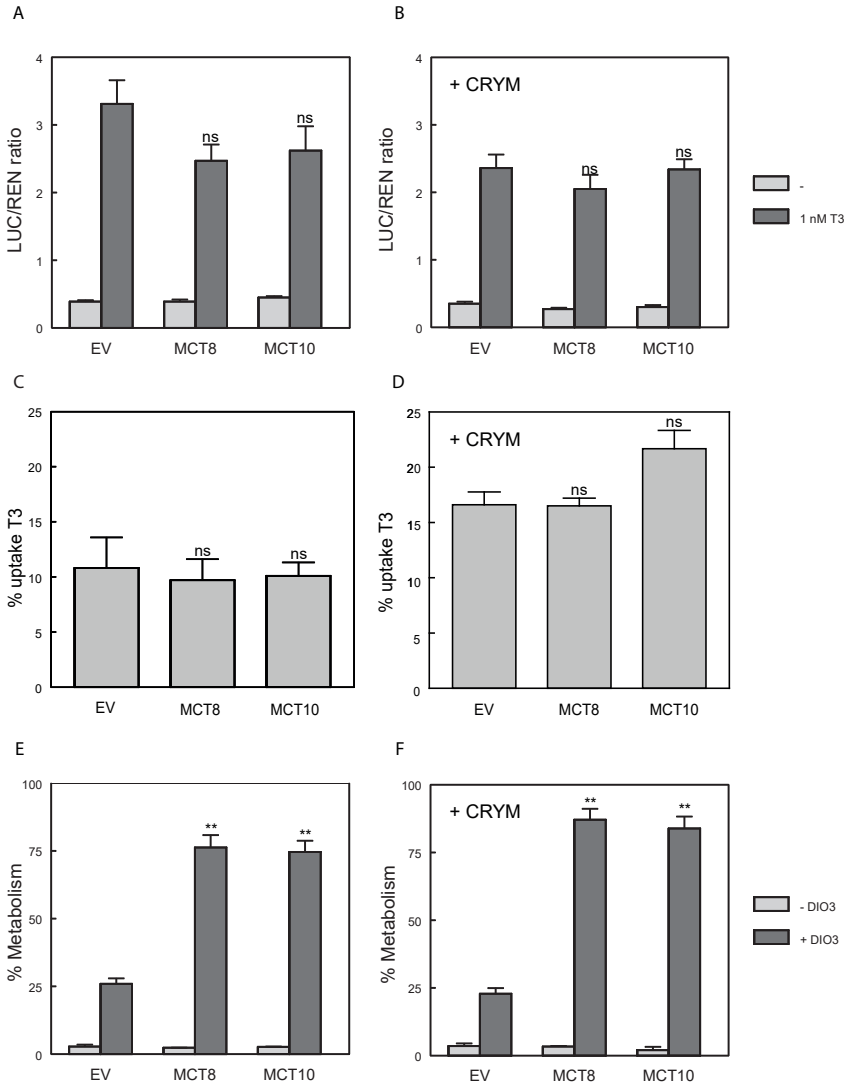

FIGURE 1. A,B Functional analysis of JEG3 cells co-transfected with a TRE-regulated LUC and control REN construct and TRβ1 in combination with either empty vector, MCT8 or MCT10, without (A) or with (B) CRYM. Cells were incubated for 24 hours without or with 1 nM T3. Values represent means ± SEM of 5 experiments performed in triplicate. ns, not significant vs. cells without transporter.
C,D 24-hour uptake of 1 nM [125I]T3 in JEG3 cells in the absence (C) or presence (D) of CRYM. Cells were transiently transfected with empty vector, MCT8 or MCT10. Values represent means ± SEM of 3 experiments performed in triplicate. ns, not significant vs. cells without transporter.
E,F 24-hour metabolism in JEG3 cells of [125I]T3 in the absence (E) or presence (F) of CRYM. Cells were transfected with empty vector, MCT8 or MCT10, with or without D3. Values represent means ± SEM of 2 experiments performed in triplicate; ** P<0.01 vs. cells without transporter.

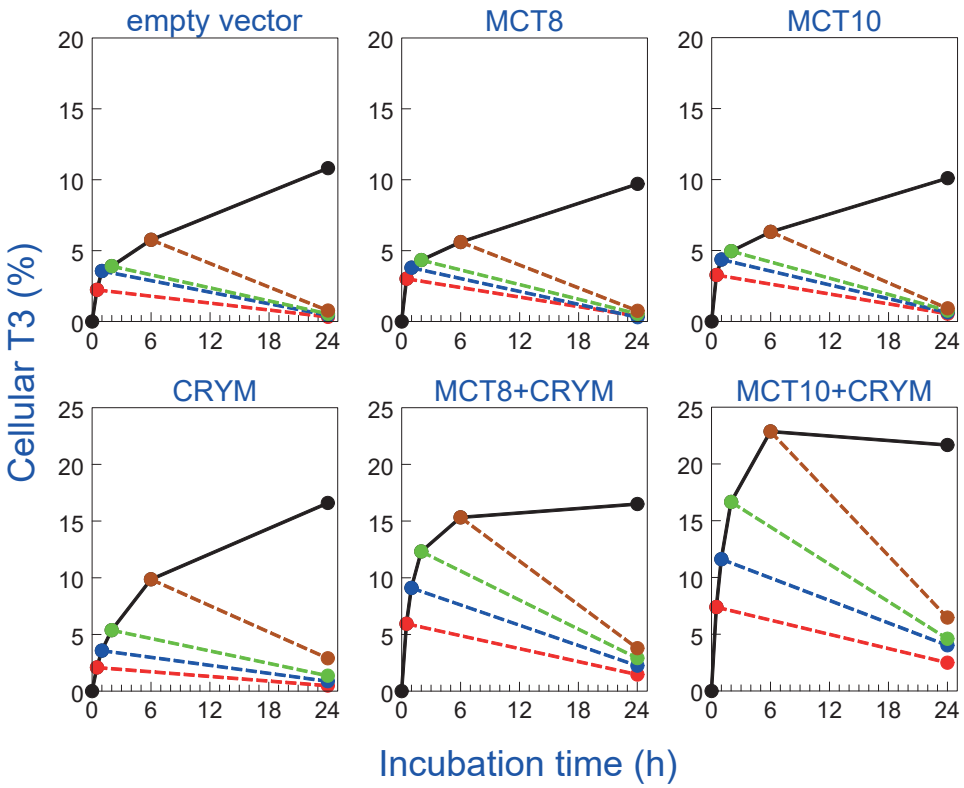

FIGURE 2. Cellular accumulation of [125I]T3 in cells transfected with empty vector, MCT8 or MCT10 without or with CRYM as function of incubation time (0.5-24 hours). Interrupted lines indicate the loss of cellular T3 when [125I]T3-containing medium is replaced at the indicated time points with T3 deplete medium and the incubation continued for a total period of 24 hours. Results are the means of 2 experiments performed in triplicate.

However, in the presence of CRYM, expression of MCT8 or MCT10 resulted in the marked stimulation of T3 uptake during at least 6 hours of incubation. Therefore, we decided to test the effects of MCT8 and MCT10 in the absence or presence of CRYM on the uptake, nuclear activity and D3-mediated metabolism during a 4-hour incubation with T3. In addition, the effects of co-transfection with D3 were studied on the cellular accumulation and transcriptional activity of T3. Figure 3A shows that expression of MCT8 or MCT10 in the absence of CRYM had no significant effect on the cellular accumulation of T3 during 4 hours of incubation.

Transfection of cells with CRYM alone did not markedly stimulate T3 uptake over the 4-hour period (Figure 3B). However, transfection of CRYM-expressing cells with MCT8

or MCT10 stimulated cellular T3 accumulation 2 and 3-fold, respectively. Expression of MCT8 or MCT10 in the absence of CRYM did not have a significant effect on the transcriptional activity induced by exposure of the cells for 4 hours to T3 (Figure 3C). Transfection of the cells with CRYM alone resulted in a significant decrease in nuclear T3 activity (Figure 3D). However, transfection of CRYM-expressing cells with MCT8 or MCT10 resulted in the stimulation of the transcriptional activity of T3 to levels at or above those observed in cells expressing MCT8 or MCT10 in the absence of CRYM.

In these short incubations, T3 metabolism was again negligible in cells transfected with empty vector, MCT8, MCT10 or CRYM alone (Figure 3E,F). About 5% of added T3 was metabolized during 4-hour incubation with cells expressing D3 in the absence or MCT8 or MCT10, and this was increased to about 30% after co-transfection with MCT8 or MCT10. The metabolism of T3 by D3 in MCT8 or MCT10-expressing cells was not affected by co-transfection of CRYM. Expression of D3 reduced the cellular uptake and transcriptional activity of T3 independent of the expression of CRYM and completely prevented the stimulation of T3 uptake and nuclear activity induced by MCT8 or MCT10 observed in the presence of CRYM (Figure 3E,F).

Finally, the transcriptional activity induced by incubation of the variously transfected cells for different time periods with 1 nM T3 was studied as a function of time-integrated cellular T3 concentration. By plotting the percentage of T3 uptake against time, the area under the curve represents the intracellular T3 concentration during the whole incubation period (Figure 2A). There was a good correlation between the integrated cellular T3 concentration and the luciferase response to T3, both in the absence and presence of CRYM. However, the intracellular T3 concentrations associated with similar T3 responses were higher in the presence than in the absence of CRYM (Figure 4).

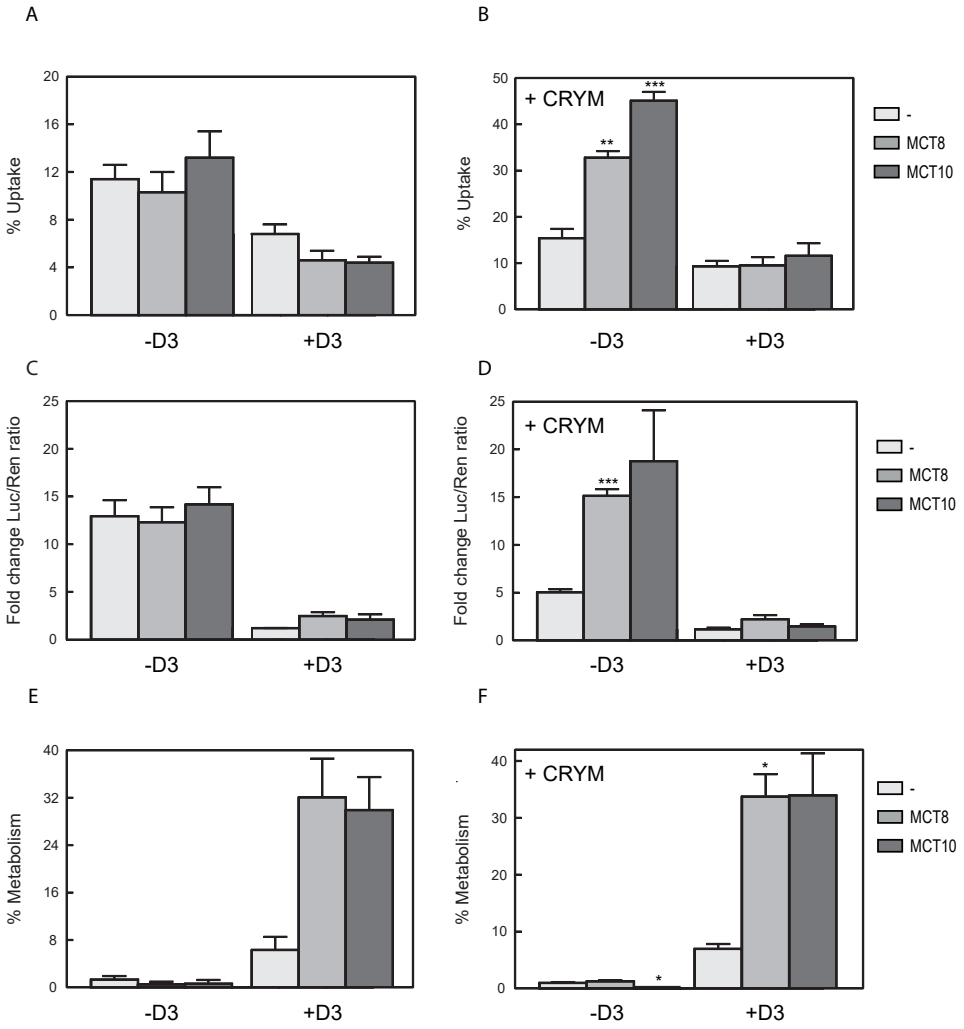

FIGURE 3. Cellular uptake (A,B), nuclear activity (C,D) and metabolism (E,F) of 1 nM T3 incubated for 4 hours with JEG3 cells co-transfected with reporter construct and Flag-TRβ1 plus MCT8, MCT10, CRYM and/or D3. In C and D, incubations were continued with T3 deplete medium until 24 hours to allow sufficient time for luciferase synthesis. Results are the means ± SEM of 2-4 experiments performed in triplicate; * P<0.05, ** P<0.01, *** P<0.001 vs. cells without transporter.

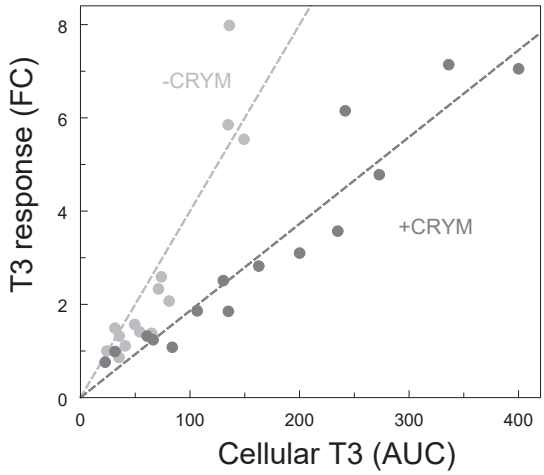

FIGURE 4. T3-stimulated reporter activity as a function of T3 accumulation in JEG3 cells transfected with reporter construct and TRβ1 plus empty vector, MCT8, MCT10, and/or CRYM. Cells were incubated for 0.5-24 hours with 1 nM T3 and subsequently until a total period of 24 hours in the absence of T3 to allow sufficient luciferase synthesis. The experiment was performed in triplicate. FC: fold change, AUC: area under the curve.

DISCUSSION

In the current study we demonstrate that transfection of JEG3 cells with MCT8 or MCT10 has widely different effects on the binding of T3 to the nuclear receptor compared with its deiodination by D3 located in the cell membrane.

A schematic overview of the data is presented in Figure 5. Figure 5A represents a non-transfected JEG3 cell where cellular T3 uptake and efflux is mediated by endogenous transporters. In MCT8 or MCT10 overexpressing cells, both T3 influx and efflux are increased. Therefore, steady-state intracellular T3 levels and thus nuclear receptor binding are hardly affected (Figure 5B). In D3 expressing cells, T3 is degraded to 3,3-T2, resulting in decreased nuclear T3 receptor activation (Figure 5C). Transfection of D3 expressing cells with MCT8 or MCT10 stimulates T3 metabolism but does not increase nuclear availability of T3 (Figure 5D). The cytoplasmic binding protein CRYM decreases T3 efflux and thus enhances the transient increase in cellular T3 accumulation in MCT8 or MCT10 overexpressing cells. Consequently, nuclear receptor activation is stimulated by expression of MCT8 or MCT10 during short T3 exposure times (Figure 5E). However,

co-transfection of D3 completely prevents T3 action even in the presence of CRYM and MCT8 or MCT10, suggesting that cellular T3 is degraded before it can reach the nuclear receptor (Figure 5F).

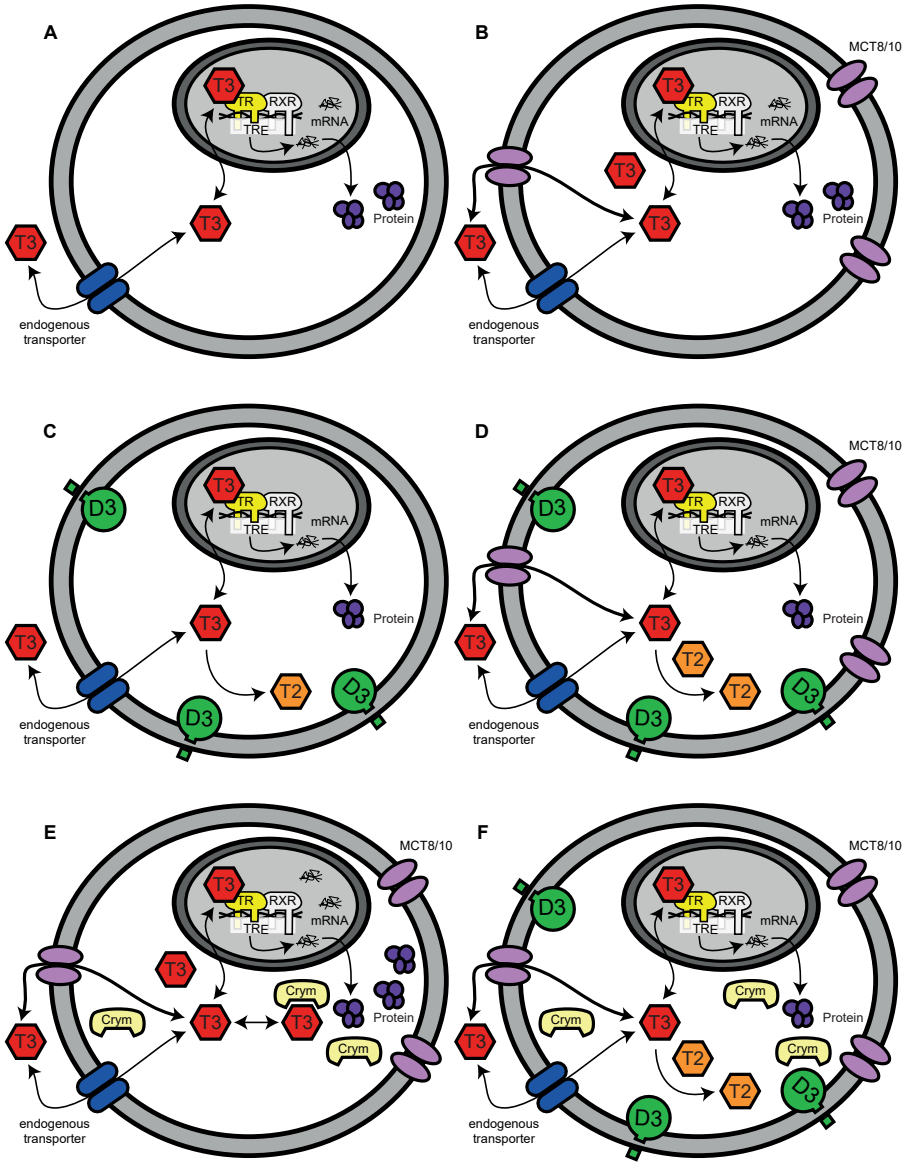

FIGURE 5. Schematic representation of a TH target cell with overexpression of MCT8/10, CRYM and/or D3. T3 responsive gene expression is controlled by a positively regulated TRE.

Inactivating mutations in MCT8 lead to AHDS [19, 20]. MCT10, the highly homologous family member of MCT8, has been studied in less detail and no patients with mutations in MCT10 have yet been identified.

Overexpression of MCT8 or MCT10 stimulates T3 uptake, resulting in a rapid rise in intracellular T3 concentration, as we have shown previously [7, 8]. Nevertheless, the increased uptake results in only a modest increase in biological activity of T3. This is explained by the fact that both MCT8 and MCT10 are also capable of T3 efflux, leading to a rapid equilibrium between influx and efflux [7, 8]. Interestingly, some patients with AHDS have a milder phenotype, which has been ascribed to a greater impact of their mutations on the efflux than on the uptake of TH by MCT8 [34, 35].

Co-transfection of either MCT8 or MCT10 greatly stimulates T3 metabolism by D3, confirming our earlier findings [8]. Rapid degradation by D3 reduces the nuclear availability of T3, irrespective of the transfection with MCT8 or MCT10. D3 is mainly present in fetal tissues and its expression level declines after birth [36, 37]. A major site of action of D3 is the central nervous system, where it acts primarily in neurons [38, 39]. It was recently shown in mice that D3 protects the cerebellum from high TH levels during fetal life and in the neonatal period [40]. Our data suggest that TH transporters can only raise intracellular T3 concentrations when D3 activity is low.

Expression of the intracellular binding protein CRYM inhibits the efflux of T3 [41]. This extends the period after addition of T3 during which MCT8 or MCT10 increase intracellular T3 before equilibrium is reached between T3 influx and efflux. We utilized this property of CRYM in our *in vitro* testing system, but the possible physiological roles for CRYM are intriguing. It has homology to enzymes involved in glutamate and ornithine metabolism, and T3 binding to CRYM depends on NADPH and thiol cofactors [41, 42]. It has been hypothesized that CRYM may play a role in the delivery of TH to mitochondria or to the nucleus, depending on the redox state of the cytoplasm [42]. CRYM has recently been identified as an enzyme with ketimine reductase activity regulated by TH [43]. The apparent Kd value of T3 for CRYM amounts to 0.3 nM [44].

CRYM is found in the inner ear, and mutations in CRYM have been associated with non-syndromic deafness [45]. Furthermore, CRYM is abundantly expressed in the central nervous system; in the cerebral cortex predominantly in the cytoplasm of neurons [46]. Co-transfection of cells with MCT8, CRYM and D3 may thus mimic to some extent the handling of TH in central neurons. The co-transfection of CRYM in addition to D3 leads to a similar T3 metabolism as D3 alone. This suggests that the rapid degradation of T3 prohibits an increase in cytoplasmic T3 levels, thus decreasing nuclear T3 availability.

The most remarkable finding in our study is the marked stimulation of intracellular metabolism of T3 by D3 if cells are co-transfected with MCT8 or MCT10. This is in contrast to the modest stimulation of T3 action as detected by the promoter-luciferase construct. D3 is located in the plasma membrane with its active center located on the cytoplasmic surface [8, 30, 47]. The biological activity of T3 is mediated by its nuclear receptor. Therefore, the action of T3 requires a much greater penetration into the cell than its deiodination (Figure 5). The extent of T3 distribution within the cell depends on a number of factors, including the rate of T3 transport back to the extracellular milieu and its binding to intracellular proteins that may facilitate its further distribution. Our results suggest that in MCT8 over-expressing cells, efflux of T3 is much more rapid than its transport to the nucleus, explaining why accumulation of T3 in the nucleus is modest despite the strong stimulation of cellular T3 uptake.

In contrast, intracellular metabolism by D3 is greatly stimulated by co-transfection with MCT8 or MCT10 irrespective of the expression of CRYM. This suggests that the available T3 concentration at the D3 active center is much less affected by the rapid T3 efflux mediated by MCT8 or MCT10. We speculate that transporters such as MCT8 or MCT10 that mediate both rapid uptake and efflux of T3 have a much greater effect on intracellular T3 availability at the cell periphery than at the cell nucleus even in the presence of the cytoplasmic high-affinity T3-binding protein CRYM.

It should be realized that JEG3 cells express endogenous TH transporters, which is reflected by the steady increase in T3 uptake even during prolonged incubations with non-transfected cells. Of course, the effects of transfected MCT8 or MCT10 in cells already expressing endogenous transporters are quite different from cells devoid of other transporters. MCT8 may be the only significant TH transporter in certain populations of central neurons where it plays a crucial role in TH metabolism and action during critical periods of brain development [22]. In addition to neurons, MCT8 is importantly expressed in endothelial cells of the blood-brain barrier (BBB). Perhaps, both TH uptake and efflux functions of MCT8 are involved in the transcellular transport of T4 and T3 across the BBB. Indeed MCT8 has been localized in the apical (blood-facing) as well as the basolateral (brain-facing) membranes of the BBB endothelial cells [10].

In conclusion, we demonstrated that over-expression of MCT8 or MCT10 in JEG3 cells induce only a transient increase in cellular T3 accumulation upon addition of T3, resulting in a modest increase in the nuclear activity of T3. In the presence of CRYM, the temporary effects of MCT8 or MCT10 on cellular accumulation and nuclear activity of T3 are enhanced. However, this effect is annulled in the presence of D3 due to rapid degradation of T3. MCT8 and MCT10 appear to have a much greater effect on the cellular

availability of T3 for D3 located in the plasma membrane than for the T3 receptor located in the nucleus. We hypothesize that this differential effect of MCT8 and MCT10 on TH availability for processes located at the cell periphery or at the nucleus are inherent to the rapid cellular TH efflux also mediated by these transporters. The implications of our findings for the pathogenic mechanism of AHDS remain to be explored.

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CHAPTER 6

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

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ABSTRACT

Thyroid hormone (TH) transporter proteins mediate transport of TH across the plasma membrane, thereby facilitating its intracellular bioavailability. As only a few transporters have been identified which are relatively specific for TH, including monocarboxylate transporter (MCT) 8 and MCT10, the need for identification of novel specific TH transporters is obvious. A possible strategy to identify TH transporters is their modification with a ligand-derived affinity-label and subsequent identification by mass spectrometry. Previously, *N*-bromoacetyl-iodothyronines have been reported as useful affinity-labels for human (h) MCT8. In the present study we reinvestigated possible BrAc[^{125}I]T3-labeling of hMCT8 and hMCT10. The present study demonstrates that hMCT8 and hMCT10 both facilitate BrAc[^{125}I]T3 transport, but are not labeled by BrAc[^{125}I]T3. We provide evidence that human protein disulfide isomerase, which molecular mass is similar to hMCT8, is labeled by BrAc[^{125}I]T3. In addition, differential inhibitory effects were observed of iodothyronines derivatives with different side chains on T3 transport by hMCT8 and hMCT10.

In conclusion, we demonstrated that not hMCT8 and hMCT10, but human protein disulfide isomerase, is labeled by BrAc[^{125}I]T3. The usefulness of BrAc[^{125}I]T3 as a tool for the identification of novel TH transporters remains to be explored.

INTRODUCTION

It has become increasingly clear that thyroid hormone (TH) transporter proteins control intracellular TH availability by regulating transport of TH across the plasma membrane [1, 2]. The molecular identification and characterization of TH transporters has increased our understanding of TH physiology. Several classes of TH transporters have been identified, of which most transporters accept a variety of substrates [3]. Transporters which are relatively specific for TH are organic anion-transporting polypeptide (OATP) 1C1, monocarboxylate transporter (MCT) 8 and MCT10 [4-6]. Mutations in TH transporters may underlie human diseases, which was clearly exemplified when mutations in *MCT8* were found in patients with severe psychomotor retardation and abnormal serum TH levels [7, 8]. As it is likely that other as-yet-unknown specific TH transporters exist, the need to discover those proteins for a full understanding of TH pathophysiology is obvious. However, the tools to identify TH transporters are currently limited. Theoretically, a valuable method may be the modification of a transporter with an affinity-label and subsequent identification of the labeled protein by mass spectrometry.

Previously, *N*-bromoacetyl (BrAc)-iodothyronines have been reported as useful affinity-labels for TH carrier proteins, the T3-receptor, the type 1 deiodinase (D1) and protein disulfide isomerase (PDI) [9-15]. Based on these findings, our laboratory tested the possible affinity-labeling of hMCT8 with BrAc[^{125}I]T3 in transfected cells [16]. In cells transfected with hMCT8 a large increase in the BrAc[^{125}I]T3 labeling of a 61-kDa protein was observed, similar to the apparent molecular mass of hMCT8. Therefore, it was concluded that hMCT8 is labeled by BrAc[^{125}I]T3. This let to the suggestion that affinity-labels such as BrAc[^{125}I]T3 are useful tools for the identification of novel TH transporters.

Recently, we found that mutation of Cys residues in hMCT8, which are likely targets of BrAc[^{125}I]T3, did not abolish affinity-labeling [17]. Furthermore, we demonstrated that incubation of human umbilical venous endothelial cells with BrAc[^{125}I]T3 resulted in labeling of a 58-kDa protein, which was identified as protein disulfide isomerase (PDI) [18]. Therefore, we reinvestigated possible BrAc[^{125}I]T3-labeling of hMCT8. The present study demonstrates that hMCT8 and hMCT10 both facilitate BrAc[^{125}I]T3 transport. In contrast with previous conclusions, our current findings indicate that hMCT8 and hMCT10 are not labeled by BrAc[^{125}I]T3.

MATERIALS AND METHODS

Materials

[3'-^{125}I]T3, [3',5'-^{125}I]T4 and BrAc[^{125}I]T3 (specific radioactivity 78.6 MBq/nmol) were prepared as previously described [16, 19]. Nonradioactive iodothyronines, BrAcT3 and triiodothyroacetic acid (Triac) were obtained from Henning GmbH (Berlin, Germany).

Plasmids

Cloning of hMCT8, hMCT10 and rat (r) D1 in the pcDNA3 expression vector has been described previously [6, 16]. pCMV-SPORT6.hPDI was obtained from Open Biosystems (Huntsville, AL).

Cell culture

COS1 cells were cultured in six-well culture dishes with DMEM/F12 medium supplemented with 9% heat-inactivated FBS and 100 nM sodium selenite. Cells were transfected for 48 h with 500 ng pcDNA3.hMCT8, pcDNA3.hMCT10, pcDNA3.rD1, pCMV-SPORT6.hPDI alone or in combination as indicated.

TH uptake assays and affinity-labeling with BrAc[^{125}I]T3

TH uptake and affinity-labeling experiments were performed as described previously [16].

Western blotting

SDS-PAGE of cell lysates was performed as reported recently, except for the use of 10% acrylamide gels (Pierce, Etten-Leur, The Netherlands) in the present study [16, 20]. SDS-PAGE for western blotting was done in parallel with the affinity-labeling experiment. A specific polyclonal PDI antibody (Cell Signaling Technology, Danvers, MA) was used for probing blots following manufacturer's protocol. The purified MCT8-specific antibody 1306 and purified MCT10-specific antibody 1758 were used for detection of hMCT8 and hMCT10 protein, respectively.

Statistical analysis

All results are the means of duplicate determinations from 2-4 experiments. Values are expressed as means ± SE. Statistical significance was determined using the Student's *t* test for unpaired observations.

RESULTS

In the present study we transfected COS1 cells with cDNA coding for hMCT8, hMCT10, rD1, hPDI alone or in combination. Intact cells were incubated with BrAc[^{125}I]T3 and, subsequently, lysates were separated by SDS-PAGE. Autoradiography of untransfected cells shows 2 major bands of 48 and 55 kDa, respectively (Figure 1A, lane1). Transfection with rD1 (lane 4) resulted in a clear labeling of a protein with an apparent molecular mass of 25 kDa, in accordance with the molecular mass of D1. Transfection of hPDI (lane 5) increased the intensity of the labeled 55-kDa protein. Parallel western blotting revealed that hPDI and hMCT8 both have an apparent molecular mass of 55 kDa (Figure 1B,C). This implicates that no distinction based on molecular mass between hPDI and hMCT8 can be made in the BrAc[^{125}I]T3-labeling experiment. To investigate whether the 55-kDa labeled protein corresponds to either hMCT8 or hPDI, cells were co-transfected with hMCT8 and hPDI. Compared to hMCT8 alone (lane 2) or hPDI alone (lane 5), co-transfection with hMCT8 and hPDI (lane 7) strongly increased the affinity-labeling of the 55-kDa protein. In addition, co-transfection of hMCT10 and hPDI (lane 9) markedly enhanced the intensity of this protein compared with hMCT10 alone. These effects were not cell-type specific as they were observed in similar experiments with JEG3 cells (data not shown). The small decrease in D1 labeling if co-expressed with hMCT8 (lane 6) or hMCT10 (lane 8) is most likely due to decreased D1 expression resulting from a limited transcription/translation capacity and/or competition for trafficking of these plasma membrane proteins. We used a complementary approach to prove that MCT8 is not labeled by BrAc[^{125}I]T3. Therefore, we used a YFP-tagged hMCT8 construct, encoding a chimeric protein ~27 kDa larger than untagged hMCT8. MCT8-YFP is normally expressed at the plasma membrane (Figure 1D) and MCT8-YFP and untagged hMCT8 equally transport T3 (Figure 1E). Cells were transfected with hMCT8, hMCT8- YFP, rD1 and hPDI alone or in combination, and incubated with BrAc[^{125}I]T3. Co-transfection of hMCT8 or hMCT8-YFP with hPDI both increased the intensity of the 55 kDa band (Figure 1F, lanes 6 and 8). No band at ~82 kDa was observed when hMCT8-YFP was transfected. These results strongly argue against a labeling of hMCT8.

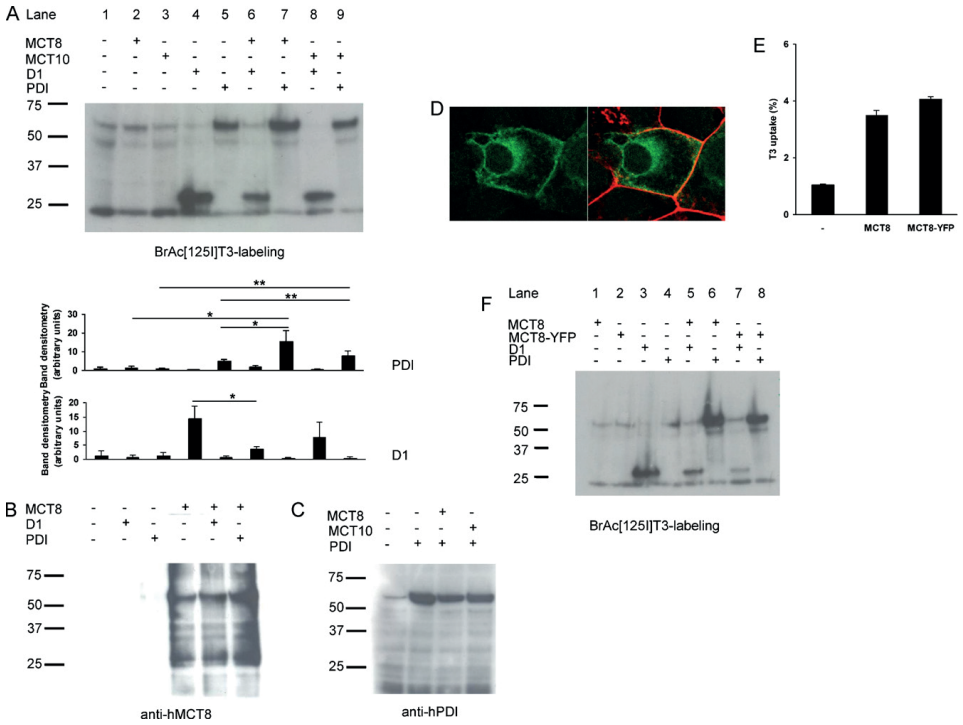

FIGURE 1. Parallel BrAc[^{125}I]T3 affinity-labeling and western blotting. (A) Affinity-labeling with BrAc[^{125}I]T3. COS1 cells were cotransfected with combinations of hMCT8, hMCT10, hD1 and rPDI. Intact cells were incubated for 6 h at 37 °C with BrAc[^{125}I]T3. Lower panels represent the relative intensities of the BrAc[^{125}I]T3 affinity-labeled proteins assessed on samples run in triplicate. *P < 0.05, **P < 0.01. Western blotting of COS1 cells co-transfected with combinations of hMCT8, hMCT10, rD1 and hPDI with a MCT8 antibody (B) and PDI antibody (C). (D) Fluorescence microscopy of cells transfected with MCT-YFP. (E) Uptake of [^{125}I]T3 in cells transfected with untagged MCT8 or MCT8-YFP. (F) Affinity-labeling with BrAc[^{125}I]T3. COS1 cells were co-transfected with combinations of hMCT8, hMCT8-YFP, rD1 and hPDI.

Since the present results and previous reports strongly suggest that hMCT8 and hMCT10 both facilitate BrAc[^{125}I]T3 transport, we tested this hypothesis directly by incubating cells transfected with hMCT8 and hMCT10 with BrAc[^{125}I]T3. Figure 2 shows a clear time-dependent increase in the uptake of BrAc[^{125}I]T3 (Figure 2A) in cells expressing hMCT8 and hMCT10, in contrast to [^{125}I]Triac uptake (Figure 2B), although less than [^{125}I]T3 (Figure 2C). These data confirm that hMCT8 and hMCT10 both facilitate transport of BrAc[^{125}I]T3, which subsequently labels intracellular hPDI and rD1 if present. Apparently, the 48-kDa protein does not represent degraded hPDI, as western blotting with an anti-PDI antibody does not detect a band of 48 kDa (Figure 1C).

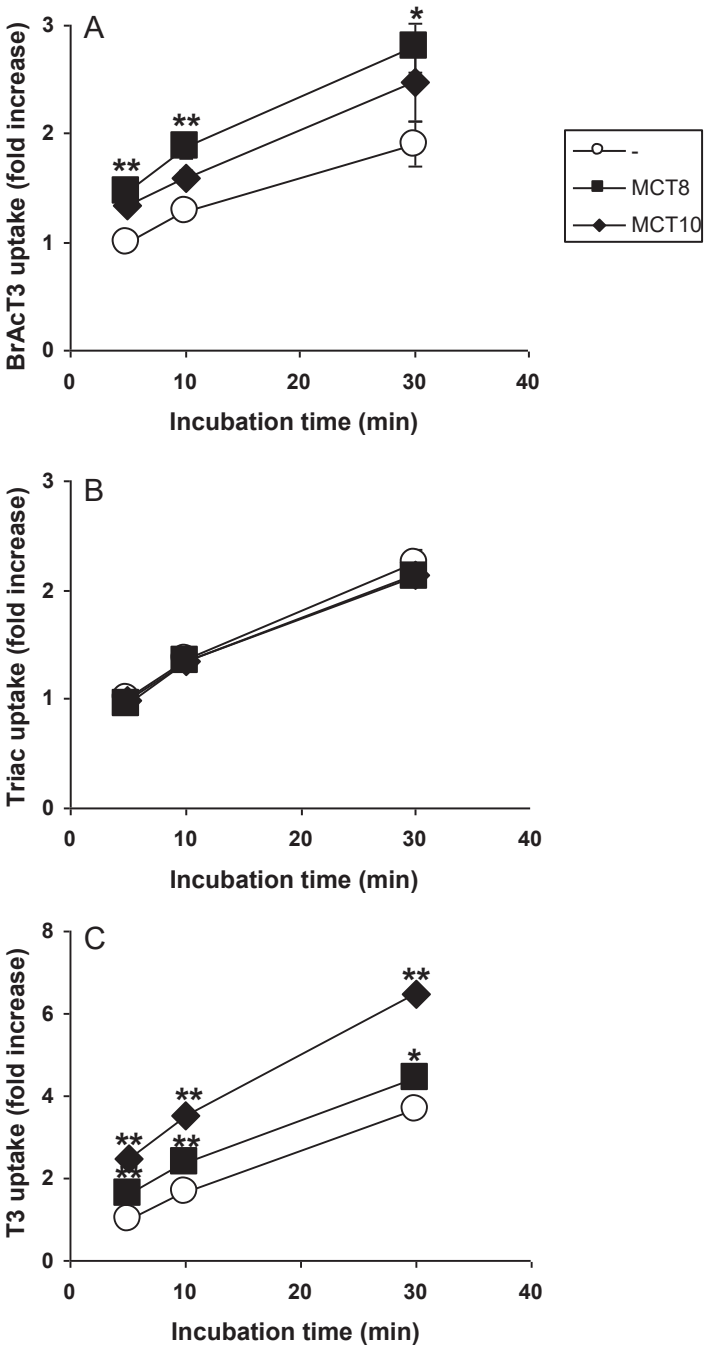

FIGURE 2. Uptake of BrAc[^{125}I]T3 (A), [^{125}I]Triac (B) and [^{125}I]T3 (C) in COS1 cells transfected with empty vector (circles), hMCT8 (squares) or hMCT10 (diamonds). *P < 0.05, **P < 0.01.

Next, we studied the effects of 10 μM T3, T4 and T3 analogs where the αNH_2 group is modified (BrAcT3) or removed (Triac) on hMCT8 and hMCT10-mediated T3 and T4 transport (Figure 3). T3 and T4 uptake by hMCT8 were equally inhibited by BrAcT3 as well as by T3 or T4 (Figure 3A,C). In contrast, no inhibitory effects of Triac were observed. hMCT10-facilitated T3 uptake was potently reduced by T3, whereas much weaker inhibitory effects were observed with T4, BrAcT3 and Triac (Figure 3B). The effects of the tested compounds on T4 uptake by hMCT10 were somewhat larger (Figure 3D). However, it should be noted that absolute T4 transport by hMCT10 is much less than T3 transport (2.97 *vs* 1.74 fold induction of T3 and T4 uptake, respectively).

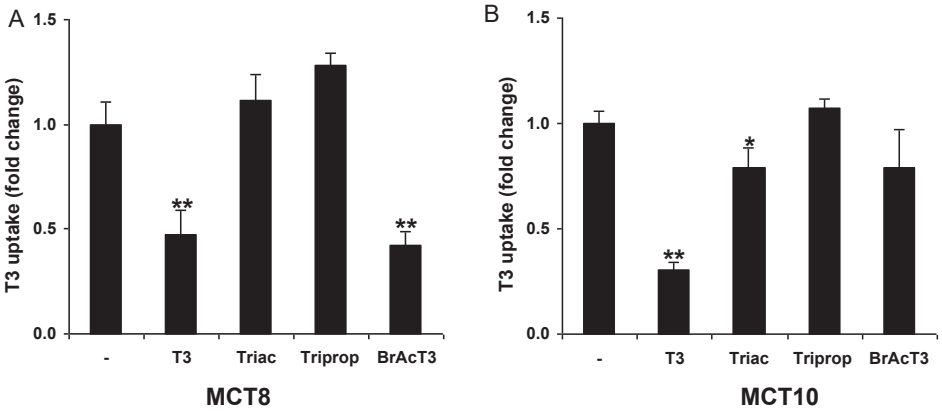

FIGURE 3. Influence of 10μM T3, Triac, Triprop or BrAcT3 on T3 uptake mediated by hMCT8 (A) and hMCT10 (B). *P < 0.05, **P < 0.01.

DISCUSSION

Efforts to discover novel TH transporters at the molecular level are currently limited by the lack of appropriate tools. Previously, the affinity-label BrAc[^{125}I]T3 was suggested to modify hMCT8 [6, 16]. It was assumed that if this key player in TH metabolism would be labeled by BrAc[^{125}I]T3, other important TH transporters might also be labelled. Combined with mass spectrometry approaches, modification of transporters by this label might be the first step to unravel the molecular identitity of such labeled proteins. In the present study we reinvestigated and extended studies concerning the BrAc[^{125}I] T3-labeling of hMCT8 and hMCT10.

The present study demonstrates that cells transfected with hMCT8, hMCT10 or hPDI all result in an increased intensity of a 55-kDa protein band labeled with BrAc[^{125}I]T3 (Figure 1). This agrees with recent work in which, retrospectively, an increased intensity of a protein of ~ 55 kDa can be observed not only in cells transfected with hMCT8, but also with hMCT10 [6]. Western blotting revealed that the apparent molecular mass of hMCT8 and hPDI is similar (Figure 1B,C), indicating that SDS-PAGE cannot differentiate between hMCT8 and hPDI.

We now demonstrate that cotransfection of hPDI with either hMCT8 or hMCT10 results in a largely increased intensity of the BrAc[^{125}I]T3-labeled 55-kDa protein compared to hPDI alone. This confirms the hypothesis that the 55-kDa protein represents hPDI and not hMCT8. For over 20 years, PDI has been shown to be readily labeled by BrAc[^{125}I]T3 [14]. It has been suggested that PDI, apart from its function in protein folding, may serve as a hormone reservoir to modulate intracellular free hormone concentrations [21]. Thus, the transporters hMCT8 and hMCT10 both facilitate BrAc[^{125}I]T3 transport, thereby increasing the intracellular availability of this affinity-label[^{125}I]T3 [6, 16, 22]. Subsequently, BrAc[^{125}I]T3 labels the intracellular protein hPDI as well as the intracellular rD1, if present. If rD1 was overexpressed, the labeling of hPDI decreased, likely representing competition of D1 and PDI for BrAc[^{125}I]T3. The closer proximity of the binding site of D1, located in the plasma membrane, to the internalized BrAc[^{125}I]T3, may result in a preferential labeling compared to PDI, which is located in the endoplasmic reticulum. Competition between two intracellular proteins is more likely than between an intracellular protein and plasma membrane transporter, supporting the concept that the 55-kDa band labeled by BrAc[^{125}I]T3 does not represent a transporter.

Consequently, hMCT8 is not modified by BrAc[^{125}I]T3, implying that our previous conclusions are not correct [16]. If hMCT8 would be labeled by BrAc[^{125}I]T3, replacement of at least one of the Cys residues, which are likely targets of BrAc[^{125}I]T3, would probably affect affinity-labeling. However, no difference in BrAc[^{125}I]T3 protein labeling was observed with any of the 10 mutated Cys residues in hMCT8 [17]. Furthermore, cells transfected with hMCT8 mutants, mimicking mutations found in MCT8 patients, resulting in a decreased BrAc[^{125}I]T3 labeling of hPDI is well explained by a reduced or absent transport capacity for the affinity-label [22]. The observation that BrAcT3 is transported by hMCT8 and hMCT10, whereas it does not modify these proteins, may be explained if the target amino acids Cys, Lys or His are not in the proximity of the substrate recognition site(s) for iodothyronine derivatives. This is supported by our previous work, which demonstrates that T3 transport in Cys>Ala hMCT8 mutants is not affected [17]. In

addition, the relatively specific TH transporter OATP1C1 appears also not to be labeled by BrAc[^{125}I]T3 (*W.M. van der Deure, personal communication*). Thus, it remains to be seen if BrAc[^{125}I]T3 is a useful tool to identify new TH transporters as suggested earlier.

In our inhibition studies, we noticed that excess T3 and T4 were similarly effective in reducing hMCT8-mediated iodothyronine uptake, with the effects on T4 uptake even exceeding those on T3 uptake. Iodothyronine transport was differentially affected by the T3 derivatives BrAcT3 and Triac, where the αNH2 group is blocked or deleted, respectively. No inhibitory effects of Triac on hMCT8-mediated T3 and T4 uptake were observed, which may suggest that the αNH2 group is important for substrate recognition by hMCT8. However, this explanation is challenged by the observation that BrAcT3 potently inhibited T3 and T4 transport. These findings are reconciled assuming that the αNH2 group is important for substrate recognition and the space-occupying Br-atom is responsible for the inhibition by BrAcT3.

Alternatively, other intrinsic characteristics of the tested inhibitory compounds such as molecular weight, charge and length of the side chain may explain different inhibitory effects. Previously, however, strong inhibitory effects were noticed of both Triac and BrAcT3 on iodothyronine uptake by rat Mct8 [5]. These different effects of various compounds on iodothyronine transport in the present study compared with the previous results may be explained by differences between human and rat MCT8 or by technical differences such as the expression system (mammalian cell lines *vs* Xenopus laevis oocytes) and incubation medium.

The most potent inhibitor of T3 uptake by hMCT10 was T3, whereas weaker inhibitory effects were observed for T4, BrAcT3 and Triac. More pronounced effects were observed on T4 uptake, although it should be noted that hMCT10 preferentially transports T3. The observation that cis-inhibitory effects of T3, T4, BrAcT3 and Triac as well as the known substrate preferences differ between hMCT8 and hMCT10 may suggest that the homologous transporters have different binding sites for the common substrate T3.

In conclusion, we demonstrated that not hMCT8 and hMCT10, but hPDI, are labeled by BrAc[^{125}I]T3, which suggest that this affinity-label should be used with caution for the identification of novel TH transporters. Furthermore, our study show different inhibitory effects of iodothyronine (derivatives) on T3 transport by hMCT8 *vs* hMCT10, suggesting different binding sites for T3 in these TH transporter proteins.

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CHAPTER 7

Overlap and differences in genes regulated by 3,5-T2, T3 and Triac

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MANUSCRIPT IN PREPARATION

ABSTRACT

Genomic action of thyroid hormone is mediated by binding of the bioactive hormone T3 to its nuclear receptors, *THRA* and *THRB*. Different thyroid hormone receptor isoforms are generated by alternative splicing and different promoter usage, with TRα1, TRβ1 and TRβ2 as highly homologous T3 binding isoforms. Besides T3 also other iodothyronines, such as 3,5-T2 and TA3, have been shown to bind to the thyroid hormone receptors. In the current study, we compared effects of T3, 3,5-T2 and TA3 on gene expression profiles in the human liver cell line HepG2, which endogenously expresses similar levels of TRα1 and TRβ1. We show that these iodothyronines affect expression of the same genes based on micro-array data. None of ligands showed a preference between the TRα1 and TRβ1 isoforms in our luciferase cell model.

INTRODUCTION

Thyroid hormone (TH) plays a key role in essential processes of the human body, such as development, growth, differentiation and metabolism [1-3]. The genomic action of thyroid hormone is mediated by binding of T3 to its thyroid hormone receptors (TRs) which are bound to different thyroid hormone response elements (TREs) in the promoter region of thyroid hormone regulated genes [4]. Depending on the gene and binding with coactivators or corepressors, T3 can have positive or negative effects on gene transcription [5]. TRs are encoded by 2 genes, *THRA* and *THRB,* located on chromosome 3 and 17 respectively [3, 5]. Different TR isoforms are generated by the usage of different transcription start sites and different splice sites, with TRα1, TRβ1 and TRβ2 as highly homologous T3 binding isoforms [5]. TRα1 and TRβ1 are both ubiquitously expressed, whereas TRβ2 has a more restricted expression pattern [1, 5]. TRα1 is predominantly expressed in brain, skeletal muscle, lung, heart, and intestines whereas TRβ1 is predominantly expressed in kidney, liver, pituitary and thyroid [1, 5-7]. Mutations and deletions in the different TR isoforms result in the clinical phenotype of Resistance to Thyroid Hormone (RTHα and RTHβ) (see [8-11] for extended reviews).

The thyroid gland mainly produces the pro-hormone T4 [3]. Only 20% of serum concentrations of the bioactive TH (triiodothyronine, T3) is derived from thyroidal secretion, whereas the remainder is produced by extrathyroidal conversion (deiodination) out of T4 [3, 12]. T4 and T3 can be further deiodinated, hereby generating other metabolites such as rT3, 3,5-T2 and 3,3'-T2 [13].

Outer-ring deiodination of T3 results in the production of the naturally occurring ligand 3,5-T2 [14, 15]. 3,5-T2 levels are detectable in healthy subjects, and can be increased during illness [15]. It has been shown that administration of 3,5-T2 in rats leads to a rapid increase in mitochondrial activity [15]. This seems to be mediated via non-genomic effects, which fits with the rapid onset of action [15]. However other data suggest that 3,5-T2 can also bind and activate the TRβ1 receptor [14-16]. In addition, it has been shown that 3,5-T2 can regulate some thyroid hormone responsive genes, although not as potent as T3 [16]. The affinity of 3,5-T2 for TRβ is >40 times weaker than T3 [12, 15].

Triac (TA3) is an alternative thyroid hormone metabolite. It is produced in the liver by deamination and decarboxylation of the alanine side chain [15], which is estimated to account for 14% of total T3 metabolism in humans [12, 15]. TA3 has a higher preference for TRβ1 (3.5 fold) and TRα1 (1.5 fold) than T3 [15, 17]. TA3 treatment of patients with

Resistance to TH due to a mutation in TRβ (RTHβ) suppresses TSH and leads to an increased metabolic rate in obese patients [12, 18]. However, due to the short half-life of TA3, higher therapeutic dosages are needed than of T3 [15, 18, 19].

Previous studies in TR knockout mouse models and in stably TR transfected cell lines reveal that TRα1 and TRβ1 largely regulate overlapping sets of target genes [4, 20, 21]. In addition, microarray analysis of liver tissues from TRβ KO mice compared to wild type mice implied a role in the liver for TRα after stimulation with T3 [22]. However, in contrast to these overexpression and knockout models, no studies have yet been performed in cells with endogenous TR expression. The liver is one of the major target organs of TH. For that reason, we studied HepG2 cells, a known liver cell line with the necessary endogenous TH machinery for transport, deiodination and metabolism [23-25], in which lipid metabolism is still regulated by genomic actions of TH [26].

In the current study, we compared effects of T3, 3,5-T2 and TA3 on gene expression profiles in the human liver cell line HepG2, with equal endogenous expression of TRα1 and TRβ1.

MATERIALS AND METHODS

HepG2 cell culture

HepG2 cells were cultured in 6 well plates containing DMEM/F12 with 9% heat-inactivated fetal bovine serum and 100 nM sodium selenite until 80% confluency. The cells were for 24h pre-incubated with DMEM/F12 with 9% charcoal treated serum to deprive the cells of thyroid hormones. After pre-incubation, the cells were incubated for either 6 or 24h with 10 or 100 nM T3, TA3, 3,5-T2 or vehicle. Direct genomic effects were studied after 6 hours of incubation. The iodothyronines used in this study (T3, 3,5-T2, and TA3) were purchased from Henning.

Array

After incubation of the HepG2 cells, RNA was isolated and biotin labeled aRNA was generated with Illumina Totalprep RNA Amplification Kit (Applied Biosystems). RNA quality (RIN) was analyzed with an Agilent's 2100 bioanalyser. Afterwards, the samples were hybridized in biological triplicates on an Illumina HumanHT-12_v3_Beadchip. After several washing steps, the slides were scanned with an Ilumina beadstation. Data were exported from GenomeStudio (Illumina) after correction for background and average normalization. Data analysis was performed in BRB-ArrayTools v3.8.1 after detection p-value selection. In total 15435 probes were analysed. All data were an average of

biological triplicates. Significance of hormone-induced changes in gene expression was determined by SAM analysis. An unadjusted parametric p-value <0.001 was considered significant. Heat maps were generated using TMEV and Venn diagrams using BioVenn [27].

Luciferase assay

Luciferase assays were performed as previously described [28]. HepG2 cells were treated as described above and incubated after transfection with 1-1000 nM ligand for 6 hours.

RESULTS AND DISCUSSION

In the current study, we analysed the effect of T3 and two alternative TR ligands (TA3 and 3,5-T2) on gene expression profiles in a human hepatic cell line, which endogenously expresses TRα1 and TRβ1 to a similar extent [4, 29]. TRα1 and TRβ1 are both ubiquitously expressed, although TRβ1 is generally considered to be the predominant isoform in liver [5-7]. A previous micro-array analysis of HepG2 cells overexpressing either TRα1 or TRβ1 revealed that T3 stimulation regulated largely overlapping sets of target genes, but to a distinct level [4]. Another study on isoform specific regulation of target genes was performed in TRα or TRβ knock-out mouse models, also showing largely overlapping sets of genes upon stimulation of the two receptors [20]. However, this study was performed in congenital knockouts, which may have influenced results and compensatory expression of the other TR isoforms may have contributed to the overlap in gene expression patterns. So far, none of the studies analyzing isoform specific effects used a model in which endogenously expressed TRs were studied.

A 6 hours incubation of HepG2 cells with 10 nM T3 resulted in a significant regulation of 92 genes, whereas 10 nM 3,5-T2 affected 154 genes and 10 nM TA3 169 genes compared to control cells (Figure 1A). 13 genes were significantly regulated by all ligands, whereas 19 genes were regulated by both T3 and TA3, 26 genes were regulated by TA3 and 3,5-T2 and 14 by T3 and 3,5-T2. Despite this relatively small overlap in significant genes, a heatmap analysis showed a very similar pattern of genomic effects for the different ligands (Figure 1B).

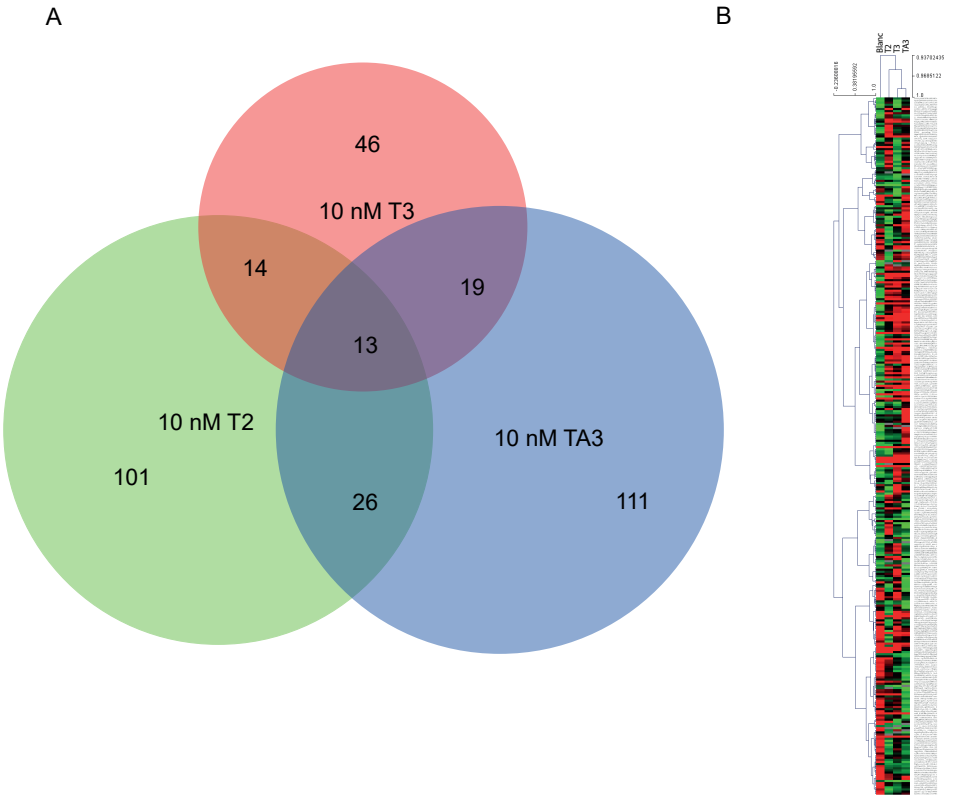

FIGURE 1. A Venndiagram with significant regulated genes by 10 nM of the ligand and its overlap. **B** Heatmap with the genomic effects of the different ligands.

A subsequent microarray was performed with higher concentrations of 100 nM 3,5-T2 and T3, showing an increased number of significantly regulated genes. 100 nM T3 regulated 201 genes and 100 nM 3,5-T2 324 genes, with an overlap of 49 genes (Figure 2A). Again, the heatmap analysis showed highly similar genomic effects for both ligands, although several genes only reached significance in one of the two incubations (Figure 2B). Extended incubations for 24 hours with 10 nM of the different ligands showed again a similar pattern with overlapping gene profiles (data not shown). Although the data from the SAM analysis might suggest differences in sets of genes regulated by the different ligands, the heatmap clearly shows great overlap in gene regulation between the ligands.

The liver is a suitable model to study changes in genomic expression by TH and its analogues, since approximately 8% of the hepatic genes are regulated by TH [21]. HepG2

cells, derived from a well differentiated hepatocellular carcinoma, are a commonly used model for the liver because they still secrete known liver specific plasma proteins [23, 30, 31]. Furthermore, HepG2 cells have the endogenous machinery to respond to changes in TH concentrations [23-25].

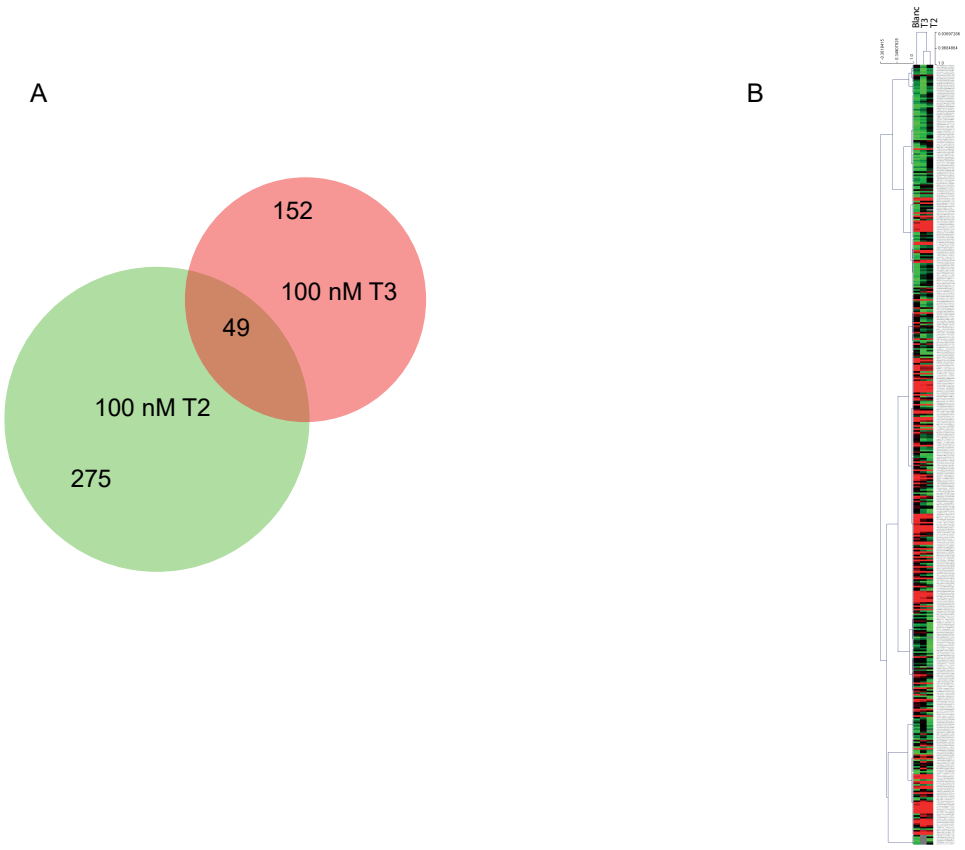

FIGURE 2. A Venndiagram with significant regulated genes by 100 nM of the ligand and its overlap. **B** Heatmap with the genomic effects of the different ligands.

In line with previous studies showing that HepG2 cells overexpressing TRα1 and TRβ1 regulate largely overlapping gene sets when stimulated by high doses of (100nM) T3 [4], our results show a great overlap in gene regulation between different TR ligands via the endogenous TRs in HepG2 cells. To correlate these expression data to *in vitro* nuclear receptor stimulation we subsequently studied a luciferase cell model. Transfected HepG2 cells with a TRE-regulated luciferase receptor and renilla control either in combination

with pcDNA3, TRα1 or TRβ1 were treated using a similar protocol as the cells analysed for gene expression above. After incubation with increasing concentrations of either T3, 3,5-T2 or TA3, luciferase activity was determined. After 6 hours of incubation, T3 stimulated the luciferase reporter signal in both TRα1 and TRβ1 transfected HepG2 cells. Concentrations of 100 nM T3 and higher were equally effective as 10 nM T3 (Figure 3A). For TA3 a similar result was seen as for T3. No effect was noted of TA3 on the cells transfected with empty vector, while increased concentrations of ligand resulted in increased stimulation of the luciferase signal (Figure 3B). A similar experiment was performed with increasing concentrations of 3,5-T2. At high concentrations, 3,5-T2 (>100 nM 3,5-T2 for TRα1 and >1000 nM 3,5-T2 for TRβ1) was also able to induce luciferase activity (Figure 3C), which is in line with an induction of gene expression as shown in the previous figures. There was no evidence for a particular receptor preference of the different ligands in our cell model.

In conclusion we analyzed the genomic action of T3, 3,5-T2 or TA3 by looking at regulation of gene expression. There was great overlap in genes regulated by the different ligands in HepG2 cells, and we did not find any evidence for a preference for specific receptor isoforms of the different ligands.

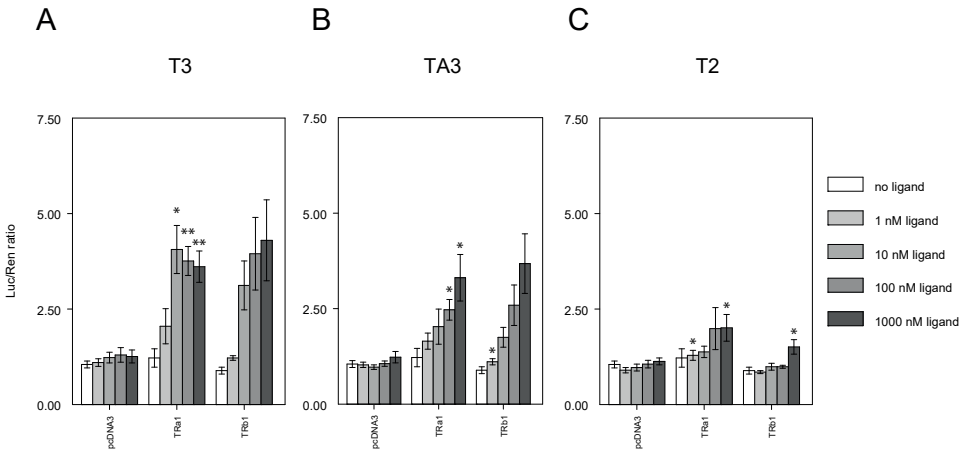

FIGURE 3. Functional analysis of T3 (**A**), TA3(**B**) or 3,5-T2(**C**) incubations of HepG2 cells transfected with a TRE-regulated luciferase and control renilla construct co-transfected with either empty vector, TRα1 or TRβ1. After 6h of incubation with increasing concentrations of the ligand, luciferase activity was determined.

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CHAPTER 8

General discussion

GENERAL DISCUSSION

TH plays a crucial role in development, differentiation and metabolism of the human body [1, 2]. TH action is mediated by binding of T3 to its nuclear receptors TRα and TRβ. Regulation of TH action at the cellular level can take place at different levels. TH action can be disturbed by defects in TRα or TRβ. Furthermore, it can be regulated at the pre-receptor level by alterations in the intracellular metabolism of TH mediated by the deiodinases, or it can be regulated via alterations in the uptake of TH into the cell. All three aspects are discussed in this thesis and this chapter, which is largely based on two reviews that we published [3] and [4].

THYROID HORMONE RECEPTORS

Resistance to TH (RTHα and RTHβ)

In 1967, a familial syndrome with reduced sensitivity to TH was first described [16]. This clinical syndrome, which was later demonstrated to be due to mutations in *THRB*, is nowadays known as resistance to thyroid hormone β (RTHβ) [16, 17]. The mutant TRβ interferes with the function of wild-type (WT) TRβ, resulting in a dominant-negative effect and dominant inheritance [18]. In contrast, RTH caused by *THRB* gene deletions has a recessive inheritance, due to a lack of dominant-negative interference with the WT receptor [19]. Homozygous mutations in TRβ are rare and result in a more severe phenotype [20]. Until now more than 1000 patients and more than 100 different mutations have been published [20-23]. The mutations identified in RTH patients are located in the C-terminus of TRβ, which is identical in TRβ1 and TRβ2. The mutations are mostly contained within three CpG rich "hot spots" in the LBD (amino acid 242-460 in TRβ1) and adjacent hinge domain (amino acid 234-243) of the receptor protein [22]. The mutant TRβ proteins have a reduced affinity for T3, and/or abnormal interaction with cofactors (decreased interaction with coactivators [24] or increased interaction with corepressors [25]).

Patients with RTHβ have high levels of TH without clinical symptoms of hormone excess in most tissues, or even with symptoms of TH deficiency in certain tissues [26, 27]. The syndrome is characterized by elevated serum TH levels and a non-suppressed TSH. Serum rT3 levels are usually high resulting from a decreased activity of D1, which gene is T3-dependent and under control of TRβ [28]. The severity of TH resistance varies not only among different tissues in an affected individual, but also among different subjects

carrying the same gene mutation, even within the same family [29, 30]. Treatment is usually not necessary in most RTHβ patients, while others will benefit from treatment with TA3 [31].

Ever since its characterization in 1987, investigators have searched for patients with mutations in *THRA* [32]. As TRα1 is not involved in the negative feedback action of TH, no major changes in serum TH levels were expected. To unravel the physiological role of TRα, different mouse models were generated. Interestingly, mice devoid of all TRs have less symptoms of hypothyroidism than wild-type hypothyroid mice, consistent with a repressive effect of unliganded TRs [33]. Unliganded TRα1 seems to play a major role in the cerebellar damage in hypothyroid mice [34-39]. In 2012 the first human patients with heterozygous inactivating mutations in TRα1 were identified, of which two of them are described in **chapter 2 and 3** [40-50]. Until now, 15 different mutations have been identified [41-53]. In the last 6 years, 28 cases from 15 different families with mutations in THRA have been identified. All described mutations are localized in the LBD of TRα, thereby impairing ligand binding without influencing DNA binding [1, 42].

As described in **chapters 2 and 3,** the phenotype of TRα1 patients with inactivating mutations includes growth retardation, delayed bone development, mildly delayed motor and mental development, abnormal thyroid function tests, low GH and IGF1 levels, and constipation [40-42]. These findings support an important role of TRα1 in bone, brain, intestine, and possible involvement in GH regulation. Interestingly, the phenotypes of heterozygous TRα1-T394fs406X (TRα1-PV) and TRα1-L400R mice, with a mutant TRα that completely lacks T3 binding, is very similar to the phenotype of the human patients, with severe growth retardation as the most prominent phenotype [35, 37]. In addition to the growth retardation, delayed bone development is an important part of the phenotype. Several of the patients identified at young age had a delayed tooth eruption, delayed closure of the skull sutures, and a clearly delayed bone age [41, 43, 44, 51, 52].

Mice with a TRα1 mutation have a very diverse phenotype, depending on the location and the severity of the mutation [35-39]. Characteristics of the various mouse models include delayed endochondral ossification resulting in dwarfism, disturbed behavior, memory impairment, locomotor dysfunction, mild bradycardia and insulin resistance [35, 37, 38, 54-59]. These diverse phenotypes are probably due to different interactions of unliganded TRα1 mutants with co-repressors, whose expression is tissue-dependent and developmentally regulated [25, 60]. Similarly, it can be expected that human mutations with a different location or a less detrimental effect on the function of TRα1, will have a different or more subtle effect on the clinical phenotype. In line with mutations in

RTHβ, the identified mutations in RTHα are in the equivalent C-terminal domain of the receptor and also show evidence for a dominant negative effect of mutant TRα1 over WT TRα1 [41, 43, 44, 46-48, 50-52]. Patients with a mutation in both TRα1 and TRα2 have a similar clinical phenotype as patients with a TRα1 mutation, except the patient with N359Y [42, 47]. In case of a combined mutation in TRα1 and TRα2, a higher dosage of TH may lead to reversibility of the dominant negative effect [43, 44, 46].

As described in **chapters 2 and 3,** RTHα patients generally have low (F)T4, high T3, low rT3, but normal TSH levels. Similarly, most adult TRα1 mutant mice are euthyroid with a mildly elevated TSH. The high T3/T4 ratio as well as the low rT3 levels in patients with RTHα suggest an altered expression of deiodinases, which are the most important mediators of peripheral metabolism of TH (**chapter 3**)**.** Indeed, TRα1-PV mutant mice, with a frame-shift mutation in TRα1 similar to some of the patients, have elevated levels of T3 in combination with a normal TSH and an increased levels of hepatic D1 [35, 61]. In contrast to TRα1-PV mutant mice, TRα1-/- mice have normal liver D1 activity [62]. Increased D1 activity results in increased T4 to T3 conversion and degradation of rT3. In addition, TRα1-/- mice have an impaired regulation of D3, which leads to a reduced production of rT3 and degradation of T3 [62, 63]. Both changes in D1 and D3 expression may contribute to the particular TH changes in patients with TRα1 mutations. In **chapter 4,** we show that TRα1-PV mice have an increased liver D1 activity compared to WT mice, while D3 activity is decreased. Furthermore, D3 activity in cerebellum of TRα1-PV mice was decreased, despite the increase in serum T3 levels. As described in **chapters 2 and 3,** treatment of the patient with a F397fs406X mutation with LT4 resulted in an initial catch-up in growth rate and bone age, while additional GH treatment had little effect. [41, 64]. A different patient, who was identified at the age of 1 year with a D211G mutation, reported an evident improvement in motor development, next to an increase of length [43]. TSH is generally suppressed during treatment, implying a normal feedback loop [43, 44, 46-48, 52]. Furthermore, as described in **chapter 3,** SHBG and IGF1 increase upon treatment, while cholesterol and serum creatine kinase levels decrease [43, 44, 46, 48, 51, 52]. Moreover, therapy also result in an improvement of the constipation, whereas the anemia remains [42]. Interestingly, in TRα1-R384C mice, whose receptor has a 10 times decreased but not completely abolished affinity for T3, growth retardation can be overcome by raising serum TH levels [38, 65, 66]. Furthermore, these mice display neurological damage which improves after T3 treatment. A delayed cerebellar development and locomotor dysfunction can be prevented by postnatal T3 treatment, whereas anxiety-like behaviour and reduced recognition memory is relieved by T3 treatment in adulthood [65].

Gene expression

TRs are bound to different TREs in the promoter region of thyroid hormone regulated genes [5]. Depending on the gene and binding with coactivators or corepressors, T3 can have positive or negative effects on gene transcription [6]. With micro-array analysis the effect of different ligands on gene expression profiles can be studied. In **chapter 7** we analysed the effect of T3 and two alternative TR ligands (TA3 and 3,5-T2) on gene expression profiles in a human hepatic cell line, which endogenously expresses TRα1 and TRβ1 to a similar extent [5, 7]. Since approximately 8% of the hepatic genes are regulated by TH, the liver is a suitable model to study changes in genomic expression by TH and its analogues [8]. HepG2 cells, derived from a well differentiated hepatocellular carcinoma, are a commonly used model for the liver because they still secrete known liver specific plasma proteins and have the endogenous machinery to respond to TH [9-13].

Triac (TA3) is an alternative thyroid hormone metabolite, which is produced in the liver by deamination and decarboxylation of the alanine side chain [14]. It has been reported that TA3 has a higher preference for TRβ1 (3.5 fold) and TRα1 (1.5 fold) than T3 [14, 15]. Therefore, TA3 has been used to treat of patients with RTHβ. In **chapter 7** we studied if the relative TRβ selectivity of TA3 resulted in differences in gene expression compared to T3 or another TH metabolite 3,5-T2 in a liver cell line (HepG2) which endogenously expresses TRα1 and TRβ1 to a similar extent [5, 7]. However, there was great overlap in genes regulated by the different ligands and we did not find any evidence for a preference for specific receptor isoforms of the different ligands.

Deiodinases

Expression of the iodothyronine deiodinases D1-3 is controlled by thyroid state. D1 expression in liver is increased in hyperthyroidism and decreased in hypothyroidism and this regulation is exerted by T3 primarily at the transcriptional level [28]. The T3 response elements (TREs) involved have been identified in the 5' flanking region of the DIO1 gene [67-69]. D2 expression in brain and other tissues is negatively regulated by TH, and this involves both transcriptional and post-translational mechanisms [70]. D3 expression in the brain is positively regulated by T3 at the transcriptional level, mediated primarily by the binding of TRα1 to a TRE half site in the upstream region of the *DIO3* gene [62]. Expression of the deiodinases is also regulated by other (patho)physiological factors, such as stage of development, nutrition and illness [71-73]. D3 expression is stimulated by a variety of factors, including hypoxia induced factor 1α, TGFβ and sonic hedgehog [70, 74-77]. Furthermore, the *DIO3* gene is located in the DLK1-DIO3 locus on mouse

chromosome 12 and human chromosome 14 comprising genes which are controlled by imprinting. At least in mice, the paternal D3 allele is more actively expressed than the maternal allele. D3 expression is thus regulated by a distant locus control region [72, 78].

In **chapter 4** we describe the altered deiodinase activity in TRα1-PV mice, with a decrease in D3 in liver and cerebellum. D3 was also decreased in cerebellum of hypothyroid mice, but not in liver. Interestingly, the hypothyroidism-induced decrease in cerebellum D3 activity was largely prevented in TRα1-/- mice but not in TRβ1-/- mice. The negative control of liver D3 by TH was still observed in TRα-/- mice and to some extent also in TRβ-/- mice. Our results suggest opposite regulation of D3 expression by T3 and T3 receptor subtypes in mouse brain and liver. We presume that TRα1 patients have an increased D1 as well as decreased D3 expression. However, the extent to which these changes in D1 and D3 expression contribute to the alterations in serum TH levels or vice versa remains elusive.

Thyroid hormone transporters

Since action and deiodination of TH take place intracellularly, transport of the hormone across the plasma membrane is required. Although many transporters accept T4 and/or T3 as a ligand, only a few transporters have been shown to be specific TH transporters [79], of which Monocarboxylate transporter 8 (MCT8, SLC16A2) is the most studied one. MCT8 specifically transports the iodothyronines T4, T3, rT3 and 3,3'-T2 [80, 81]. The highly homologous MCT10 (SLC16A10) was initially designated as a T-type aromatic amino acid transporter, but has later been shown to transport TH, with a preference for T3 over T4 [82, 83]. In contrast to MCT8, no patients with mutations in MCT10 have been identified so far. Both MCT8 and MCT10 are widely expressed. The organic anion transporting polypeptide 1C1 (OATP1C1) is importantly expressed at the blood brain barrier and transports T4 [84-86]. Another known TH transporters are Na+/taurocholate-cotransporting polypeptide (NTCP), also known as SLC10A1, several organic anion transporting polypeptides (OATPs) and L amino acid transporters capable of TH transport [87-89]. Other yet unknown specific TH transporters are likely to be important for human physiology as well [90].

The clinical importance of TH transporters was established by the discovery of mutations in the *MCT8* gene, which is located on the X-chromosome, as a cause of psychomotor retardation accompanied by TH abnormalities [91-95]. Affected males display a severe delay in motor and neurological development [96]. Soon after the description of the first patients, it was realized that the phenotype had similarities to the Allan-Herndon-Dudley syndrome (AHDS), the first X-linked mental retardation syndrome described in 1944. Genetic analysis in these families revealed that *MCT8* mutations are the

genetic basis of AHDS [96]. The complete pathogenesis of AHDS is not yet completely understood, but it is hypothesized that AHDS is due to a hypothyroid state of the MCT8-expressing neurons as a result of the inactivating mutations in MCT8 [90, 95, 97-99]. All AHDS patients have a very characteristic and remarkable combination of serum TH abnormalities. Serum T4 concentrations are low or low-normal, while serum T3 levels are markedly elevated. TSH levels are in the high normal range. Serum T3 levels are far above the upper reference limit, particularly during childhood. Serum rT3 levels are largely reduced. Consequently, T3/rT3 ratios are strongly increased. This biochemical profile is very similar to the thyroid function tests seen in patients with *THRA* mutations although it appears that serum T3 levels are less elevated than in AHDS patients.

Our *In vitro* studies in **chapter 5** showed that MCT8 only increased intracellular availability of TH for its nuclear receptor in the absence of D3. If D3 was co-transfected along with MCT8, there was a marked stimulation of intracellular T3 metabolism. D3 is mainly present in fetal tissues and its expression level declines after birth [100, 101]. D3 is located in the plasma membrane with its active center located on the cytoplasmic surface [81, 102, 103]. T3 action is mediated by binding to its nuclear receptor. Therefore, the action of T3 requires a much greater penetration into the cell than its deiodination. The extent of T3 distribution within the cell depends on a number of factors, including the rate of T3 transport back to the extracellular milieu and its binding to intracellular proteins, such as cytoplasmic TH-binding protein μ-crystallin (CRYM) that may facilitate its further distribution [104].

Labeling

Previous research showed that labeling can be a useful method to identify TH transporters [105]. The transporter is labeled with an affinity label and subsequently identified by mass spectrometry. The transporters hMCT8 and hMCT10 both facilitate BrAc[^{125}I]T3 transport, thereby increasing the intracellular availability of this affinity-label[^{125}I]T3 [81, 106, 107]. Our results in **chapter 6** show that the affinity label did not modify hMCT8 and hMCT10. This may be explained by the fact that its target amino acids Cys, Lys or His are not in the proximity of the substrate recognition site(s) for iodothyronine derivatives. Instead of the transporters themselves, it turned out that an intracellular binding protein was labeled [105]. It therefore remains to be determined if labeling remains a useful method for the identification of TH transporters.

CONCLUSION

This thesis focuses on the clinical and molecular aspects of nuclear thyroid hormone action. We identified one of the first patients with a mutation in TRα1 (RTHα) and subsequently studied their phenotype and possible underlying mechanisms. The phenotype of patients with RTHα includes growth retardation, delayed bone development, mildly delayed motor and mental development, abnormal thyroid function tests, low GH and IGF1 levels, and constipation. Treatment with levothyroxine results in clinical improvement. The phenotype of the RTHα patients identified so far is highly similar but does not completely overlap with the various TRα1 mice models. This is probably dependent on the location and the severity of the mutation. Presumably, further research will reveal new mutations, the role of binding with coactivators or corepressors and improvement of therapy options. Furthermore, we explored the effect of TR ligands TA3 and 3,5-T2, on gene expression profiles in a human hepatic cell line and found a great overlap between the ligands.

Another important factor in TH homeostasis is transport of TH across the plasma membrane. We show that this particularly increases TH metabolism mediated by deiodinases. Furthermore, we demonstrate that affinity labelling results in an intracellular binding protein instead of the TH transporters, MCT8 or MCT10.

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CHAPTER 9

SUMMARY

Thyroid hormone (TH) is important for normal development, differentiation and metabolism. Untreated congenital hypothyroidism leads to cretinism with brain damage, dwarfism, constipation, lethargy and feeding difficulties. TH is synthesized by the thyroid gland, which mainly produces the prohormone T4 and to a lesser extent the biological active T3. Multiple TH transporters have been recognized, an example of a highly specific transporter is monocarboxylate transporter 8 (MCT8). Three deiodinating enzymes (D1-3) have been identified which catalyze the activation of T4 to T3 or the inactivation of T4 to rT3 and of T3 to 3,3'-T2. TH action is mediated by binding of T3 to its nuclear receptors TRα and TRβ.

TH action at the cellular level can be regulated at different levels. TH action can be disturbed by defects in TRα or TRβ. Furthermore, it can be regulated at the pre-receptor level by alterations in the intracellular metabolism of TH mediated by the deiodinases, or it can be regulated via alterations in the uptake of TH into the cell.

This thesis focuses on the clinical and molecular aspects of nuclear thyroid hormone action. **Chapter 1** is a general introduction about the regulation of TH action, which also contains the general aims and outline of this theses. In **chapter 2** we describe the phenotype of a girl and her father with an inactivating mutation in TRα1 leading to growth retardation, delayed bone development, mildly delayed motor and mental development, abnormal thyroid function tests, low GH and IGF1 levels, and constipation. In **chapter 3** we report about the effect of LT4 treatment in two patients (described in **chapter 2**) with a heterozygous mutation in TRα1. Treatment with LT4 leads to an improvement of certain features of the phenotype, such as serum markers, hypothyroid features and growth. These findings support an important role of TRα1 in bone, brain, intestine, and possible involvement in GH regulation.

RTHα patients generally have low (F)T4, high T3, low rT3, but normal TSH levels, which suggests an abnormal deiodinase activity. In **chapter 4** we investigate the effects of mutation of TRα1, as well as the consequences of hypothyroidism in KO and wild-type mice on deiodinase activity. TRα1-PV mice have an increased liver D1 activity compared to WT mice, while D3 activity is decreased. Furthermore, D3 activity in cerebellum of TRα1-PV mice was decreased, despite the increase in serum T3 levels. In **chapter 4** we show opposite regulation of D3 expression by T3 in brain versus liver and kidney. In **chapter 5** we analyse the role of MCT8 and MCT10 on biological availability of TH for either D3 or the nuclear TR. Both TH transporters increase intracellular availability of TH for D3 and for its nuclear receptor, but genomic action needs more deep penetration

into the cell. The availability of TH is influenced by intracellular binding proteins such as cytoplasmic TH-binding protein μ-crystallin (CRYM). In **chapter 6**, we show that affinity labelling does not modify MCT8 and MCT10, but an intracellular binding protein. If labeling is an useful method for TH transporter identification remains to be determined. In **chapter 7** we explore the effect of TR ligands (TA3 and 3,5-T2) on gene expression profiles in a human hepatic cell line compared to the biological active T3. There was great overlap in genes regulated by the different ligands and we did not find any evidence for a preference for specific receptor isoforms of the different ligands. Finally, in **chapter 8** we discuss about the findings in this thesis and its implications in view of the literature.

This thesis focused on the clinical and molecular aspects of nuclear thyroid hormone action. We identified one of the first patients with a mutation in TRα1 and subsequently studied their phenotype and possible underlying mechanisms. The phenotype of the RTHα patients identified so far is highly similar but does not completely overlap with the various TRα1 mice models. This is probably dependent on the location and the severity of the mutation. Presumably, further research will reveal new mutations, the role of binding with coactivators or corepressors and improvement of therapy options.

SAMENVATTING

Schildklierhormoon (SKH) is belangrijk voor een normale ontwikkeling, differentiatie en metabolisme van het menselijk lichaam. Kinderen met een aangeboren niet werkende schildklier (congenitale hypothyreoïdie) die niet worden behandeld hebben hersenschade, dwerggroei, obstipatie, lethargie en voedingsproblematiek. SKH wordt geproduceerd in de schildklier, welke voornamelijk het voorloperhormoon T4 maakt en in mindere mate ook het biologisch actieve hormoon T3. Voordat SKH zijn werking kan hebben, moet het eerst worden opgenomen door de cellen in het lichaam. Deze opname wordt geregeld door zogeheten SKH transporters. Er zijn meerdere SKH transporters bekend, een specifieke SKH transporter is bijvoorbeeld monocarboxylaat transporter 8 (MCT8). De omzetting van SKH in de cel gebeurt door de zogeheten dejodases. Er zijn drie dejodase enzymen (D1-3) geïdentificeerd. Deze enzymen catalyzeren het activeren van SKH en zetten T4 om naar T3, of inactiveren SKH door T4 om te zetten naar rT3 of van T3 naar 3,3'-T2. Omdat T3 bindt aan zijn kernreceptor TRα of TRβ kan SKH effect hebben op de expressie niveaus van genen.

Regulatie van de SKH actie kan op meerdere niveaus plaatsvinden. SKH actie kan beïnvloed worden door afwijkingen in TRα en TRβ. Verder kan SKH actie gereguleerd worden door veranderingen in het metabolisme van SKH door veranderde dejodase expressie of door veranderingen in SKH opname via de transporters.

Dit proefschrift focust op de klinische en moleculaire aspecten van SKH actie in de kern. **Hoofdstuk 1** bevat een algemene introductie over SKH regulatie. Daarnaast worden de algemene doelen en opbouw van het proefschrift beschreven. In **hoofdstuk 2** beschrijven we het klinisch fenotype van een jonge patiënte en haar vader met een inactiverende mutatie in TRα1, welke leidt tot groeivertraging, vertraagde bot ontwikkeling, mild vertraagde motorische en mentale ontwikkeling, abnormale SKH waarden in het bloed, een verlaagd groeihormoon en IGF1 level en obstipatie. In **hoofdstuk 3** bespreken we het effect van levothyroxine (LT4) behandeling van de twee patiënten (beschreven in **hoofdstuk 2**) met een heterozygote TRα1 mutatie. Behandeling met LT4 geeft een verbetering van verschillende facetten van het fenotype, zoals serumwaarden, hypothyreote kenmerken en groei. Deze bevindingen bevestigen de belangrijke rol van TRα1 in bot, hersenen, darmen en mogelijk ook in groeihormoon regulatie. RTHα patiënten hebben een laag (F)T4, hoog T3, laag rT3, maar wel een normaal TSH level, wat suggereert dat er sprake kan zijn van abnormale activiteit van de dejodase enzymen. In **hoofdstuk 4** onderzoeken we het effect van een mutatie in TRα1, net als het effect van hypothyreoïdie in knock-out (KO) en wild-type (WT) muizen op de dejodase activiteit. TRα1-PV muizen hebben, vergeleken met WT muizen, een

verhoogde D1 activiteit in de lever terwijl D3 activiteit verlaagd is. Verder is D3 activiteit in het cerebellum van TRα1-PV muizen verminderd ondanks de toegenomen T3 levels in het serum. In **hoofdstuk 4** beschrijven we dat er in het brein ten opzichte van de lever en nier een tegengestelde regulatie van D3 expressie is door T3. In **hoofdstuk 5** analyseren we de rol van MCT8 en MCT10 op de biologische beschikbaarheid van SKH voor de kernreceptor. Beide transporters verhogen de intracellulaire beschikbaarheid van SKH voor D3 en voor de kernreceptor, hoewel de genomische actie een verdere penetratie van SKH in de cel benodigd heeft. De beschikbaarheid van SKH wordt beïnvloed door intracellulaire bindingseiwitten zoals bijvoorbeeld cytoplasmic TH-binding protein μ-crystallin (CRYM). In **hoofdstuk 6** tonen we aan dat affinity labelling niet MCT8 of MCT10 modificeert, maar een intracellulair bindingseiwit. In hoeverre labellings onderzoek bruikbaar is voor SKH transporter onderzoek moet nog verder uitgezocht worden. In **hoofdstuk 7** verkennen we het effect van SKH liganden TA3 en 3,5-T2 op genetische expressie profielen in een humane lever cellijn in vergelijking met het profiel van T3. Er was een grote overlap in de genen gereguleerd door de verschillende liganden en we hebben geen voorkeur gevonden van de liganden voor een specifieke receptor isovorm. In **hoofdstuk 8** bediscussiëren we de bevindingen van dit proefschrift in het licht van de literatuur.

Dit proefschrift focust op de klinische en moleculaire aspecten van SKH actie in de kern. We hebben één van de eerste patiënten geïdentificeerd met een mutatie in TRα1 en hebben vervolgens het fenotype en onderliggende mechanisme bestudeerd. Het fenotype van RTHα patiënten is in grote lijnen vergelijkbaar met de verschillende TRα1 muismodellen. Waarschijnlijk is dit verklaard door de locatie en ernst van de mutaties. Vermoedelijk zal verder onderzoek nieuwe mutaties aantonen, meer duidelijkheid geven over de binding van TRα1 met co-activatoren en co-repressoren en verbetering van therapeutische opties.

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A. A. van Mullem, A. L. M. van Gucht, W. E. Visser, M. E. Meima, R. P. Peeters and T. J. Visser (2016). "Effects of thyroid hormone transporters MCT8 and MCT10 on nuclear activity of T3." Mol Cell Endocrinol 437: 252-260.

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W. E. Visser, **A. A. van Mullem**, T. J. Visser and R. P. Peeters (2013). "Different causes of reduced sensitivity to thyroid hormone: diagnosis and clinical management." Clin Endocrinol (Oxf) 79(5): 595-605.

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Other publications

W. E. Visser, W. S. Wong, **A. A. van Mullem**, E. C. Friesema, J. Geyer and T. J. Visser (2010). "Study of the transport of thyroid hormone by transporters of the SLC10 family." Mol Cell Endocrinol 315(1-2): 138-145

C. Groba, S. Mayerl, **A. A. van Mullem**, T. J. Visser, V. M. Darras, A. J. Habenicht and H. Heuer (2013). "Hypothyroidism compromises hypothalamic leptin signaling in mice." Mol Endocrinol 27(4): 586-597.

E. Porcu, M. Medici, G. Pistis, C. B. Volpato, S. G. Wilson, A. R. Cappola, S. D. Bos, J. Deelen, M. den Heijer, R. M. Freathy, J. Lahti, C. Liu, L. M. Lopez, I. M. Nolte, J. R. O'Connell, T. Tanaka, S. Trompet, A. Arnold, S. Bandinelli, M. Beekman, S. Bohringer, S. J. Brown, B. M. Buckley, C. Camaschella, A. J. de Craen, G. Davies, M. C. de Visser, I. Ford, T. Forsen, T. M. Frayling, L. Fugazzola, M. Gogele, A. T. Hattersley, A. R. Hermus, A. Hofman, J. J. Houwing-Duistermaat, R. A. Jensen, E. Kajantie, M. Kloppenburg, E. M. Lim, C. Masciullo, S. Mariotti, C. Minelli, B. D. Mitchell, R. Nagaraja, R. T. Netea-Maier, A. Palotie, L. Persani, M. G. Piras, B. M. Psaty, K. Raikkonen, J. B. Richards, F. Rivadeneira, C. Sala, M. M. Sabra, N. Sattar, B. M. Shields, N. Soranzo, J. M. Starr, D. J. Stott, F. C. Sweep, G. Usala, M. M. van der Klauw, D. van Heemst, **A. van Mullem**, S. H. Vermeulen, W. E. Visser, J. P. Walsh, R. G. Westendorp, E. Widen, G. Zhai, F. Cucca, I. J. Deary, J. G. Eriksson, L. Ferrucci, C. S. Fox, J. W. Jukema, L. A. Kiemeney, P. P. Pramstaller, D. Schlessinger, A. R. Shuldiner, E. P. Slagboom, A. G. Uitterlinden, B. Vaidya, T. J. Visser, B. H. Wolffenbuttel, I. Meulenbelt, J. I. Rotter, T. D. Spector, A. A. Hicks, D. Toniolo, S. Sanna, R. P. Peeters and S. Naitza (2013). "A meta-analysis of thyroid-related traits reveals novel loci and gender-specific differences in the regulation of thyroid function." PLoS Genet 9(2): e1003266.

A. Anik, S. Kersseboom, K. Demir, G. Catli, U. Yis, E. Bober, **A. van Mullem**, R. E. van Herebeek, S. Hiz, A. Abaci and T. J. Visser (2014). "Psychomotor retardation caused by a defective thyroid hormone transporter: report of two families with different MCT8 mutations." Horm Res Paediatr 82(4): 261-271.

S. Kersseboom, A. L. M. van Gucht, **A. van Mullem**, G. Brigante, S. Farina, B. Carlsson, J. M. Donkers, S. F. J. van de Graaf, R. P. Peeters and T. J. Visser (2017). "Role of the Bile Acid Transporter SLC10A1 in Liver Targeting of the Lipid-Lowering Thyroid Hormone Analog Eprotirome." Endocrinology 158(10): 3307-3318.

CURRICULUM VITAE

Aaltje Ariënne Alies van Mullem was born on March 16th, 1985 in Nederlek. In 2003 she completed secondary school at the Wartburg College in Rotterdam. Afterwards, she started her study of Medicine at the Erasmus University in Rotterdam. After obtaining her medical degree in 2009, she started at the thyroid laboratory of Prof. Dr. Ir. Theo Visser and Prof. Dr. Robin Peeters. The results of her PhD research are presented in this thesis. In 2013 she started her training residencies in Internal Medicine at the Sint Franciscus Gasthuis in Rotterdam under supervision of Dr. Arie Rietveld. In 2017 she continued her residency in Internal Medicine at the Erasmus MC under supervision of Dr. Stephanie Klein Nagelvoort and Dr. Adrienne Zandbergen. Alies is married with Simon in September 2007.

PHD PORTFOLIO

Name PhD student:	drs. A.A.A. van Mullem
Erasmus MC Department:	Internal Medicine - Endocrinology
Research School:	Molmed
PhD period:	2009 -2018
Promotor:	Prof. Dr. Ir. T.J. Visser
	Prof. Dr. R.P. Peeters

1. PhD training	Year	Workload
Courses		
Course on radioactivity, hygiene and rules, Erasmus MC	2008	3 days
Basic data analysis on gene expression arrays (BAGE)	2009	1.1 ECTS
Bioinformatics workshop, Molmed	2010	0.6 ECTS
Basic introduction course on SPSS, Molmed	2010	1 ECTS
Basic and translational endocrinology, Molmed	2011	2.2 ECTS
Photoshop and Illustrator CS5 workshop, Molmed	2012	0.3 ECTS
Indesign CS5, Molmed	2012	0.15 ECTS
Biomedical Scientific English Writing, Molmed	2013	2 ECTS
Presentations		
Overlap and differences in genes regulated by T3 and Triac. 14th International thyroid congress, Paris, France	2010	poster
The effect of MCT8 on the biological activity of T3 13th European Congress of Endocrinology, Rotterdam, the Netherlands	2011	oral
Effects of MCT8 and MCT10 on the biological activity of T3 35th European Thyroid Association, Krakow, Poland	2011	oral
The effect of MCT8 on the biological activity of T3 15th Annual Molecular Medicine Day, Rotterdam, the Netherlands	2011	oral
Effects of MCT8 and MCT10 on the biological activity of T3 Symposium Dutch Thyroid Club, Amsterdam, the Netherlands	2011	oral
The effect of MCT8 on the biological activity of T3 Internal Medicine science days, Antwerp, Belgium	2011	poster
Clinical phenotype associated with mutation of thyroid hormone receptor alpha 1 (TRα1) Dutch Endocrine meeting, Noordwijkerhout, the Netherlands	2012	oral
Clinical phenotype associated with mutation of thyroid hormone receptor alpha 1 (TRα1) 94th Endocrine society's annual meeting, Houston, USA	2012	oral
Clinical phenotype of a new type of thyroid hormone resistance caused by mutation of the T3 receptor TRα1 36th European Thyroid Association ETA, Pisa, Italy	2012	oral

Clinical phenotype associated with mutation of thyroid hormone receptor alpha 1 (TRα1) Dutch Internist days, Maastricht, the Netherlands	2012	oral
Clinical phenotype associated with mutation of thyroid hormone receptor alpha 1 (TRα1) Annual Molecular Medicine Day, Rotterdam, the Netherlands	2012	oral
Clinical phenotype associated with mutation of thyroid hormone receptor alpha 1 (TRα1) Internal Medicine science days, Antwerp, Belgium	2012	poster
Clinical phenotype of a new type of thyroid hormone resistance caused by mutation of the T3 receptor TRα1 Dutch Endocrine meeting, Noordwijkerhout, the Netherlands	2013	oral
Clinical phenotype of a new type of thyroid hormone resistance caused by a mutation of the T3 receptor TRα1 Internal Medicine science days, Antwerp, Belgium	2013	poster
(Inter)national conferences		
Symposium Dutch Thyroid Club, Rotterdam, the Netherlands	2009	0.5 day
1th Dutch Thyroid Workshop, Rotterdam, the Netherlands	2009	0.5 day
14th Annual Molecular Medicine Day, Rotterdam, the Netherlands	2010	0.3 ECTS
14th International thyroid congress, Paris, France	2010	5 days
Internal Medicine science days, Antwerp, Belgium	2010	2 days
13th European Congress of Endocrinology, Rotterdam, the Netherlands	2011	5 days
35th European Thyroid Association, Krakow, Poland	2011	5 days
15th Annual Molecular Medicine Day, Rotterdam, the Netherlands	2011	0.3 ECTS
Symposium Dutch Thyroid Club, Amsterdam, the Netherlands	2011	0.5 day
Internal Medicine science days, Antwerp, Belgium	2011	2 days
Dutch Endocrine meeting, Noordwijkerhout, the Netherlands	2012	2 days
94th Endocrine society's annual meeting, Houston, USA	2012	4 days
36th European Thyroid Association ETA, Pisa, Italy	2012	5 days
Dutch Internist days, Maastricht, the Netherlands	2012	2.5 days
Annual Molecular Medicine Day, Rotterdam, the Netherlands	2012	0.3 ECTS
Internal Medicine science days, Antwerp, Belgium	2012	2 days
Dutch Endocrine meeting, Noordwijkerhout, the Netherlands	2013	2 days
Annual Molecular Medicine Day, Rotterdam, the Netherlands	2013	0.3 ECTS
17th Annual Symposium Dutch Thyroid Club	2013	1 day
Internal Medicine science days, Antwerp, Belgium	2013	2 days
Symposium Dutch Thyroid Club, Amsterdam, the Netherlands	2013	0.5 day
Dutch Internist days, Maastricht, the Netherlands	2014	2.5 days
Dutch Internist days, Maastricht, the Netherlands	2015	2.5 days
Rotterdam Internal Medicine Day, Rotterdam, the Netherlands	2015	1 day

Dutch Internist days, Maastricht, the Netherlands	2016	2.5 days
Rotterdam Internal Medicine Day, Rotterdam, the Netherlands	2016	1 day
Dutch Internist days, Maastricht, the Netherlands	2018	2.5 days
Awards and grants		
NvE Goodlife healthcare travelgrant ECE (€625)	2011	
Research award Internisten dagen (€500)	2012	
NvE Goodlife healthcare travelgrant ENDO (€2200)	2012	
Outstanding abstract award Endocrine Society ($2200)	2012	
Travel grant ETA (€500)	2012	
Young Investigator's award (€750)	2012	
2. Teaching		
Teaching of medical students about thyroid diseases	2010	
Teaching of medical students about thyroid diseases	2011	
Teaching of medical students about thyroid diseases	2012	
Basic and translational endocrinology	2013	

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