

CLINICAL STUDY

Extended clinical phenotype, endocrine investigations and functional studies of a loss-of-function mutation A150V in the thyroid hormone specific transporter MCT8

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Abstract

Objective: Thyroid hormones, besides having other functions, are known to be essential for the development of the human brain. Recently the monocarboxylate transporter 8 (MCT8) was identified as a thyroid hormone transporter which is expressed in different regions of the human brain. Here we describe in detail the clinical and biochemical features in response to thyroid hormone administration of a boy carrying an MCT8 mutation (A150V) in the second transmembrane domain.

Methods: To study the functional impact of the mutation we performed triiodothyronine (T3) uptake, immunofluorescence and dimerization studies.

Results: Thyroid hormone (L-thyroxine (LT4) and LT3) administration did not result in any significant clinical changes; however, with high doses of LT4, alone or in combination with T3, TSH suppression was achieved. We could show a robust uptake of ¹²⁵I-T3 for wild type (WT) MCT8, whereas no specific uptake could be detected for the mutant A150V. Subcellular localization of WT and mutant MCT8 revealed a strong cell surface expression for the WT MCT8, in contrast to A150V, which is mostly retained intracellularly with only weak cell surface expression. We could also demonstrate for the first time that WT MCT8 as well as the mutant are able to form multimers.

Conclusion: Our findings open a wide field of possible interaction within the central nervous system and will help to understand the crucial role of MCT8 in early fetal brain development.

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Introduction

Thyroid hormones are important for the development of many tissues and their metabolic function throughout life. Of utmost importance is the critical role of thyroid hormones in brain and nervous system development (1). The biological effects of thyroid hormones are mediated through the binding of triiodothyronine (T3) to nuclear receptors, which leads to a change in the interaction of the receptors with T3-responsive elements in regulatory regions of many genes (2, 3). Therefore, cellular uptake of thyroxine (T4) and T3 is critical for the biological activity of thyroid hormones.

Until recently it was assumed that thyroid hormones are transported by non-specific, ubiquitous transporters, such as organic anion co-transporting polypeptides. Recently the monocarboxylate transporter 8 (MCT8) was identified as the first active and specific thyroid hormone transporter, which induces a more than 10-fold increase in the uptake of T3

and to a lesser extent of T4 into cells (4). The X-chromosome-located gene encodes the transporter of 539 amino acids (when counted from the second putative start ATG), which is a putative 12 transmembrane domain protein with the N- and C-termini located intracellularly. After the identification of MCT8 as a specific thyroid hormone transporter, human patients with mutations in MCT8 were described who presented with severely retarded development (5, 6). The core neurological phenotype is associated with severe muscular hypotonia, absence of developmental milestones and lack of cognitive function. The described endocrine findings included elevated bioactive T3 and decreased T4 levels in the presence of a normal thyrotropin (TSH) secretion. It is assumed that this newly described syndrome of X-linked mental retardation is due to a defect of thyroid hormone uptake into cells, especially into neurons, thereby affecting the development of the central nervous system (CNS) pre- and early postnatally.

Here, we describe in detail the effect of thyroid hormone administration in a patient carrying the mutation A150V who presented with a complex phenotype of severe congenital mental retardation, lack of motor development, muscular hypotonia, nystagmus, strabismus and elevated bioactive T3 as well as decreased T4 levels. Moreover, we show that MCT8 A150V is only poorly expressed at the plasma membrane. In addition, we demonstrate for the first time multimerization of both wild type (WT) and mutated MCT8.

The subject

The patient is the first son of non-consanguineous parents. There is no family history of thyroid disorders or disturbed motor or mental development.

The patient was noticed to have a lack of development at the age of 4–6 months. His growth and weight gain were grossly normal (Fig. 1 and Table 1). There were no feeding difficulties, especially no difficulties in swallowing. Neurological investigations revealed

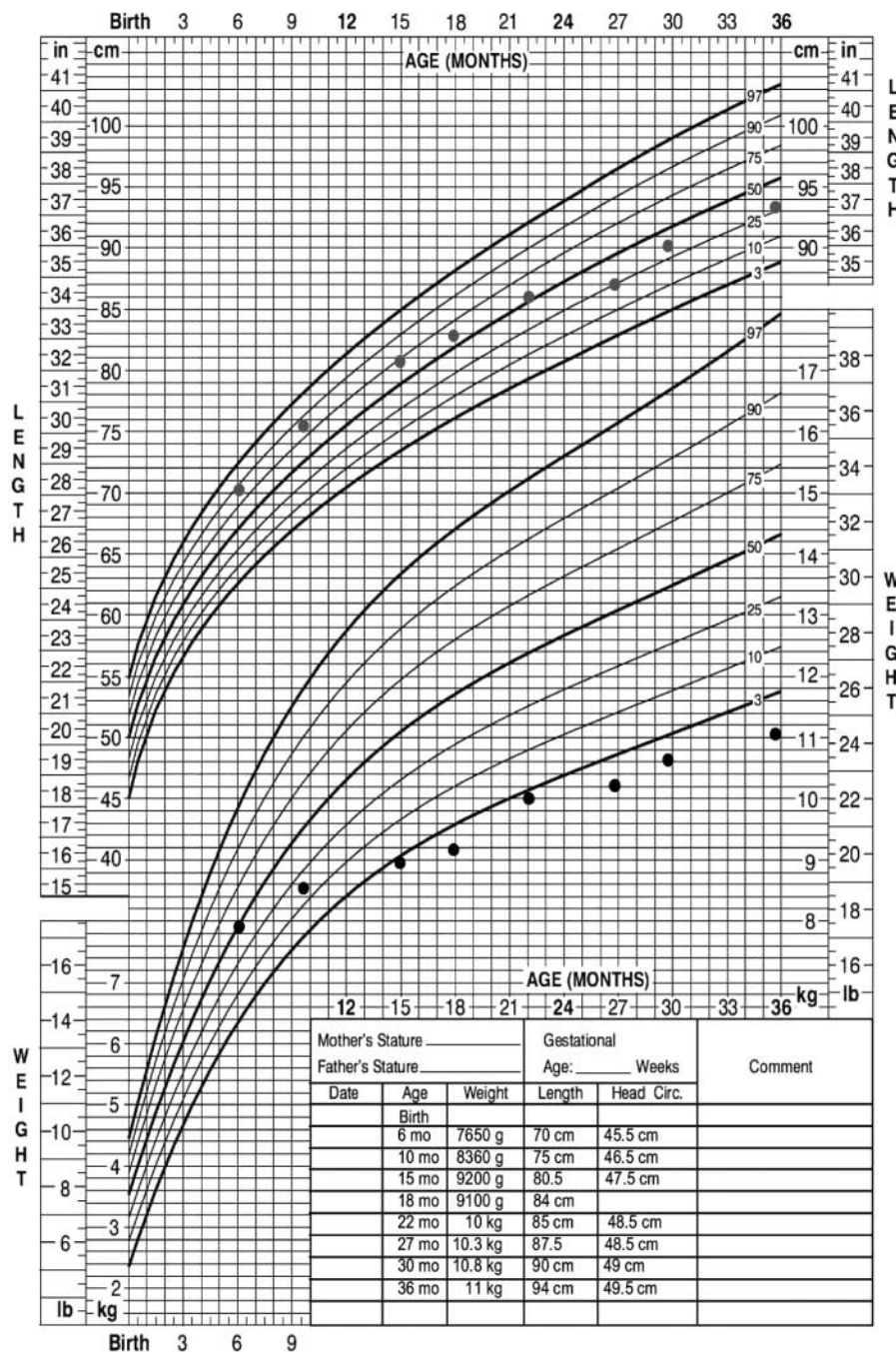


Figure 1 Growth and weight gain of the investigated patient. The weight increase is indicated in black circles and growth increase in gray circles.

Table 1 Summary of phenotypic findings in the patient.

	Normal	Pathological
Muscles	Biopsy:normal, CK normal	Severe hypotonia
Reflexes	+	
Eyes	VEP	Strabismus, nystagmus, no fixation
Hearing	AEP	
Cognitive function	Griffith's test	Severe mental retardation
Speech development		Lacking
Growth	+	
Gastrointestinal function		Constipation
Cardiovascular function (ECG, RR, Echocardiography)	+	
Pituitary	+	

+ normal. CK: creatinine kinase; VEP: visual evoked potential; AEP: acoustic evoked potential; RR: blood pressure.

a severe muscular hypotonia and decreased reflexes as well as lack of fixation of the eyes. There were some involuntary, dyskinetic movements. Acoustic as well as visual evoked potentials were normal. Extensive biochemical testing for metabolic diseases including tandem mass spectrometry gave normal results. Lactate and pyruvate concentrations in serum and spinal fluid were repeatedly found to be normal. Furthermore, investigations of the spinal fluid for inflammation or metabolic disease were normal (see Table 1). Cranial magnetic resonance imaging (cMRI) was performed at 14 months, showing only subtle cortical and subcortical atrophy (Fig. 2).

In this patient a mutation of the MCT8 gene leading to an amino acid change of alanine to valine in the second transmembrane domain (A150V, numbering from the second start ATG) of the transporter was identified (5). This mutation of the MCT8 transporter was associated with a distinct biochemical phenotype of the patient with significantly elevated serum levels for T3 and free T3 (fT3) and decreased T4 and free T4 (fT4) concentrations in the presence of normal TSH levels. Moreover, TSH secretion could be stimulated normally in a standard thyrotropin-releasing hormone (TRH) test (Table 2). Furthermore, basal TSH levels were readily suppressible by administration of high doses of LT4 alone or in combination with LT3, but not by LT3 alone, and were then not more stimulated by the administration of TRH (see Table 2). However, with a high age-adjusted LT3 in addition to a doubled dose of LT4 for 1 week, impaired weight gain and increased sweating was observed, but no other signs or symptoms of hyperthyroidism, especially no effect on the cardiovascular system, were apparent (see Table 2). Thyroid auto-antibodies were negative.

Although T4 as well as T3 administration in high dosage was able to suppress serum TSH concentrations, reflecting responsiveness of the pituitary to the increased thyroid hormone concentrations, no change

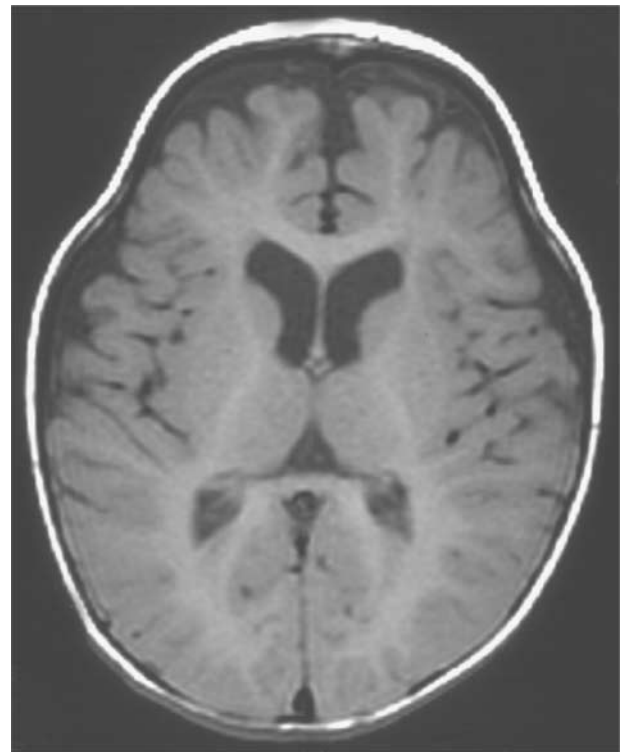


Figure 2 cMRI. T1-weighted axial SE 400/11 image showing slight cortical and subcortical atrophy.

of the clinical picture, especially of the mental and psychomotor development was observed. There was never an improvement of neurological findings, motor or cognitive functions or muscle tone. A developmental test at 4 years of age revealed a developmental age of not more than 2–4 months. Cardiovascular indices remained unchanged with T4 treatment up to 100 µg/day at the age of 4 years (10 µg/kg is three times the normal substitution), but slight tachycardia was observed after the addition of 30 µg T3/day. With this treatment also body weight slightly decreased and increased sweating was observed. Therefore this treatment was stopped after 1 week.

Baseline cholesterol levels were low and did not change with thyroid hormone treatment. Sex hormone-binding globulin (SHBG) levels were very high (comparable with thyrotoxic patients) and did not significantly increase with increasing LT4 doses. However, an increase from 264 to 459 nmol/l was observed after the addition of T3, indicating a normal hepatic response to T3.

Further endocrine studies including basal growth hormone, insulin-like growth factor-I, insulin-like growth factor-binding protein-3, gonadotropins, prolactin, cortisol, adrenocorticotropin and testosterone as well as corticotropin-releasing hormone, growth hormone-releasing hormone and luteinizing hormone-releasing hormone tests were normal.

Table 2 Summary of the findings of thyroid function tests performed at baseline at with different regimens of thyroid hormone administration.

	8 months	1 year	15 months	17 months (Period 1)	18 months (Period 1)	2 years	25 months (Period 2)	26 months (Period 2)	3 years (Period 3)	42 months (Period 3)	4 years (Period 3)	4 years (Period 4)	Mother	Father
TSH (mU/l) (0.5–6.3) (TRH test)	2.65/13.2	4.2	3.3	0.1	<0.1/0.4 (TRH test)	4.4	1.65	1.35	<0.1	<0.1	<0.1	<0.1	1.2	1.1
T4 (nmol/l) (95–165)	45	52	58	86	96	71	35	28	95	89	143	152	122	123.4
T3 (nmol/l) (1.4–3.2)	5.9	6.4	6.3	7.9	7.5	5.2	5.3	5.8	10.8	6.9	10.3	16.1	2.1	2.7
fT4 (pmol/l) (15–32)	12.1	—	10.2	10.5	12.9	—	9.8	8.7	12.6	—	—	20.5	—	—
fT3 (pmol/l) (3.9–7.2)	14.2	—	10.8	12.9	9.5	—	9.4	12.5	12.2	—	—	20.1	—	—
TG (ng/ml)	—	—	30	—	—	—	—	—	—	—	—	—	—	—
Cholesterol (mmol/l)	—	—	—	—	17.8	18.6	20.4	20.8	20.2	19.8	18.8	18	—	—
SHBG (nmol/l)	—	—	—	—	298	321	296	264	305	330	264	459	—	—
Therapy	—	—	—	LT4 75 µg	LT4 75 µg	—	LT3 20 µg	LT3 20 µg	LT4 100 µg	LT4 100 µg	LT4 100 µg	LT4 100 µg + LT3 30 µg	—	—

Period 1: From 17 to 18 months, l-thyroxine 75 µg/day (10 µg/kg/day).

Period 2: From 25 to 26 months, l-triiodothyronine 20 µg/day (2 µg/kg/day).

Period 3: From 3 to 4 years, l-thyroxine 100 µg/day (10 µg/kg/day).

Period 4: At 4 years (for 1 week), l-thyroxine 100 µg/day (10 µg/kg) + l-triiodothyronine 30 µg/day (3 µg/kg).

TG, triglyceride. Normal ranges are given in brackets.

Methods

Molecular studies

DNA of the patient and his parents was extracted from peripheral white blood cells using the QIAGEN Amp Blood Kit (Qiagen, Hilden, Germany). In the patient, sequence analysis of all six exons coding for MCT8 revealed a mutation in exon 2, a C to T transition resulting in a change of an alanine residue to valine in the second transmembrane domain (amino acid position 150 when numbered from the second start ATG).

In vitro functional analysis of WT and mutant MCT8

T3 uptake assay For investigation of T3 uptake, CHO-K1 cells were seeded into six-well plates (5×10^5 cell/well). WT and A150V mutant MCT8 (1 μ g plasmid DNA/well) were transiently transfected into CHO-K1 cells by Metafectene (Biotex, Munich, Germany) according to the manufacturer's protocol. Three days later the cells were washed three times in uptake solution (100 mmol/l NaCl; 2 mmol/l KCl; 1 mmol/l CaCl₂; 1 mmol/l MgCl₂; 10 mmol/l HEPES; 10 mmol/l Tris pH 7.5) and were then incubated with 1 nmol/l ¹²⁵I-labeled T3. Ten minutes later the reaction was stopped by addition of 0.1% BSA to the uptake solution. The cells were lysed in 0.1 mol/l NaOH and T3 uptake was measured in a gamma counter.

Immunofluorescence studies Stably transfected thyroid follicular carcinoma cell line 238 (FTC-238) with either N-terminally hemagglutinin (HA)-tagged WT MCT8 or A150V mutant were seeded on sterile coverslips. After 48 h, cells were fixed with 4% formaldehyde in PBS and were washed quickly with PBS. Cells were permeabilized with 0.1% Triton-X in PBS. After blocking in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS), cells were incubated with mouse anti-HA antibody 12CA5 (Roche, Mannheim, Germany), washed three times with PBS and were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma, Taufkirchen, Germany). After careful washing, the coverslips containing the cells were mounted onto glass slides and viewed with a fluorescence microscope (Axiovert10, Zeiss, Jena, Germany).

Dimerization studies To investigate receptor dimerization a sandwich ELISA was performed with N-terminally HA (N-HA) and C-terminally FLAG-tagged WT MCT8 (C-FLAG) as well as the differentially tagged A150V mutant (N-HA-MCT8-A150V and C-FLAG-MCT8-A150V). As dimerization control, co-transfection of WT MC4R-N-HA and WT MC4R-C-FLAG was used (7). COS-7 cells were seeded in 6 cm dishes and transfected with 3 μ g DNA using Metafectene according to

the manufacturer's protocol (Biotex). Three days after transfection, cells were washed, harvested and solubilized in a lysis buffer containing 50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1 mmol/l EDTA, 0.5% deoxycholate, 1% Nonidet P-40, and 0.2 mmol/l phenylmethylsulfonyl fluoride overnight at 4 °C. Plates with 96 wells were coated with monoclonal FLAG antibody (10 μ g/ml; Sigma) in 0.15 mol/l sodium tetraborate, pH 8 overnight at 4 °C. Subsequently plates were blocked with 10% FCS in DMEM. After removal of cell debris the cell lysates were incubated in the FLAG antibody-coated 96-well plate in triplicates for 2 h. Thereafter plates were washed three times with PBS containing 0.05% Triton X-100 (PBS-T). For detection a biotin-labeled monoclonal anti-HA antibody (1 μ g/ml in PBS-T; Roche) was added, and incubated for 2 h. Plates were again washed three times with PBS-T and incubated with peroxidase-conjugated streptavidin antibody (0.2 μ g/ml in PBS-T; Dianova, Hamburg, Germany) for 1 h. After washing three times with PBS-T to remove excess unbound conjugate, H₂O₂ and *o*-phenylenediamine (2.5 mmol/l each in 0.1 mol/l phosphate-citrate buffer pH 5.0) served as substrate and chromogen. After approximately 5 min the enzyme reaction was stopped by adding 1 mol/l H₂SO₄ containing 0.05 mol/l NaSO₃. The color intensity was measured bichromatically at 492 and 620 nm using an ELISA reader.

For statistics we conducted a one-way ANOVA to investigate global differences between the dimerization of the differentially tagged positive control (MC4R-N-HA + MC4R-C-FLAG), the WT-MCT8 and the mutant A150V-N-HA co-transfected with WT-MCT8-C-FLAG or A150V-C-FLAG and the negative control (MC4R-N-HA + MCT8-C-FLAG) followed by subsequent Tukey tests for post-hoc comparisons between the negative control and the different constructs. Statistical tests were performed using SPSS.

Results

For functional characterization, WT and mutant MCT8 were transfected into CHO-K1 cells and ¹²⁵I-T3 uptake was measured. The A150V mutation of the investigated patient led to a complete loss of specific T3 uptake (Fig. 3) after 10 min incubation with ¹²⁵I-labeled T3. In order to investigate the reason for the loss of function of the mutant MCT8 we performed immunofluorescence studies. We could demonstrate that the subcellular localization of the mutated transporter revealed a more reticular expression pattern than expression on the cell membrane, which could be clearly demonstrated for the WT MCT8 (Fig. 4). Therefore the loss of T3 transport of the mutant MCT8 is due to a reduced cell surface expression of the mutated transporter in comparison with WT MCT8 (see Fig. 4).

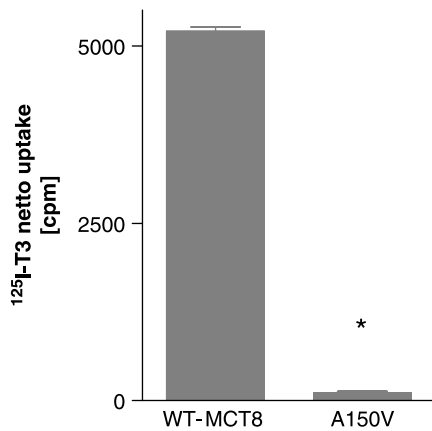


Figure 3 T3 uptake of WT and mutant MCT8. CHO-K1 cells were transiently transfected with the WT and mutant MCT8. Three days after transfection cells were incubated in a final concentration of 1 nmol/l ¹²⁵I-T3 and specific T3 uptake was measured as described in the Methods section. The result of one out of three independent experiments performed in quadruplicates is shown. Statistical analysis was performed with the one-sample *t*-test and resulted in **P* < 0.001. Error bars indicate the intraassay variation.

Very recently it has been shown that a glutamate transporter functioned as a trimer (8). These findings prompted us to investigate dimerization of MCT8. Using an established sandwich ELISA approach (7) with differentially tagged WT or mutant MCT8, we could show for the first time that WT MCT8 is able to form dimers (Fig. 5). Moreover, we could show for the mutant A150V MCT8 that dimerization is comparable with the WT MCT8 (see Fig. 5). Although this situation is not physiologically present either in the patient or in the heterozygous mother due to X-chromosome inactivation, we could additionally demonstrate that the mutant MCT8 is able to heterodimerize with WT MCT8. The dimerized WT MCT8 and mutant as well as WT with mutant differed significantly in their optical density in comparison with the negative control (MC4R-N-HA + MCT8-C-FLAG) (for all tested variants *P* < 0.001).

Discussion

Although human MCT8 had been already cloned in 1994 (9), until today its physiological role has never been elucidated. With the description of the crucial role for thyroid hormone transport across the cell membrane (4), the role of MCT8 in thyroid hormone-dependent development of the CNS has become evident. Severe deficits of normal motor and cognitive functions were observed in boys with loss-of-function mutations of the X-chromosome transporter gene (5, 6). These findings of severe mental and psychomotor retardation in male individuals affected with loss-of-function mutations of the X-linked MCT8 gene imply that MCT8 is involved in the development and function of the CNS.

Complete loss of function of the MCT8 gene is most probably responsible for the severe neurological and developmental symptoms in male patients, because large gene deletions and mutations resulting in aberrant or truncated proteins produce the same phenotype. Interestingly, although thyroid hormone access to cells is impaired, the typical symptoms observed in patients with congenital hypothyroidism, especially regarding skeletal growth, gastro-intestinal function, cardio-vascular function and metabolism, are not observed in these patients. On the other hand, despite very high serum T3 levels, signs of hyperthyroidism like diarrhea, tachycardia, tremor and sweating are also not present. Symptoms of hyperthyroidism were observed in the patient of this study only when high doses of T3 were added to a high-dose T4 treatment, which alone had not resulted in hyperthyroid symptoms over a period of 16 months. This could be explained by an MCT8-independent T3 transport mechanism when the circulating T3 concentrations exceed a certain threshold. TSH serum levels were normal without treatment, despite the presence of very high bioactive T3 concentrations in serum, suggesting that the amount of T3 entering the pituitary cell is not sufficient to suppress TSH secretion. This could be explained by the fact that low serum

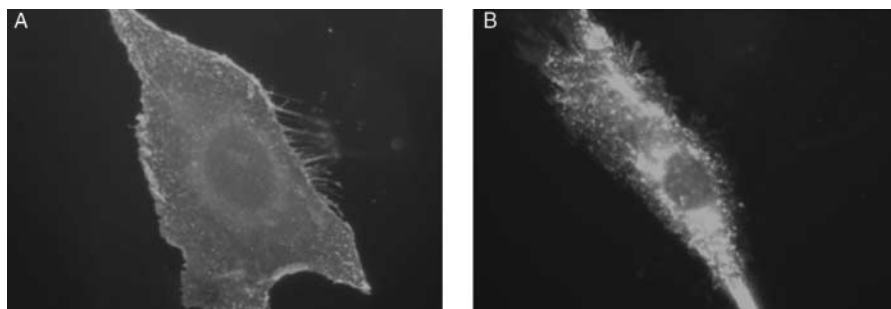


Figure 4 Immunofluorescence studies of WT and mutant MCT8. Immunocytochemistry of N-terminally HA-tagged WT MCT8 and A150V mutant. Constructs were stably expressed in FTC-238 cells. Two days after seeding, cells were permeabilized and WT and mutant MCT8 was localized by detecting the HA tag using mouse anti-HA as primary and anti-mouse FITC conjugate as secondary antibody. (A) WT MCT8 is localized at the cytoplasmic membrane. (B) A150V mutant is retained intracellularly. Two representative cells out of three independent experiments are shown.

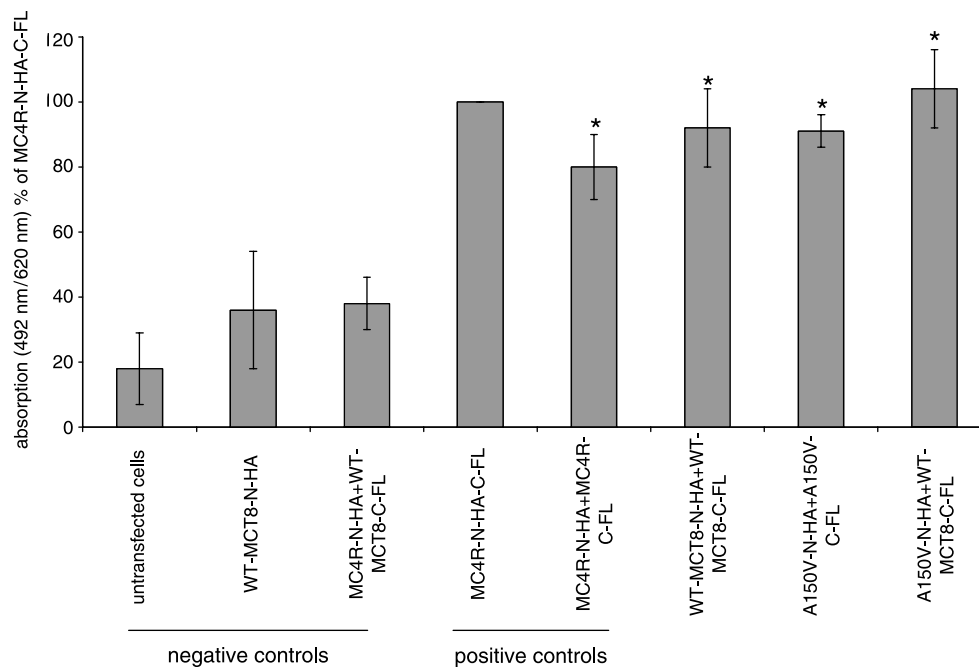


Figure 5 Investigation of dimerization of WT and mutant MCT8. MCT8 dimerization was investigated by a sandwich ELISA approach. COS-7 cells were co-transfected with differentially tagged MCT8 constructs, solubilized overnight and incubated in FLAG (FL) antibody-coated 96-well plates. In the case of transporter dimerization, determination of the N-terminal HA epitope was measured as an increase in optical density. Untransfected cells, WT MCT8-N-HA and co-transfection of MC4R-N-HA with MCT8-C-FLAG serve as negative controls, double-tagged MC4R and co-transfection of MC4R-N-HA and MC4R-C-FLAG were used as positive controls. Three independent experiments performed in triplicates are shown. OD values are normalized as percentage of the double-tagged MC4R (MC4R-N-HA-C-FLAG) with a mean OD (492 nm/620 nm) of 1.01 ± 0.07 . One-way ANOVA and Tukey tests were applied to test for statistically significant differences between the negative control (MC4R-N-HA + MCT8-C-FLAG) and four different constructs ($*P < 0.001$). The result of three independent experiments is shown. Error bars are standard deviation scores of mean values.

T4 levels stimulate the pituitary type II deiodinase to convert T4 to T3. Moreover, T3 is less efficient at suppressing TSH compared with T4 (see Table 2, Period 2), which is supported by the suppression of TSH to undetectable concentrations with high-dose thyroxine treatment and lack of TSH suppression with monotherapy with triiodothyronine. These findings are suggestive of an MCT8-independent transport mechanism of T4 into the pituitary.

SHBG levels were already elevated without thyroid hormone administration and increased significantly with a high-dose combined T4 and T3 treatment. We propose that MCT8 has possibly no critical role for hepatic thyroid hormone transport. Hepatic type I deiodinase stimulated the conversion of T4 to T3. This is supported further by the finding of low T4/fT4 serum concentrations without T4 administration and high circulating T3 levels. This effect is further enhanced by T4 replacement therapy (see Table 2, Periods 1, 2 and 4). Compensation by an increase of T4 production of the thyroid does not occur, because pituitary TSH levels do not increase.

Although MCT8 expression has been shown in many tissues in mice and humans (9–11), an impaired postnatal thyroid hormone transport via MCT8 into other cells than the nervous system does not seem to cause

relevant clinical symptoms and points to a more important role of MCT8-specific thyroid hormone transport into neurons during embryonic and fetal development. The normal heart rate suggests that MCT8 is important for T3 transport, e.g. into myocardial and endocardial cells, but at the same time this finding indicates that other transporters might also be capable of transporting thyroid hormone into these cells or other compensatory mechanisms exist, because no bradycardia is observed. The investigated patient presented with periods of constipation in the presence of elevated thyroid hormone levels. This could indicate that MCT8 is important for the transport of thyroid hormones into intestinal cells; however, since constipation is a frequent finding in retarded and immobile patients this finding alone does not prove a role for MCT8 in the gut. The transport of thyroid hormones by MCT8 into bone cells seems not to be of crucial importance, because no alteration of growth was observed pre- and postnatally and no retardation of bone age was observed. We therefore assume that thyroid hormone transport via MCT8 is critical for the developing CNS and that possibly redundancy of other tissue-specific thyroid hormone transport mechanisms in the postnatal period may lead to less prominent postnatal symptoms in other organs.

Although the mutation of alanine to valine at amino acid position 150 does not appear to be very dramatic it could be assumed that the change of the conserved alanine residue at this position resulted in alteration of transporter structure. For other membrane proteins like G protein coupled receptors it was shown that the change of an alanine to a valine residue within the transmembrane segment destroys the close interhelical contact of the transmembrane domains. That interhelical contact is crucial for the inactive state of the receptor. In this view also, apparent minimal changes like the change of alanine to valine within transmembrane domains may result in altered expression and function. In accord with this interpretation is the description of an change from alanine to valine in the first transmembrane domain of the luteinizing hormone receptor, which resulted in reduced cell surface expression and constitutive activity (12).

For a huge variety of transmembrane proteins like receptor tyrosine kinases, G protein coupled receptors, ion channels and transporters, di- or oligomerization is one important functional feature (13). The finding of MCT8 dimerization now opens a wide spectrum of possible interactions not only with other transporters but also other membrane proteins within the CNS. These findings are the first step towards a better understanding of the physiological role of MCT8 in CNS development and in its peripheral function for T3 transport.

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