

Doctorate Program in Molecular Oncology and
Endocrinology
Doctorate School in Molecular Medicine

XXIV cycle - 2008–2011
Coordinator: Prof. Massimo Santoro

**“Changes in thyroid status during early
development of Mct8 deficient mice”**

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“Changes in thyroid status during early development of Mct8 deficient mice”

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LIST OF PUBLICATIONS

This dissertation is based on the following publication:

- 1) Ferrara AM, Changes in thyroid status during early development in Mct8 deficient mice. Manuscript in preparation (main body of the Dissertation)

Original publications:

- 2) A somatic gain-of-function mutation in the thyrotropin receptor gene producing a toxic adenoma in an infant.
Kohn B, Grasberger H, Lam LL, Ferrara AM, Refetoff S
Thyroid. 2009 Feb;19(2):187-91 (attached at the end of Dissertation)
- 3) Mutations in TAZ/WWTR1, a co-activator of NKX2.1 and PAX8 are not a frequent cause of thyroid dysgenesis
Ferrara AM, De Sanctis L, Rossi G, Capuano S, Del Prete G, Zampella E, Gianino P, Corrias A, Fenzi GF, Zannini M, Macchia PE
J Endocrinol Invest, 2009 Mar; 32(3): 238-41 (attached at the end of Dissertation);
- 4) The DREAM protein is associated with thyroid enlargement and nodular development.
Rivas M, Mellström B, Torres B, Cali G, Ferrara AM, Terracciano D, Zannini M, Morreale de Escobar G, Naranjo JR.
Mol Endocrinol. 2009 Jun;23(6):862-70. Epub 2009 Mar 19 (attached at the end of Dissertation).
- 5) Benign hereditary chorea: clinical and neuroimaging features in an Italian family.
Salvatore E, Di Maio L, Filla A, Ferrara AM, Rinaldi C, Saccà F, Peluso S, Macchia PE, Pappatà S, De Michele G.
Mov Disord. 2010 Jul 30;25(10):1491-6 (attached at the end of Dissertation).
- 6) Screening for mutations in the ISL1 gene in patients with thyroid dysgenesis.
Ferrara AM, Rossi G, Zampella E, Di Candia S, Pagliara V, Nettore IC, Capalbo D, De Sanctis L, Baserga M, Salerno MC, Fenzi G, Macchia PE.
J Endocrinol Invest. 2011 Jul-Aug;34(7):e149-52. Epub 2010 Nov 8 (attached at the end of Dissertation).

- 7) Homozygous thyroid hormone receptor β gene mutations in resistance to thyroid hormone: three new cases and review of the literature.
Alfonso Massimiliano Ferrara, Kazumichi Onigata, Oya Ercan, Helen Woodhead, Roy E. Weiss, Samuel Refetoff [submitted]

List of abbreviations

5'D	5'-deiodination
5D	5-deiodination
AHDS	Allan-Herndon-Dudley syndrome
Aldh1a3	Aldehyde dehydrogenase 1 family, member A3
BAT	Brown Adipose Tissue
CNS	Central Nervous System
D1	deiodinase type I
D2	deiodinase type II
D3	deiodinase type III
HPT	hypothalamus-pituitary-thyroid axis
Hr	Hairless
IRD	inner ring deiodination
Klf9	Kruppel-like factor 9
LAT1	L-aminoacid transporter type 1
LAT2	L-aminoacid transporter type 2
LoI/MMI/CIO ₄	Low iodine diet/methimazole/ sodium perchlorate
MCT8	monocarboxylates transporters type 8
MCT10	monocarboxylates transporters type 10
Nr4a1	Nuclear receptor subfamily 4, group A, member 1
OATP1C1	organic anions transporters type 1C1
OATP3A1	organic anions transporters type 3A1
ORD	outer ring deiodination
P0, 3, 7, 11, 14, 18, 21, 100	postnatal day 0, 3, 7, 11, 14, 18, 21, 100
PTU	propylthiouracil
rT ₃	reverse T ₃
Sema 7a	semaphorin type 7a
Shh	Sonic hedgehog
T ₂	3,3'-diiodothyroine
T ₃	3,3',5-triiodothyronine
T ₄	thyroxine (3,3',5,5'-tetraiodothyronine)
TH	thyroid hormone
TR	TH receptors
TRE	thyroid hormone response elements
TRH	thyrotropin releasing hormone
TSH	thyrotropin

ABSTRACT

The MCT8 deficiency syndrome is an X-linked disease presenting with severe psychomotor retardation. A feature of the syndrome is a peculiar thyroid phenotype characterized by low levels of serum T₄ and rT₃ and high levels of serum T₃. Mice models for Mct8 deficiency replicate the thyroid tests abnormalities of humans but do not manifest gross neurological phenotype.

This work demonstrates for the first time that the thyroid status in Mct8 deficient mice is highly dynamic since it changes during postnatal life from birth to adulthood. Indeed, *Mct8KO* mice show hyperthyroxinemia at birth, followed by a short euthyroid state during the first few days of life before manifesting the classical features of Mct8 deficiency. Moreover we found that this initial hyperthyroxinemia is accompanied by altered gene expression both in cerebral cortex and in liver suggestive of hormone excess.

It is conceivable that the neonatal thyroid status of Mct8 deficient mice has a role in preserving brain function.

INTRODUCTION

Thyroid hormone physiology

Regulation of thyroid hormone synthesis

Thyroid hormone (TH) plays an important role in the development and proper function of multiple organs. Of particular interest is the role of TH in brain development (1) as well as in the regulation of the metabolic activities of the tissues and in the thermogenesis throughout life.

The essential role of TH in fetal and early postnatal development is well recognized in both animals and humans. During embryogenesis TH promotes differentiation and attenuates proliferation. Both insufficient supply of T₃ and premature exposure of embryos to adult levels of T₃ are detrimental and result in abnormal development. The serious consequences ensuing from maternal hypothyroidism and early childhood hormone deprivation have been documented in endemic areas of iodine deficiency (2) and in inherited and acquired hypothyroidism occurring with high frequency in the Western world (3). TH deficiency causes severe brain malfunction that, if not treated in early postnatal life, is responsible for irreversible mental retardation. This together with stunted growth is known in humans as cretinism. The mental dysfunction is, in part, due to hypomyelination and to defects of neuronal cell migration and differentiation (4).

TH [thyroxine (3,3',5,5'-tetraiodothyronine, T₄) and 3,3',5-triiodothyronine (T₃)] are produced by the thyroid gland. Iodine is an

indispensable component of the TH, comprising 65% of the T_4 molecular weight, and 58% of T_3 's. TH is the only iodine-containing compound with established physiologic significance in vertebrates. Most of the circulating TH is bound to serum proteins. The free circulating fraction of the hormone levels is that available to tissues and thus correlates best with its biological effect.

TH synthesis and secretion is under the control of thyrotropin (TSH) produced by the thyrotrophs, located in the anterior pituitary gland. TSH is a circulating glycoprotein made of two subunits that binds to a G-protein coupled receptor located on the membrane of follicular cells of the thyroid gland. In turn, circulating levels of TH (mainly T_4 , which is locally deiodinated to T_3) regulates TSH levels through a negative feedback system (for further details, see **Figure 1**). Thus, under normal circumstances, a high serum TSH concentration is indicative of TH deficiency, while TH excess suppresses TSH. Thyrotropin releasing hormone (TRH), a tripeptide secreted by the hypothalamus, stimulates the synthesis and secretion of TSH.

The effects of TH are dependent on the quantity of the hormone that reaches tissues and the availability of unaltered TH receptors (TR) in cell nuclei. The intracellular concentration of T_3 is determined by the supply of circulating T_4 and to a lesser degree circulating T_3 . The intracellular generation of T_3 by outer ring deiodination (5'-deiodination) of T_4 is a hormone activating process, while the degradation of T_4 and T_3 by

inner ring deiodination (5-deiodination) is effectively a hormone inactivating process producing 3,3',5'-triiodothyronine (reverse of rT_3) and 3,3'-diiodothyroine (T_2), respectively (**Fig. 1**) (5).

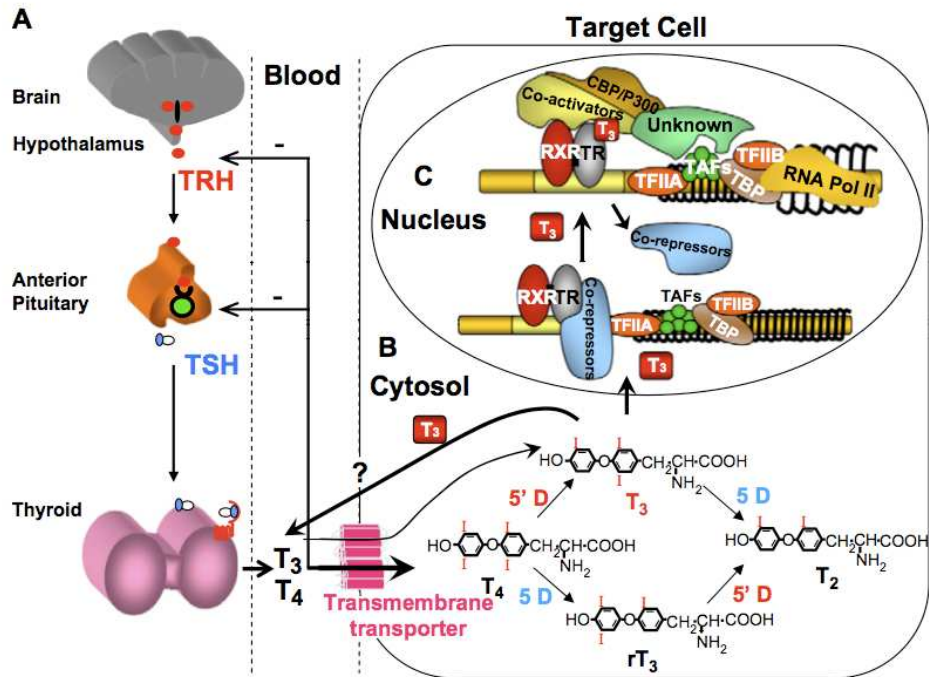


Figure 1. Regulation of thyroid hormone (TH) supply, metabolism, and genomic action. (A) *Feedback control that regulates the amount of TH in blood.* TRH produced by the hypothalamus stimulates the synthesis and secretion of TSH by the thyrotrophs, located in the anterior pituitary gland. TSH acting on its receptor located on the follicular cells of the thyroid gland, stimulates TH synthesis and secretion, principally T_4 . Constant supply of TH is controlled through a negative feedback system, regulating TH through the TRH/TSH sensing mechanism. (B) *Intracellular metabolism of TH, regulating TH bioactivity.* After active cellular uptake of TH through transmembrane transporters, the precursor T_4 is converted into the active T_3 hormone or inactive rT_3 . The iodothyronine deiodinases, D1 and D2, are the principal enzymes that catalyze 5'-deiodination (5'D), converting T_4 to T_3 and rT_3 to T_2 , while D3 catalyzes 5-deiodination (5D), converting T_4 to rT_3 and T_3 to T_2 . (C) *Mechanism of TH receptor action.* The thyroid hormone receptors (TR) form homo/heterodimers [with TR or, as in the example, retinoid X receptor (RXR)] that bind specifically to thyroid hormone response elements (TRE) in the promoter regions of target genes. In the absence of hormone, TR binds co repressor proteins that silence gene expression. When T_3 enters the nucleus, T_3 binding dissociates co-repressor from TR and Co-activators are recruited to the T_3 -bound receptor. Finally gene expression is activated. 5'D, deiodination by removal of an iodine from the 5' position in a iodothyronine; 5D, 5-deiodination; CBP/P300, cAMP-binding protein/general transcription adaptor; rT_3 reverse T_3 ; RXR, retinoid X receptor; T_2 , diiodothyronine; T_3 , triiodothyronine; T_4 , thyroxine; TAF, TBP-associated factor; TBP, TATA-binding protein; TFIIA and TFIIB, transcription intermediary factor II, A and B; TR, TH receptor; TRH, TSH releasing hormone; TSH, thyroid stimulating hormone (thyrotropin). From (6).

Thyroid hormone receptors

The nuclear pathway is the most important mechanism of TH action, although extranuclear or cytosolic pathways have also been described. The active TH, T₃, binds to the nuclear receptors and modulates gene expression (7). In vertebrates, T₃ receptors are the products of two genes, *TRα* and *TRβ*. They encode nine protein products, which arise by alternative splicing of mRNA and differential promoter usage. The *TRα* gene encodes five protein products (TRα1, TRα2, TRα3, and the truncated products ΔTRα1 and ΔTRα2) of which only TRα1 binds T₃. The *TRβ* gene encodes four T₃-binding proteins TRβ1, TRβ2, TRβ3 and ΔTRβ3, of which TRβ1, TRβ2 and TRβ3 also bind to responsive elements on DNA. In addition, the truncated protein ΔTRβ3 binds T₃ but not DNA. There are therefore two types of receptors, α and β, and four different receptor isoforms (α1, β1, β2, β3).

TH receptors, both in the unliganded (aporeceptor) and the liganded states, bind to hexameric sequences known as T₃ responsive elements (TRE) located in regulatory elements of the target genes. The aporeceptor usually represses transcription of genes positively regulated by TH, through association with corepressors (SMRT, NcoR, Alien), which recruit histone deacetylases, maintaining the chromatin in the compact, deacetylated state. After hormone binding, corepressors are released and coactivators and histone acetylases are recruited, so that transcription is allowed (**Fig. 1**). With specific regarding to the nervous system, TH

receptors have a distinct expression pattern in the developing brain. In man, the receptor is already present by week 10, before onset of fetal thyroid gland function, and increases by several-fold during the second trimester, coinciding with major events in cortical and basal ganglia development (8). Also in rats, the receptor is present in brain by E14 and even earlier, several days before onset of fetal thyroid gland function.

In the fetal brain, TR α is quantitatively the principal receptor (9, 10). The receptor expression pattern is mainly neuronal, although oligodendrocytes and, to a much lesser extent astrocytes also express the receptors. The use of knock-out mice to examine the role of receptors during development has given paradoxical results (11). In contrast to the profound effects of TH deprivation on brain development, deletion of receptor genes did not result in obvious alterations in brain maturation. For example, it is well known that cerebellar structure is normal in TR α 1-deficient mice, with no apparent differences in granular cell migration (under TR α 1 control) or Purkinje cell differentiation (dependent on both TR α 1 and TR β 1). In fact, the cerebellum of TR α 1 knockout mice is protected from the effects of TH (12). This apparent paradoxical effect is explained by the constitutive effect of the aporeceptor, repressing the expression of genes positively regulated by TH.

Thyroid hormone metabolism

The thyroid gland secretes predominantly the prohormone T_4 together with a small amount (about 15% in humans) of the bioactive hormone T_3 . Most T_3 is produced by enzymatic outer ring deiodination (ORD) of T_4 in peripheral tissues. Alternatively, inner ring deiodination (IRD) of T_4 yields the metabolite rT_3 , the thyroidal secretion of which is negligible (**Fig 1**). Normally, about one-third of T_4 is converted by ORD to T_3 and about one-third to rT_3 . The remainder of T_4 is metabolized by different pathways, in particular glucuronidation and sulfation. T_3 is further metabolized largely by IRD and rT_3 largely by ORD, yielding in both cases the metabolite 3,3'- T_2 . Thus, ORD is regarded as an activating pathway and IRD as an inactivating pathway (13).

Three enzymes catalyzing these deiodinations have been identified (**Table 1**), type I (D1), type II (D2) and type III (D3) iodothyronine deiodinases (14). They differ in catalytic properties, patterns of tissue expression and mechanisms of regulation. All three deiodinases have been cloned and characterized in different species. Deiodinases are a family of homologous selenoproteins consisting of 250-280 amino acids, containing an essential selenocysteine residue in the active center (15). It is remarkable, therefore, that production and metabolism of TH is dependent on two trace elements, namely iodine and selenium.

D1 is expressed mainly in liver, kidneys and thyroid. The hepatic enzyme is thought to be responsible for the bulk of peripheral T_3

production as well as for the clearance of rT_3 from the circulation. These are mediated by the ORD activity of D1, but the enzyme has also IRD activity directed especially towards sulfated T_4 and T_3 . A property that distinguishes D1 from the other two deiodinases is its sensitivity to inhibition by the anti-thyroid drug propylthiouracil (PTU). The important role of D1 in the production of plasma T_3 by deiodination of T_4 in peripheral tissues has been demonstrated by the marked decrease in plasma T_3 levels in T_4 -substituted athyreotic subjects treated with PTU (16).

D2 has been studied extensively in the central nervous system, pituitary and brown adipose tissue of experimental animals. D2 has only ORD activity and its expression shows adaptive changes in response to alterations in thyroid state, which serves to maintain tissue T_3 levels in the face of varying plasma T_4 and T_3 concentrations. These findings have led to the general opinion that D2 is important for the generation of local T_3 in these tissues but does not contribute much to the production of plasma T_3 . However, the recent identification of D2 in human skeletal muscle (17) has pointed to the species differences and provided evidence that a significant proportion of plasma T_3 in humans may be generated by an extra-hepatic, PTU-insensitive mechanism, particularly in subjects with reduced plasma T_4 levels. D2 has also been localized in the human thyroid gland (18).

D3 mediates the inactivation of TH since it has only IRD activity. The brain is the predominant D3-expressing tissue in adult animals, and may thus be the main site for the clearance of plasma T_3 and for the

production of plasma rT₃. However, high D3 activities have been demonstrated in the placenta and the pregnant uterus as well as in different fetal tissues. The high D3 activities at these sites appear to prevent exposure of fetal tissues to high T₃ levels, allowing the growth of these tissues. T₃ is only required at the differentiation stage of tissue development.

Expression of D1 and D3 is under positive control and that of D2 is under negative control by TH. Therefore, the relative contribution of D1 and D2 to T₃ production in peripheral tissues varies with thyroid state; D1 prevailing in the hyperthyroid and D2 in the hypothyroid state. Moreover, the D2 protein is unique in that it can be switched off and on via an ubiquitin (Ub)-regulated mechanism, triggered by catalysis of T₄ (19-21).

Table 1 summarizes most of the characteristics of the deiodinases

- Deiodinase deficiencies in rodents:

Important insights into the role of **D1** for TH metabolism were obtained from two genetically modified animal models: the C3H strain, with inherited low D1 expression in liver and kidney, and *Dio1*KO (D1KO) mice, with targeted disruption of the *Dio1* gene (22-24). The reduced D1 activity in C3H mice correlates with a CGT repeat insertion into the 5'-flanking region of the *Dio1* gene that seems to impair C3H promoter potency (22). Both genotypes have elevated serum total and free T₄ (FT₄) and rT₃ but

normal serum free T_3 (FT_3) and TSH concentrations. The normal FT_3 concentration in these mice could be explained by the fact that, although the fractional conversion of T_4 to T_3 per day would be reduced, the higher FT_4 concentration permits normal daily T_3 production. In addition, serum T_3 levels were significantly higher in C3H than in the wild-type (Wt) mice of the C57BL strain, given the same dose of exogenous T_3 , suggesting a reduction in T_3 clearance that might contribute to the maintenance of serum T_3 concentrations (25). However, the rate of disappearance of T_3 was comparable in euthyroid Wt and D1KO mice (24). A potential source of serum T_3 in both C3H and D1KO mice is D2-catalyzed T_4 to T_3 conversion. Indeed, it has been shown that, despite the higher serum FT_4 , the levels of D2 activity in brown adipose tissue of C3H mice are comparable to those observed in C57BL animals (26). The normal serum FT_3 levels would lead to an euthyroid state in peripheral tissues and can account for the observed euthyroid genotypes in both phenotypes. Of note, no cases of inherited D1 deficiency have been documented so far in human.

D2 is the first deiodinase for which a null allele was created through homologous recombination, removing 72% of the coding region, encompassing the active site of the enzyme, the selenocysteine codon (27). Except for mild growth retardation in males (9%), no gross phenotypic abnormalities were observed in *Dio2*KO mice (D2KO). No D2 activity has been observed in these animals under basal conditions or

under stimuli, such as cold-exposure or hypothyroidism. Reproductive function appeared normal with normal serum levels of T₃. However, T₄ and TSH levels were significantly elevated, 40% and 100% respectively, suggesting that the pituitary gland of the D2KO mice is resistant to the feedback effect of plasma T₄, caused by the inability to produce intracellular T₃ through 5'-deiodination by D2. In support of this is the fact that the increase in TSH was corrected by T₃ administration only. In the last decade, animal models with combined deficiencies of D1 and D2 enzymes were generated (28, 29). The C3H-D2KO mouse (targeted disruption of the *Dio2* gene and genetically low *Dio1* expression) has hepatic and renal D1 activities lower than those observed in Wt mice (C57BL/6) but unexpectedly higher than those of the C3H mice. These mice present normal serum T₃ levels and serum T₄ concentrations even higher than those of C3H mice. Serum TSH is increased, which could be important to maintain euthyroid serum T₃ concentrations. The double D1/D2KO mice are also able to maintain a normal serum T₃ level and their general health, growth, and reproductive capacity are seemingly unimpaired (29). These findings led to the interpretation that D1 and D2 enzymes might not be essential to the maintenance of normal plasma T₃ levels in the rodent as long as the hypothalamic–pituitary–thyroid axis is intact. These alterations, however, might not perfectly reflect those putatively found in humans, because the rat thyroid is responsible for 50% of the circulating T₃, whereas humans depend more on peripheral tissue

deiodination, given that only 15% of the T₃ is derived from the thyroid gland.

D3 has been inactivated in mice, by replacing the Sec codon with a cysteine, thus losing the characteristic enzymatic activity (30). Mice heterozygous for the mutation showed decreased D3 activity when the null allele was inherited from the father and no change when it was inherited from the mother, as *Dio3* gene is imprinted in mice, being predominantly paternally expressed. Later, the genetically engineered mouse model of **D3** deficiency has been instrumental in understanding the role of D3 with regard to fertility and developmental processes. Both male and female *Dio3*KO (D3KO) mice showed impaired fertility, significant perinatal mortality, and growth impairment (31). In addition, the developmental programming of the thyroid axis is perturbed in the D3KO mouse, presumably due to the overexposure of the animal to excessive levels of TH *in utero* and during the first weeks of perinatal life. Thus, the D3KO mouse manifests marked abnormalities in thyroid status and physiology, which underscores the critical role of the D3 enzyme in the development and in the function of the hypothalamic–pituitary–thyroid (HPT) axis (32, 33). Although much remains to be explored regarding the phenotypic abnormalities in the D3KO mouse, studies conducted to date clearly demonstrate the importance of D3 during development.

Table 1. Characteristics of deiodinases.

Characteristics	D1	D2	D3
Physiological role	- rT ₃ degradation - source of plasma T ₃	- provide intracellular T ₃ in some tissues - source of plasma T ₃	- inactivate T ₄ and T ₃
Reaction catalyzed	5' or 5-deiodination	5'-deiodination	5-deiodination
Substrate preference	rT ₃ > T ₄	T ₄ > rT ₃	T ₃ > T ₄
Inhibitors PTU	++++	+	+/-
Tissue	liver, kidney, thyroid, pituitary, not CNS (?)	CNS, pituitary, BAT, placenta, thyroid, skeletal muscle, heart	placenta, CNS, fetal liver, hemangiomas, pregnant uterus
Monomer size	29kDa	30.5kDa	3.5kDa
Subcellular localization	plasma membrane	ER	plasma membrane

CNS: Central Nervous System; BAT: Brown Adipose Tissue; ER: endoplasmic reticulum

Transmembrane transport of thyroid hormone

The intracellular actions of TH are predominantly mediated by the binding of T_3 to its nuclear receptors, thereby regulating gene expression. Since T_3 -dependent gene transcription takes place intracellularly, TH has to cross cell membranes in order to be metabolized and to interact with its receptors. There is a tight correlation between serum free T_4 and T_3 concentrations and the activity level of TH-dependent processes. This apparent equilibrium between the intracellular and serum free fraction of the hormone has perpetuated the hypothesis of passive TH diffusion into target cells (34). Nevertheless, several classes of membrane transporters with different kinetics and substrate preferences have been identified as likely candidates for transmembrane TH carriers (35). Their physiological role, however, remains unknown. This is principally because, in contrast to the common defects of TH synthesis and action, until recently, no defects of membrane transport proteins have been recognized.

Several TH transporters have been identified that belong to different families of solute carriers, including organic anions (OATP), amino acids, and monocarboxylates (MCT) transporters (36-40). The wide range of endogenous and xenobiotic molecules for most of these transporters hampers deduction of the putative phenotype in transporter deficiency. Their wide distribution and the role played by their ligands would predict multiple organ involvement. The presence of multiple TH

transporters suggests an overlapping, redundant function. On the other hand, their characteristics in terms of different tissue distribution (**Table 2**) and kinetics, as well as the binding of other possible ligands, qualify them to play distinctive roles in the fine tuning of organ-specific TH availability.

OATPs represent a large family of transporters responsible for Na⁺-independent transmembrane transport of amphipathic organic compounds, including bile salts, bromosulfophthalein (BSP), steroid hormones, numerous drugs, and many have been shown to transport different iodothyronines (41, 42). So far, ~ 40 OATPs have been identified in humans, rats, and mice. All OATPs are large proteins of 652-848 aminoacids in length with 12 transmembrane domains (TMDs). Most OATPs proteins are expressed in multiple tissues, including liver, kidney, brain (brain endothelial cells, choroids plexus), lung, heart, placenta, testis, eye, and small intestine (43). However, some members show a tissue-specific distribution. OATP1B1 and OATP1B3 are exclusively expressed in liver (44, 45), whereas OATPC1C1 is only present in the brain and in the Leyding cells of the testis (38). OATP1B1 and OATP1B3 transport the iodothyronine sulfates, T₄S, T₃S, and rT₃S but transport less the corresponding nonsulfated analogues (46, 47). OATP1C1 (also known as OATP14) is most intriguing as it shows a high specificity and affinity towards T₄ and rT₃, whereas uptake of T₃ is about 5-fold less than that of T₄ and rT₃ (T₄ ~ rT₃ > T₃) (**Table 3**).

In brain, OATP1C1 is localized preferentially in capillaries, suggesting that it may be important for transport of T_4 across the blood-brain barrier (38). Moreover, it has been shown that T_4S uptake is also facilitated by OATP1C1, although to a lesser extent than T_4 (48). These properties, together with the almost exclusive expression at the blood-brain barrier, suggest that OATP1C1 is critical for T_4 uptake into the brain. Intriguingly, the expression of OATP1C1 in isolated rat brain capillaries is regulated by TH, being up-regulated in hypothyroid rats and down-regulated in hyperthyroid rats (49). Together with changes in D2 expression (50), OATP1C1 counteracts the effect of alterations in serum T_4 to ensure stable TH concentrations in the brain. Nevertheless despite the high level of homology in structure and substrate specificity between rodent and human OATP1C1, the expression of OATP1C1 is higher in rodent brain compared with that of humans (51), showing that in humans OATP1C1 is less involved in brain T_4 economy than in rodents.

Table 2. Tissue distribution of TH transporters and the escort protein 4F2hc. (52)

Protein	Localization	References
MCT8	liver, kidney, brain heart, skeletal muscle, placenta, thyroid, testis	(37, 53-60)
MCT10	intestine, kidney, liver, placenta, pancreas, brain, testis, cochlea skeletal muscle, heart,	(58-60)
OATP1C1	brain, testis, cochlea	(38, 49)
OATP3A1	brain, liver, kidney, tumors (bone, breast, liver)	(61-65)
LAT1	multiple (tumors, brain, spleen, placenta, testis, testis, colon, kidney, intestine, stomach, ovary, thymus, not liver)	(66-72)
LAT2	kidney, placenta, brain, intestine, testis, ovary, liver, heart, skeletal muscle, lung, stomach	(66, 71, 73-75)
Escort Protein	Localization	References
4F2hc (CD98)	multiple (tumors, brain, intestine, kidney, liver, skeletal muscle, ovary, placenta, testis	(66)

Table 3. Uptake of iodothyronines by TH transporters across the blood-brain-barrier. The level of TH transport are indicated as follows: +++ high uptake rate; ++ modest uptake rate; + low uptake rate; n.d. not determined. (52)

Protein	Iodothyronines				Species	References
	T ₂	T ₃	rT ₃	T ₄		
MCT8	+/+	+++/>+++	+/>++	++/>++	human/rat	(37, 76)
OATP1C1	n.d.	+	+++	+++	human/rat	(38, 49, 77)
LAT1	+++	++	++	+	human	(78)
LAT2	+++	++	++	+	mouse	(78)

NTCP (SLC10A1) is expressed only in hepatocytes and has seven transmembrane domains. It is the major transporter for unconjugated bile acids and also transports TH (79).

Amino acid transporters - Iodothyronines are a particular class of amino acids built up from two tyrosine residues. Therefore, it is not surprising that amino acid transporters, in particular the L and T type, have been shown to be involved in TH uptake into several tissues. L type transporters mediate uptake of large neutral, branched-chain and aromatic amino acids, whereas T type transporters are specific for the aromatic amino acids Phe, Tyr and Trp.

Two L type transporters (LAT1 and LAT2) have been identified among the members of the heterodimeric amino acid transporter (HAT) family. HATs consist of a heavy chain and a light chain, linked through a disulfide bond (40). There are two possible heavy chains (4F2hc and rBAT) belonging to the SLC3 gene family, and seven possible light chains belonging to the SLC7 gene family. The 4F2 or CD98 cell surface antigen is expressed in many tissues, especially on activated lymphocytes and tumor cells. 4F2hc is glycosylated protein with a single transmembrane domain, whereas the light chains are not glycosylated and have 12 transmembrane domains. The LAT1 and LAT2 light chains form a functional transporter with the 4F2hc (4F2 heavy chain).

LAT1 and **LAT2** are expressed in various tissues, e.g. in luminal and abluminal membranes of brain capillary endothelial cells, placenta

and intestine, whereas LAT1 also shows high expression levels in tumor cells (**Table 2**). Functional analysis of LAT1 demonstrated the iodothyronine uptake, which decreased in order $3,3'\text{-T}_2 > r\text{T}_3 \sim \text{T}_3 > \text{T}_4$. Smaller increments in iodothyronine uptake were noted in transport mediated by LAT2 (**Table 3**).

Monocarboxylate transporters (MCT) - Monocarboxylates, such as lactate, pyruvate, and ketone bodies, play an important role in energy metabolism in different tissues, in particular brain (80). The transport of these substances across the cell membrane is carried out by the proton-linked MCT1–4 (58). They belong to a larger family, consisting of 14 homologous proteins, but the function of most other family members remains to be elucidated (37, 81). However, among those transporters, **MCT8** and **MCT10** have been well characterized.

The human *MCT8* gene [(h)MCT8, also known as *SLC16A2* (SLC16 member A2) or XPCT (X-linked PEST containing transporter)] is located on chromosome Xq13.2 and consists of six exons and five introns, the first of which is about 100,000 kb in size (58, 82). The mature mRNA is about 4.4 kb large and contains two possible translation start sites (TSSs). Depending on which of these TSSs is used, proteins of 613 or 539 amino acids are generated. The most upstream TSS is absent in most species, including mouse and rat, and the importance of the additional N-terminal sequence in the longer hMCT8 protein is as yet unknown. The N-terminal domain of the shorter

hMCT8 protein contains one PEST-domain and the longer hMCT8 protein contains two PEST domains (82). Based on this feature, MCT8 was initially named XPCT (X-linked PEST-containing transporter). PEST domains are rich in Pro, Glu, Ser and Thr residues and are often associated with rapid protein degradation, but the function of the PEST domain(s) in MCT8 is unknown.

As with all other MCTs and irrespective of the length of the N terminus, hMCT8 contains 12 putative transmembrane domains, and both the N and C terminus are located intracellularly. The hMCT8 amino acid sequence does not contain consensus glycosylation sites, and immunoblots of cells transfected with hMCT8 cDNA show a major protein with a molecular mass corresponding to the nonglycosylated protein (83). In addition, a weaker band is observed with a molecular mass suggestive of the homodimer (83).

Initially, MCT8 was characterized as a TH transporter in studies where rat MCT8 was expressed in *Xenopus* oocytes (37). This resulted in an approximately 10-fold increase in the uptake of the iodothyronines T₄, T₃, rT₃, and 3,3'-T₂, but it had no effect on the uptake of T₄ sulfate, the aromatic amino acids Phe, Tyr, and Trp, and lactate. Subsequent studies in mammalian cells indicated marked stimulation of T₄ and T₃ uptake after transient transfection with hMCT8, but the magnitude of the response was much smaller than that observed with rat MCT8 in oocytes (83). This was explained by the high rate of iodothyronine efflux

from *hMCT8*-transfected cells, which could be prevented by cotransfection with the high-affinity cytoplasmic TH-binding protein μ -crystallin (CRYM). In the presence of CRYM, net uptake of T_4 and T_3 was greatly stimulated by *hMCT8* expression in mammalian cells (83).

Metabolism of iodothyronines in intact deiodinase-transfected cells is strongly enhanced by cotransfection with *hMCT8* (83). This has been shown for T_4 metabolism by D2 or D3, for T_3 metabolism by D3, for rT_3 metabolism by D1 or D2, and for 3,3'- T_2 metabolism by D3. These findings, therefore, demonstrate that *hMCT8* greatly increases the intracellular availability of the different iodothyronines.

As demonstrated by Northern blotting and RT-PCR, *MCT8* mRNA is widely expressed in human and rat tissues, including brain, heart, liver, kidney, adrenal gland, and thyroid (59, 84). Mouse *MCT8* protein has been localized renal tubule cells, and thyrocytes (85). In thyroid has been demonstrated that *MCT8* is involved in the secretion of TH into the bloodstream (57, 86).

Analysis of the *MCT8* mRNA expression pattern in the mouse brain by *in situ* hybridization revealed a distinct localization of this transporter in specific neuronal populations known to be highly dependent on proper TH supply (53). For instance, pronounced transcript levels were detected in hippocampal pyramidal neurons and granule cells, in layers 2–3 and 5 of the cerebral cortex, in basal ganglia, throughout the amygdala, and in cerebellar Purkinje cells,

indicating that MCT8 may play a critical role in the uptake of T₃ into neuronal cells. Moreover, high transcript levels for MCT8 were observed in choroid plexus structures and in capillary endothelial cells, suggesting that MCT8 also contributes to the passage of THs via the blood-brain barrier and/or via the blood-cerebrospinal fluid barrier (51, 53)

The human *MCT10* gene [(h)MCT10 also known as *SLC16A10* (SLC16 member A10)] is located on chromosome 6q21–q22 and consists of six exons and five introns, of which the first intron is particularly large, i.e. ~ 100 kb. This gene structure is identical to that of the human *MCT8* gene and the protein is a membrane transport with 12 putative transmembrane domains (TMDs). The MCT8 and MCT10 proteins are highly homologous with an amino acid identity of 49% (81). This homology is highest in the TMDs and lowest in the N- and C-terminal domains that are both located intracellular. MCT10 has been characterized (87) as a T-type amino acid transporter, facilitating the cellular uptake and efflux of aromatic amino acids. Later it has been shown that MCT10 is an active iodothyronine transporter, facilitating both the cellular uptake and efflux TH (81).

Thyroid hormone transporters and brain

Brain development represents a very good example of a fine coordination of deiodinases action and transporters function in order to deliver TH to proper sites. For regulating brain maturation, several membrane barriers have to be overcome until T_3 finally binds to its receptor in the central nervous system (CNS). At first, TH has to be taken up into the brain either via the blood-brain barrier or the choroid plexus-cerebrospinal fluid (CSF) barrier. In rodents, entry of T_4 and T_3 via the blood-brain barrier appears to be the preferred route for the overall distribution of TH to the brain whereas the uptake via the choroids plexus-CSF barrier may be especially important to provide TH to specific areas (88). The MCT8 transporter has been shown to be critically involved in the transport of T_3 into the brain as in Mct8 deficient mice uptake of radiolabeled T_3 is severely reduced (89). However T_4 can still enter the brain even in the absence of MCT8 suggesting that at least in mice another transport mechanism exists for mediating the uptake of T_4 at the blood-brain barrier. Recently it has been demonstrated that Oatp1c1 exhibits a high degree of substrate specificity towards T_4 and rT_3 but is also strongly expressed in capillary endothelial cells of the CNS (51). After being released from endothelial cells into the brain parenchyma, T_4 has to be taken up by astrocytes where it is converted to T_3 by D2. The transporters involved in the

astrocytic TH processing are still unknown. From astrocytes, T₃ is thought to be transported into the brain parenchyma from which it can be taken up by neurons and oligodendrocytes. MCT8 mediates the transport of T₃ in certain neuronal populations where high MCT8 expression was detected by in situ hybridization (53). At least for murine CNS one has to postulate the existence of additional transporters in neurons, since T₃ transport in these cells was only mildly affected in the *Mct8KO* mice (89, 90). Putative candidates include Lat1, Lat2, Mct10, Oatp1c1. After T₃ has exerted its effect in neurons, it is metabolized by D3. **Figure 2** shows a schematic representation of TH uptake and metabolism in brain.

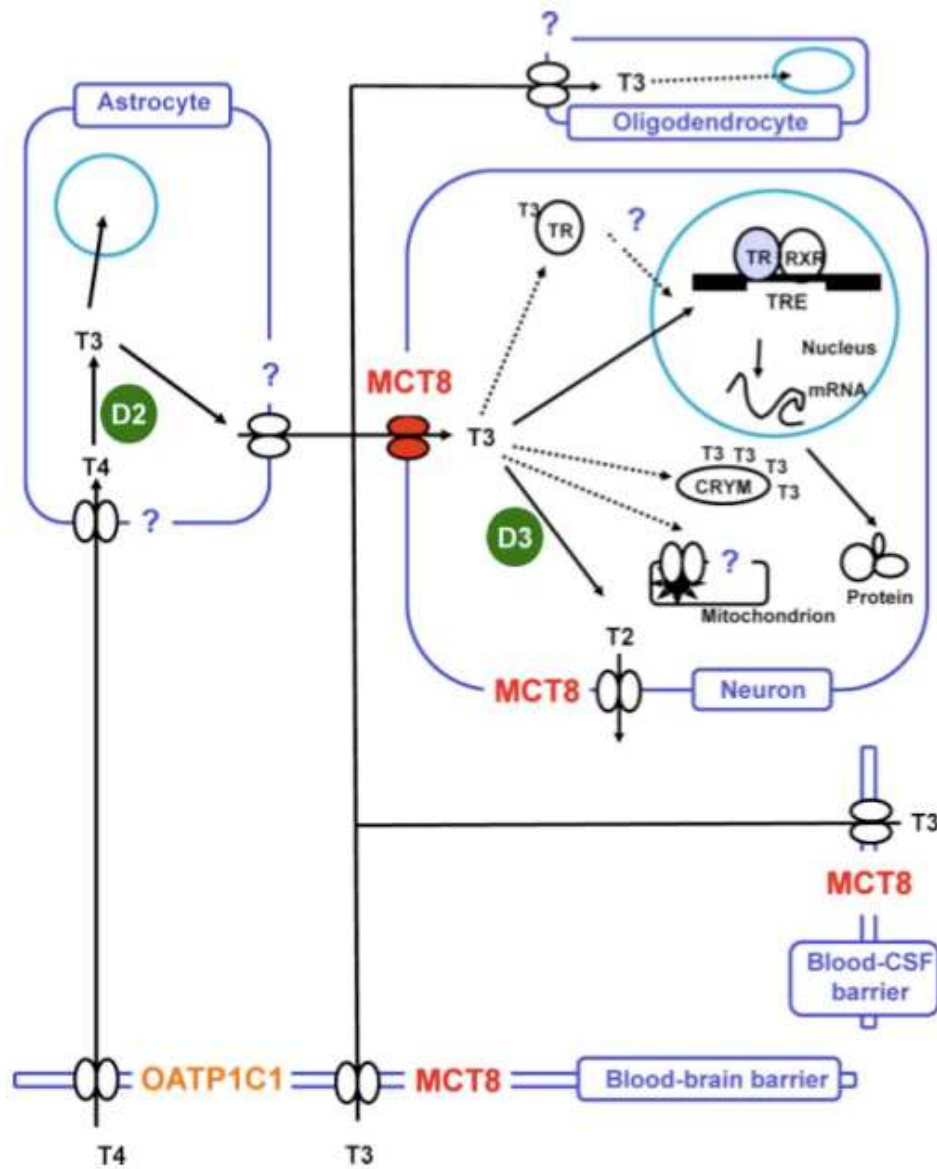


Figure 2. Model of TH regulation in brain (91)

MCT8 deficiency

MCT8 deficiency in humans

Mutations in *MCT8* gene are responsible for the Allan-Herndon-Dudley syndrome (AHDS) described in 1944 (MIM 309600) as one of the first syndromic X-linked mental retardation entities. Males are referred for medical investigation during infancy or early childhood because of neurodevelopmental abnormalities. They present with hypotonia, motor delay, feeding problems, inability to walk, and no speech development. Parents were not consanguineous and gestation and delivery were normal. Infants were normal in length, weight, and head circumference. They do not show typical signs of hypothyroidism. An early sign of the defect, manifesting in the first few weeks of life, was hypotonia and feeding difficulties. With advancing age weight gain lags behind and microcephaly becomes apparent, but linear growth proceeds normally. Although truncal hypotonia persists, there is progressive development of limb rigidity, leading to spastic quadriplegia. Muscle mass is diminished and there is generalized muscle weakness with typical poor head control. Purposeless movements and characteristic paroxysms of kinesigenic dyskinesia are common. These are usually triggered by somatosensory stimuli, such as changing of clothes or lifting the child. The attacks consist of extension of the body, opening of the mouth and stretching and flexing of the limbs lasting less than 1 to 2 minutes (92). In addition to these nonepileptic events, true

seizures can also occur. Reflexes are usually brisk, clonus is often present but nystagmus and extension plantar responses are less common. Most affected children are never able to sit by themselves or to walk; those who manage to do so, lose this ability with time, indicating progressive deterioration. Cognitive impairment is severe. Individuals never develop speech or, at most, acquire the ability to emit garbled sounds. Their behavior tends to be non-aggressive. Although early death has occurred in some families, most often due to aspiration pneumonia, some individuals live beyond the age of 70 years. Normally females heterozygous for *MCT8* gene mutations are carriers and do not manifest any of the psychomotor abnormalities described above. However, one female has been described with AHDS due to chromosomal translocation with breakpoint in *MCT8* gene on one X chromosome and unfavorable inactivation of the intact X chromosome (93).

Characteristic for patients with *MCT8* gene mutations is the remarkable combination of serum TH concentrations. These are the high serum T_3 and low T_4 and rT_3 concentrations (**Fig. 4**). Only in one instance normal thyroid function tests (TFTs) were reported in a patient with *MCT8* deficiency (94), while in another case T_4 concentration was normal (95). TSH levels are normal or slightly elevated, rarely above 6 mU/L. Regarding those TFTs, it is important to consider: a) abnormalities may be subtle, depending on the severity of the disease;

b) values must be interpreted relative to age-specific reference ranges. This is essential for serum T_3 concentrations, which are higher in infants; c) as the combination of the above-mentioned TFTs are pathognomic of MCT8 defects, thus it is best to measure all iodothyronines and TSH rather than only T_3 or T_4 (**Fig. 3**). Heterozygous female carriers have on the average serum TH concentrations intermediate between affected males and unaffected family members. Although brain magnetic resonance imaging is often normal, atrophy of the cerebrum, thalamus, and basal ganglia have been reported, reflecting dysmyelination (94, 96, 97).

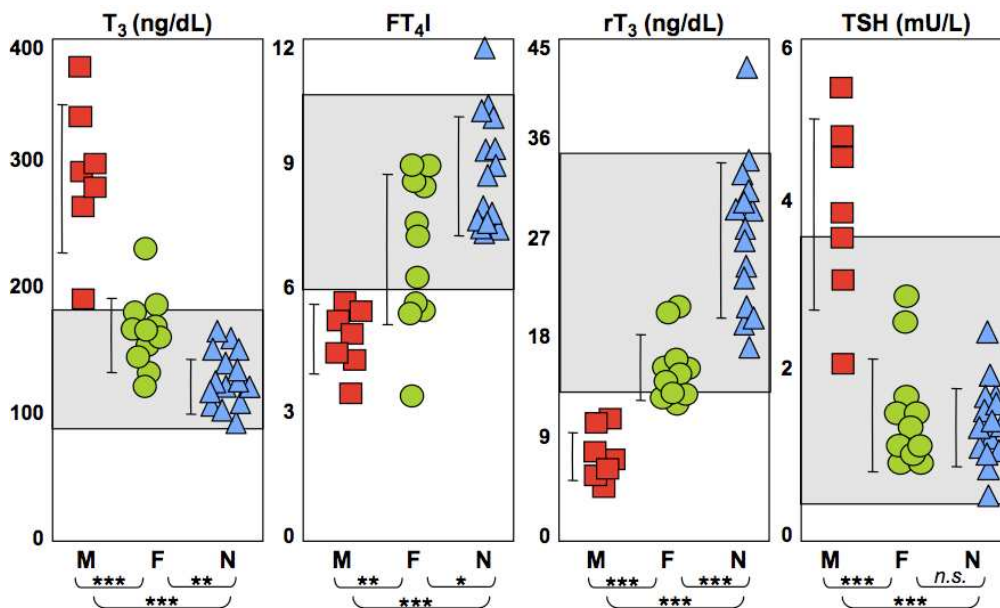


Figure 3. Thyroid function tests from several families studied at the University of Chicago: affected males are in red, female carriers are in green, unaffected subjects are in blue. Shaded areas represent the normal range for each test. *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$ [modified from (6)].

So far, *MCT8* gene mutations have been identified in over than 80 families 54 of which have been published (**Table 4**) (98). This suggests that *MCT8* mutations are not that rare even if the prevalence is currently unknown. Mutations are of different nature. Approximately 30% have deletions or mutations introducing premature stop codons. Such mutations conceivably result in complete loss of TH-transport function. However, to deduce the impact of single amino acid substitutions, small inframe insertions, or deletions on *MCT8* function is not simple. Two models have been used to study the effects of *MCT8* gene mutations on its transport capacity: mammalian cells (transiently or stably) transfected with mutant *MCT8* cDNA and fibroblasts derived from patients with *MCT8* defects. Cells overexpressing WT and mutant *MCT8* cDNA alone or in combination with D2, D3 or a crystalloid, were tested for T₃ and T₄ transport and metabolism. Most mutants were shown to be inactive, although some exhibited some residual activity. Complete loss-of-function is mostly explained by a decreased plasma membrane expression as evidenced from a predominant cytosolic localization or by a diminished or absence of protein expression (99). Trafficking of mutant *MCT8* to the plasma membrane appears cell-type dependent, probably reflecting differences in the cellular machinery, such as regulatory proteins or the capacity to form heterodimers with WT *MCT8* (100, 101). A second system to investigate the effects of

MCT8 gene mutations in skin fibroblasts obtained from patients (102, 103). Although *MCT8* gene expression was not detected by Western blot analysis or immunocytochemistry, the transporter appears functionally important, because T₃ and T₄ uptake were reduced by 50 – 60% in fibroblasts from patients as compared with normal controls (102, 103).

Table 4. *MCT8* gene mutations identified in patients with AHDS from 2004 to 2009.

Mutation		Effect on hMCT8	Reference
Nucleotide	Protein		
(1-252,553)-(652+83,793) del	delEx1	Predicted complete inactivation	(103)
(1-9338)-(652+14,538) del	delEx1	Predicted complete inactivation	(104)
	delEx2-4	Predicted complete inactivation	(105)
	delEx3-5	Predicted complete inactivation	(103, 104)
971-(1392+1008)del	M1L	N/A (hMCT8L)	(93)
1A>T		Complete inactivation	(99, 106)
565insATC	insI189	Not tested	(105)
575A>G	H192R	Complete to partial inactivation	(99, 101, 107)
581C>T	S194F	Predicted complete inactivation	(93)
630insA	N210fsX30	Predicted complete inactivation	(108)
630insG	N210fsX30	Predicted complete inactivation	(109)
631-644del	R211fsX25	Complete inactivation	(105, 107)
661G>A	G221R	Complete inactivation	(99, 104)
671C>T	A224V	Complete inactivation	(107, 109)
689-691delTCT	delF230	Complete inactivation	(99, 107)
703G>A	V235M	Complete to partial inactivation	(101, 110)
706insGTG	insV236	Complete inactivation	(99, 104)
733C>T	R245X	Complete inactivation	(109)
798-1G>C	del267-370	Complete inactivation	(101, 109)
812G>A	R271H	Partial inactivation	(105)
962C>T	P321L	Complete inactivation	(111)
1003C>T	Q335X	Predicted complete inactivation	(112)
1201G>A	G401R	Not tested	(102)
1212delT	A405fsX12	Predicted complete inactivation	(99, 101, 107)
1301T>G	L434W	Complete to partial inactivation	(105)
1333C>T	R445C	Not tested	(99, 107)
1343G>A	S448X	Complete inactivation	(113)
1343-1344insGCCC	S448fsX5	Predicted complete inactivation	(99, 104)
1412T>C	L471P	Complete inactivation	(103)
1500-1502delCTT	delF501	Complete inactivation	(102)
1535T>C	L512P	Complete inactivation	(105)
1558C>T	Q520X	Predicted complete inactivation	(114)
1610C>T	P537L	Complete inactivation	(112)
1649delA	Y550fsX17	Predicted complete inactivation	(93)
1673G>A	G558D	Partial inactivation	(103)
1690G>A	G564R	Complete inactivation	(99, 107)
1703T>C	L568P	Partial inactivation	(105)
1826delC	P609fsX70	Not tested	

N/A: not available (98).

Mct8 deficiency in mice.

Two different *Mct8KO* mouse models have been created to understand the mechanisms of the disease in patients with *MCT8* gene mutations (89, 90, 115, 116). Compared to Wt animals, *Mct8KO* mice have markedly decreased serum T_4 and rT_3 levels, increased T_3 and TSH levels normal or slightly elevated (**Fig. 4**), thereby recapitulating the TFTs observed in *MCT8* deficient patients (89, 90). Despite the loss of *Mct8* expression in liver, T_3 uptake into this organ was not different in mutant vs Wt mice, suggesting the presence of other TH transporters. This explains the increased T_3 concentration in liver tissue and the elevated T_3 -sensitive D1 expression and activity. Increased D1 activity in liver of *MCT8* deficient patients explains the decreased serum levels of rT_3 , as rT_3 is the preferred substrate of D1, resulting in an increased rT_3 degradation (117). Moreover, rT_3 production is probably low because of the low T_4 substrate levels and decreased access to D3 expressing cells. The high TSH levels in *MCT8* deficient patients and in *Mct8KO* mice, is associated with a very high TRH levels in the mice (89) and suggests that the feedback loop was affected. High doses of T_4 , but not of T_3 , reversed the abnormal TRH expression. Therefore, the hypothalamus of *Mct8KO* mice still responds to local T_4 to T_3 conversion. Presumably, an impaired *Mct8*-mediated T_3 uptake is responsible for the increased TRH expression. Expression of several T_3 -responsive genes is not affected in the pituitary of *Mct8KO* animals,

indicating adequate intracellular T_3 levels. This is most likely ensured by the increased pituitary D2 activity, which compensates for the decreased serum T_4 , resulting in the maintenance of local T_3 production. Pituitary uptake of T_3 may also be diminished in *Mct8KO* mice, although Mct8 is not markedly expressed in hormone-producing cells of the mouse pituitary. In *Mct8KO* mice rendered hypothyroid, 8-fold the T_3 and 2.5-fold the T_4 doses given to Wt animals are required to restore TSH levels (163). This may be explained in part by the reduced effect of T_3 on the hypothalamus. Thus, the studies in *Mct8KO* mice indicate that the hypothalamus and, consequently, the pituitary are relatively insensitive to TH. Together with the finding that MCT8 is expressed in human hypothalamus and pituitary, the animal studies provide a plausible explanation for the elevated TSH levels in patients with MCT8 mutations despite high serum T_3 concentrations.

The most interesting finding in *Mct8KO* mice is the absence of an overt neurological phenotype (89, 90). Only subtle behavioral abnormalities in *Mct8KO* mice have been recently reported (116). These results are surprising since T_3 uptake, and consequently T_3 content, in brains of *Mct8KO* mice is decreased (**Fig. 5**) (89, 90). The decreased D3 activity, which is inversely regulated by its substrate T_3 , probably reflects a beneficial response aimed to counteract the harmful effects of decreased T_3 transport. Parallel with the decreased serum T_4 levels, brains of *Mct8KO* mice contain less T_4 than the Wt mice. In

brains of *Mct8KO* mice, D2 activity, which is inversely regulated by its substrate T_4 , is increased (**Fig. 5**). Thus local T_3 production in brain may be relatively normal due to the decrease in T_4 levels and increase D2 activity. Brain T_3 levels are nevertheless markedly decreased despite high serum T_3 levels and decreased T_3 degradation, due to a dramatic reduction in brain T_3 uptake (115). Indeed, expression of the T_3 -sensitive gene *Hairless* in *Mct8KO* mice is lower than in Wt animals (**Fig. 5**) (115). In cultured neurons from *Mct8KO* mice, T_3 uptake was diminished compared with Wt mice (116), but *Hairless* expression did not differ between *Mct8KO* and Wt mice. Also, the effect of T_3 on dendritic outgrowth in cultured neurons was similar in *Mct8KO* and Wt animals (89). This might be explained by differences in neuronal cell populations used in the different studies, where subsets of neurons may be more vulnerable to lack of functional Mct8 than others. Indeed, in contrast to other regions, neuronal cells in the striatum are somewhat hypothyroid, as assed by the decrease in T_3 -responsive gene RC3 (89). So the above-mentioned data suggest that Mct8 is important for T_3 transport across the blood-brain barrier in mice, while T_4 transport into brain is not as dependent on Mct8 or is compensated by alternative transporters. It seems that the level of T_3 in brain of *Mct8KO* mice is sufficient to prevent gross neurological dysfunction in mice.

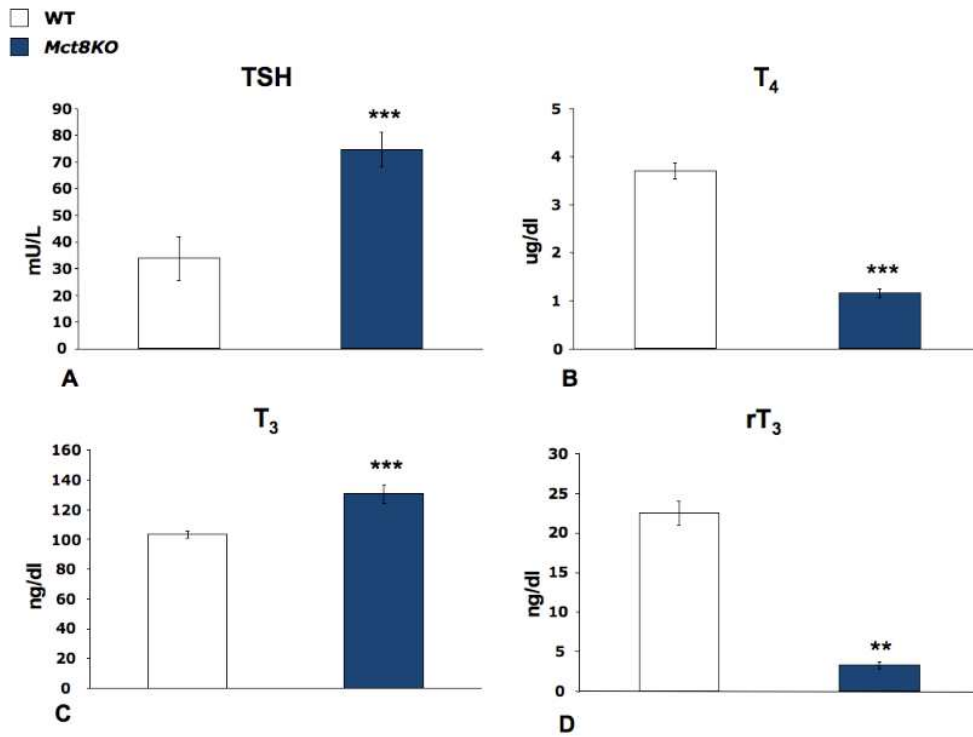


Figure 4. TFTs in adult *Mct8KO* and Wt mice. A, TSH levels, B, T₄ levels; C, T₃ levels; D, rT₃ levels. ** $P < 0,01$, *** $P < 0,001$, NS, not significant

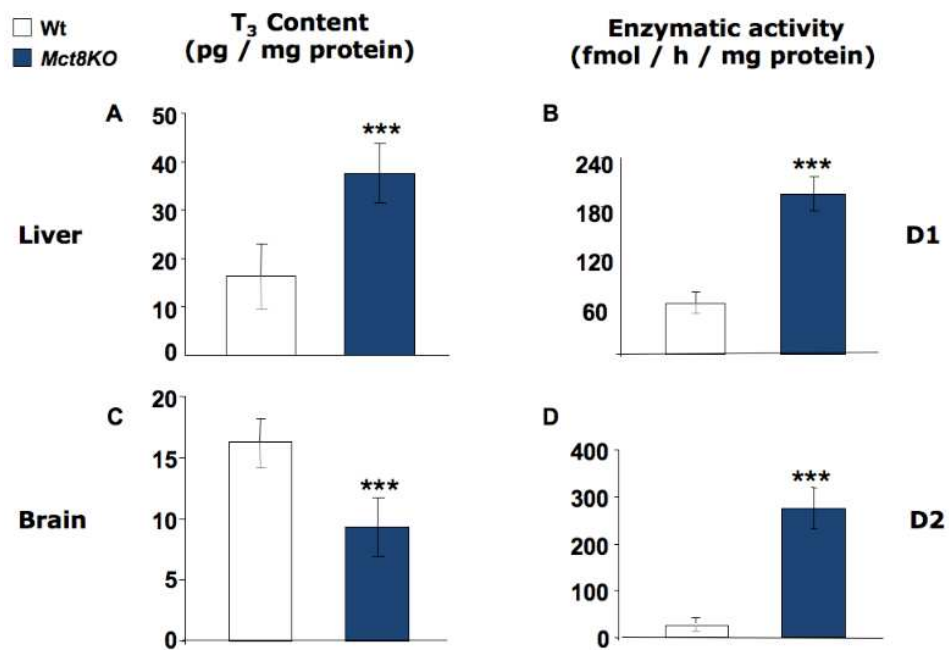


Figure 5. Effects of Mct8 deficiency in cerebrum and in liver of adult mice. A, baseline liver T₃ content. B, Baseline D1-specific enzymatic activity, C, baseline cerebrum T₃ content; D, Baseline D2-specific enzymatic activity. *** $P < 0.001$.

AIM OF THE STUDY

The identification of TH transmembrane transport defects caused by MCT8 deficiency has provided new insights into the physiology of TH action. Significant progress was made in understanding cellular TH transport and the consequences of MCT8 deficiency in humans by studying the *Mct8KO* mice. However there are some gaps of knowledge that need to be elucidated. One such question is when do the thyroid tests abnormalities typical of MCT8 deficiency first manifest.

The aim of the present study is to characterize the thyroid phenotype of *Mct8KO* mice throughout life, from early postnatal life into adulthood in terms of circulating levels of THs and TSH, TH actions in cortex and in liver.

These results are important in understanding the mechanisms that lead to the brain and metabolic defects seen in patients with *MCT8* gene mutations. They might also provide a possible explanation as to why the *Mct8* deficient mice do not manifest any gross neurological phenotype, a question that has puzzled researchers in this field.

MATERIALS AND METHODS

Experimental animals

Procedures carried out in mice and described below were approved by the University of Chicago Institutional Animal Care and Use Committee. Animals were housed in temperature ($22 \pm 2^\circ\text{C}$) and light (12 h light, 12 h dark cycle; lights on at 0700 h) controlled conditions and had free access to food and water. *Mct8KO* mice were generated as described previously (90). Experiments were carried out on post partum (P) days P0, P3, P7, P11, P14, P18, P21, P100 (adult) male Wt (*Mct8*^{+/y}) and knockout (*Mct8*^{-/y}) littermates derived from more than 10 back-crossing of heterozygous females (*Mct8*^{-/+}) with Wt males (*Mct8*^{+/y}) of the C57BL/6J strain. The genotype was confirmed by PCR of tail DNA (38 cycles at 55°C annealing temperature) using the following primers: forward common, 5'-ACAACAAAAGCCAAGCATT-3'; reverse Wt specific, 5'-GAGAGCAGCGTAAGGACAAA-3'; reverse knockout specific, 5'-CTCCCA AGCCTGATTTCTAT-3'. Using this procedure the Wt allele generated a 476-bp products and the null allele a 239-bp PCR product.

Measurements in serum

Serum total T₄, T₃ concentration were measured by coated tube

RIAs (Diagnostic Products, Los Angeles, CA) and rT₃ using reagents from Adaltis Italia (Rino, Italy), as previously described (90, 118). Procedures were adapted for measurements in mouse serum. TSH was measured using a sensitive, heterologous, disequilibrium, double-antibody precipitation RIA (118).

Measurement of specific mRNA content in tissues

Total RNA was extracted using phenol/guanidine isothiocyanate (TRIZOL; Invitrogen, Carlsbad, CA), and 2 µg total RNA was reverse transcribed using Superscript III ribonuclease H reverse transcriptase kit (Invitrogen) in the presence of 100 ng random hexamers. Reactions for the quantification of mRNAs by real-time quantitative PCR were performed in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA), using SYBR Green I as detector dye. The oligonucleotide primers were designed to cross introns. Primers used for the quantitative PCR of iodothyronine deiodinases (*Dio2* and *Dio3*), Hairless (*Hr*), Sonic hedgehog (*Shh*), semaphorin type 7a (*Sema7a*), Kruppel-like factor (*Klf9*), Aldehyde dehydrogenase 1 family, member A3 (*Aldh1a3*), Nuclear receptor subfamily 4, group A, member 1 (*Nr4a1*), *Lat1*, *Lat2*, *Oatp1c1*, *Oatp3a1*, *Mct10* mRNAs. Primer sequences are provided in **table 4**. Amplification of the housekeeping gene RNA polymerase II (*RPII*) and 18S ribosomal RNA (*Rn 18s*) was

used as internal control for cortex and liver, respectively (119).

D2 enzymatic activity

D2 enzymatic activity was performed as described (90) with the following modifications: 100 µg tissue homogenates in 100 µl reaction mixture containing 0.1 m phosphate buffer (pH 7), 1 mm EDTA, 20 mm dithiothreitol, 1 mm propylthiouracil, 100,000 cpm [¹²⁵I]T₄, and 2 nm unlabeled T₄ were incubated at 37 C for 1 h. Saturating levels of unlabeled T₃ (1 µm) were added to the reaction mixture to inhibit the D₃ enzyme.

Tissue T₃ content

Before tissue collection, mice were perfused with heparinated phosphate buffered saline (PBS) through a needle placed in the left ventricle, after severing the inferior vena cava. Tissues were rapidly collected on dry ice and stored at -80°C. T₃ was extracted from brain using a method previously described (120, 121) and T₃ content was measured by RIA. Recovery was monitored in every batch of extraction by addition of the corresponding labeled iodothyronines to the tissues before homogenization.

Induction of hypothyroidism and treatment with T₄ in pregnant mice

In pregnant mothers, a baseline blood sample was obtained from

the tail before initiation of experiments. Starting at E10 of pregnancy, endogenous production of TH was suppressed with low iodine diet (Harlan Teklad Co., Madison, WI) and the addition of 0.5% perchlorate and 0.02% methimazole in the drinking water (Lol/MMI/CIO₄). At E12 of pregnancy, together with the Lol/MMI/CIO₄ treatment, pregnant mothers were injected sc with L-T₄ (2µg/100 g body weight) once per day. At birth, blood sample was again obtained from the tail of each pregnant mother. Pups were anesthetized and, after a blood sample was obtained from the jugular vein were euthanasized by decapitation. Tissues were collected and frozen on dry ice at once.

Statistics

Statistical analysis was performed using a Student-*t* test. Data are represented as mean ± SE. Logarithmic transformation of data were performed when SDs for different groups varied by more than 20-fold and sometimes by 1000-fold (see TSH). $P \geq 0.05$ was considered not to be significant (NS).

Table 4. Primers sequences of genes used for Real-Time PCR.

Gene	F Primer	R Primer
RPII	GCACCACGTCCAATGACAT	GTGCGGCTGCTTCCATAA
Rn 18s	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
Dio2	GTTGCTTCTGAGCCGCTC	GCTCTGCACTGGCAAAGTC
Dio3	CCGACCTGATGGCTTCCA	CGCGCCATGAACGGTGGTCA
Hairless	CCAAGTCTGGGCCAAGTTTG	TGTCCTTGGTCCGATTGGAA
Shh	CGGCTGATGACTCAGAGGTG	ATGATGGCCGTCCTCATCCC
Sema7a	ACACACCGTGCTTTTCCATGA	CCTTTGTGGAGCCGATGTTT
Klf9	GGCTGTGGGAAAGTCTATGG	AAGGGCCGTTACCTGTATG
Aldh1a3	ACA ACGACTGGCACGAATCC	TCCACATCGGGCTTATCTCC
Nr4a1	TTGAGTTCGGCA AGCCTACC	GTGTACCCGTCCATGAAGGTG
Lat1	CTGCTGACACCTGTGCCATC	GGCTTCTTGAATCGGAGCC
Lat2	CCAGTGTGTTGGCCATGATC	TGCAACCGTTACCCCATAGAA
Oatp1c1	AATTCTAGTGTGGCCGGACTGA	CAGCAAGACAAGCCGACACAT
Oatp3a1	TCTGAGTGCGCCTTGATGC	AGAGAATCAGCGCCAGGTTG
Mct10	GGCCGCATTGCTGACTATTT	CAATGGGCGCCATGATAGA

RESULTS

Serum thyroid hormones and TSH levels in Mct8KO versus Wt mice from birth into adulthood.

Serum TH and TSH concentrations were measured at birth, conventionally called postnatal day 0 (P0), and data are shown in **Fig. 6**. In *Mct8KO* mice, mean serum TSH levels were reduced to 51% compared to those in Wt mice. The mean serum T₄ levels were increased to 178% and serum rT₃ levels were decreased to 40% of the corresponding (100%) values in Wt mice. In contrast, no significant difference was seen between T₃ serum levels in *Mct8KO* and Wt mice at P0.

This pattern of TFTs was most surprising as it was opposite to that of adult *Mct8KO* mice which show the typical serum thyroid tests abnormalities observed in humans, namely high T₃, low T₄, low rT₃ and a slightly elevated TSH (**Fig. 4**). This indicated a dynamic evolution of the TFTs caused by Mct8 deficiency.

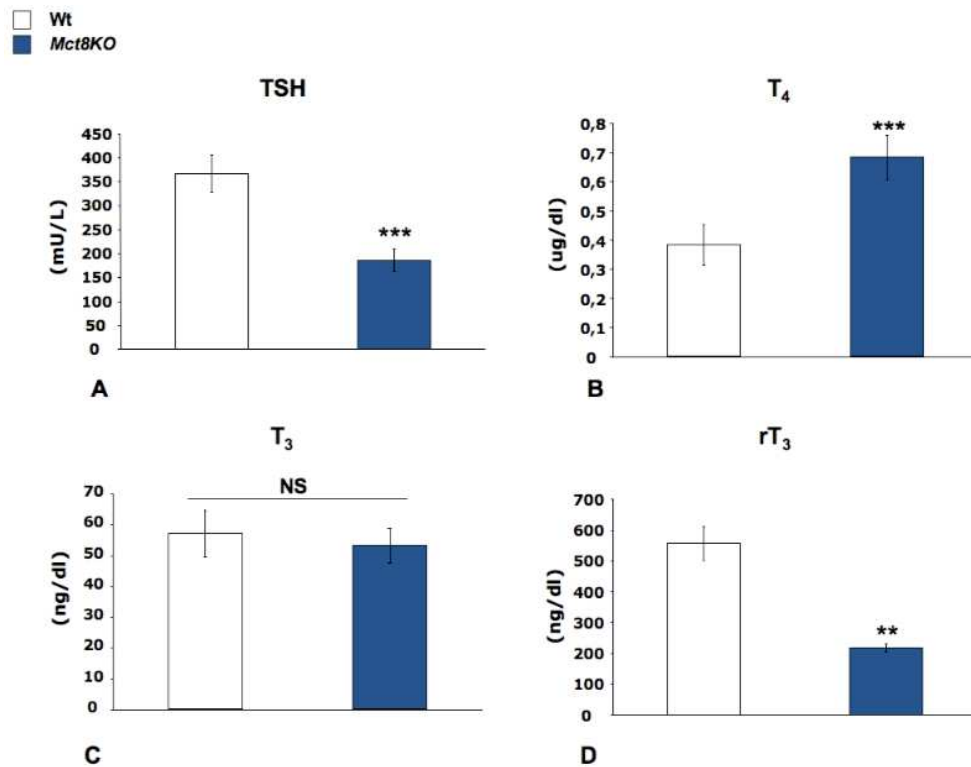


Figure 6. Serum TH levels and TSH in *Mct8KO* and Wt mice at P0. A, serum TSH levels; B, serum T₄ levels; C, serum T₃ levels; D, serum rT₃ levels. **, $P < 0.01$; *** $P < 0.001$

Next, TFTs were measured at different ages: P3, P7, P11, P14, P18, P21 and P100 both in *Mct8KO* and Wt male mice, in order to dissect the evolution of the TFTs, summarized in **Figure 7**. Several different phases in the thyroid status of the *Mct8KO* mice can be identified, based on their TFTs. Early in life, *Mct8KO* mice were relatively “hyperthyroid” with low serum TSH and high T₄ concentrations compared to Wt littermates, while T₃ was similar in both genotypes. From P3 to P7, a pattern of “euthyroidism” is apparent with TSH, T₃ and T₄ levels similar in both *Mct8KO* and Wt mice. By P11 the serum T₄ in

Mct8KO mice becomes significantly lower than in Wt littermates, while the TSH, and T₃ levels are maintained normal. By P14, the serum TSH rose above the age and sex matched Wt mice while T₄ remained low. By P18 the T₃ increased in *Mct8KO* mice compared to Wt, thus generating the thyroid phenotype of the Mct8 deficiency observed in adult mice. Of note, serum rT₃ levels are significantly lower in *Mct8KO* vs Wt mice throughout lifetime.

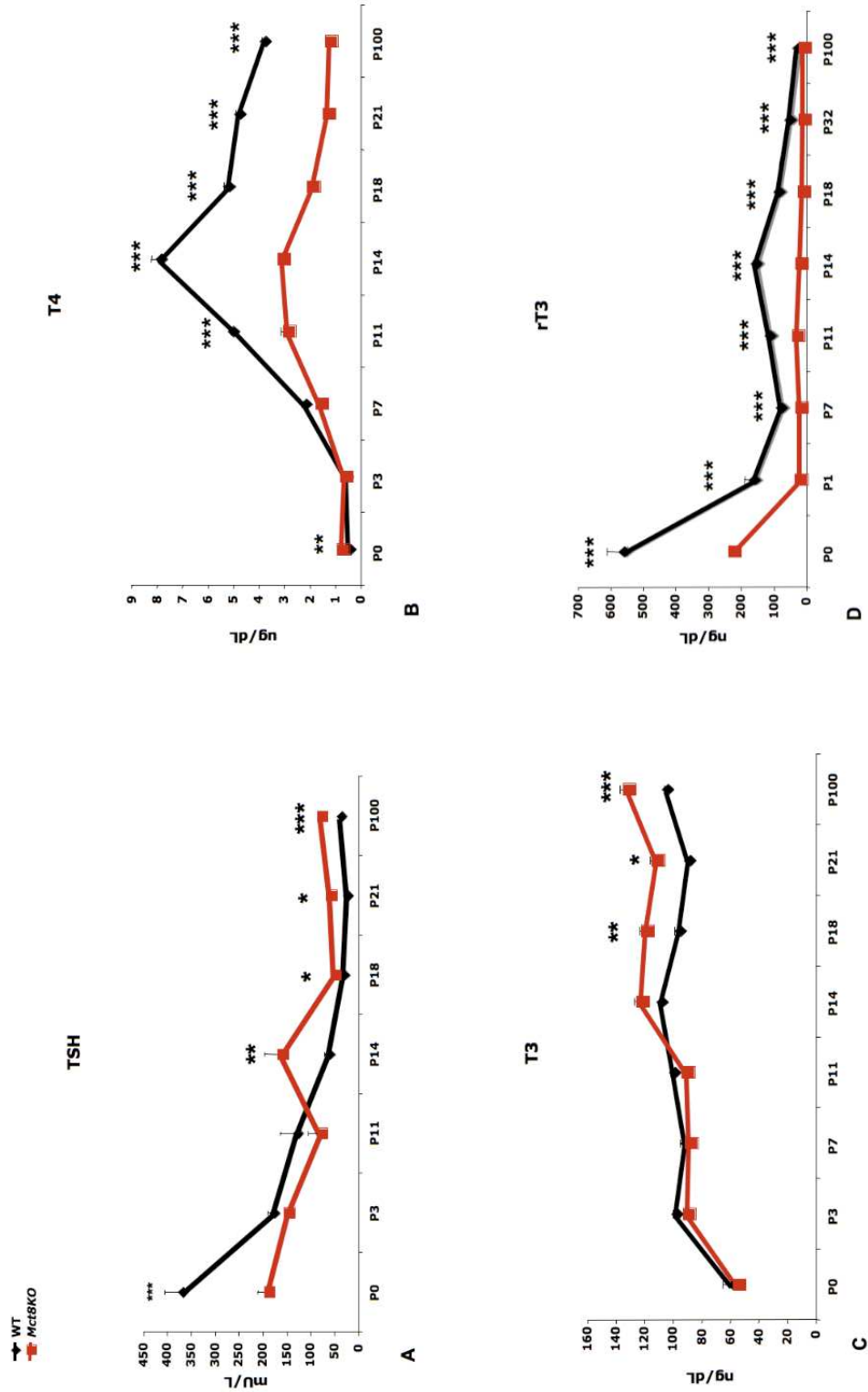


Figure 7. Serum TFTs of *Mct8*KO and Wt mice from P0 to adult life. A, serum TSH levels; B, serum T₄ levels; C, serum T₃ levels; D, serum rT₃ levels; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$

Effect on cortex.

Considering the unexpected finding of high circulating levels of T₄ and low TSH at birth, the thyroid status in brain was evaluated. The T₃ content in cortex was measured at P0, shown in **Figure 8A**. There is no significant difference in the overall cortical T₃ content between *Mct8KO* mice and *Wt* mice at birth, if anything, the trend is to a higher rather than a lower T₃ content in the *Mct8KO* animals. This is in contrast with results in adult *Mct8KO* mice, which had 50% lower T₃ content in cortex compared with *Wt* mice (**Fig. 8B**), indicating an overall local hypothyroidism.

D2 enzymatic activity in the cortex of P0 mice was measured to assess the overall thyroid status in brain (**Fig. 9A**). No difference was observed between the *Mct8KO* and the *Wt* mice. *Dio2* and *Dio3* mRNA expression were also not different (**Fig. 9B and C**, respectively).

The lack of differences in deiodinases between the two genotypes at P0 is also discordant with the results in adult mice. Adult *Mct8KO* mice have levels of D2 activity higher than those of *Wt* mice (**Fig. 5D**) while D3 enzymatic activity is lower in the KO mice (89).

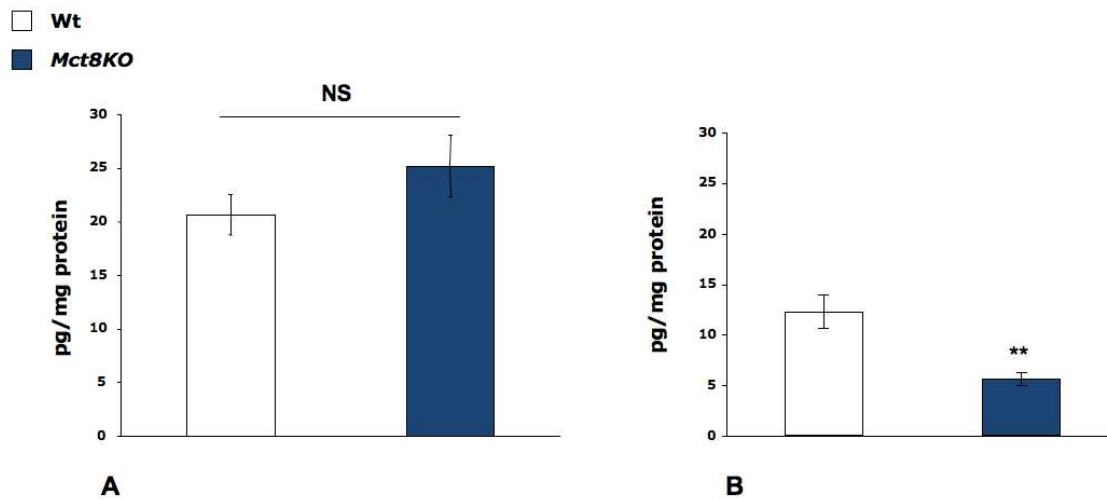


Figure 8. Tissue T₃ content A. Cortex of *Mct8KO* and Wt mice at P0 day; B. Brain of *Mct8KO* and Wt adult mice; **, $P < 0.01$; NS not significant.

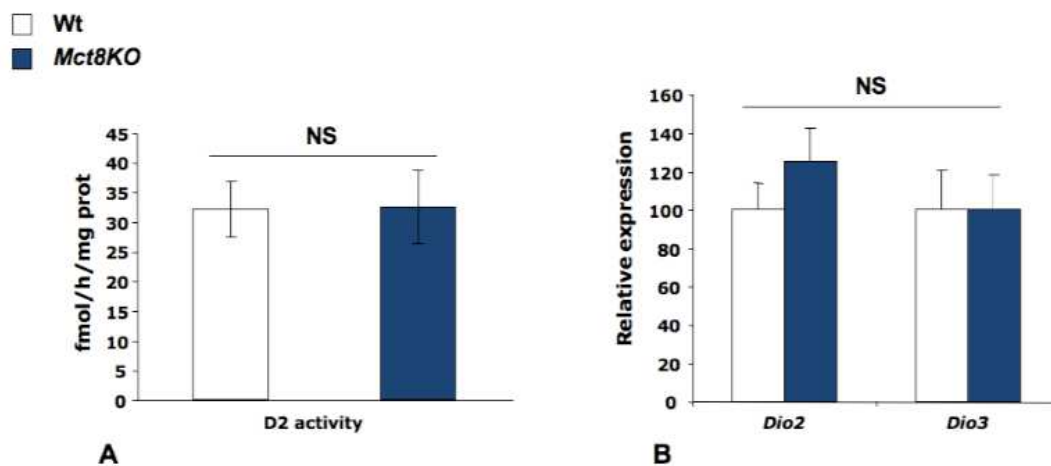


Figure 9. Cortex deiodinases activity and expression in *Mct8KO* and Wt mice at P0. A, D2 activity; B, *Dio2* and *Dio3* mRNA expression. NS, not significant.

To further investigate TH action in the brain of these mice, the expression of TH responsive genes was assessed by quantitative PCR. Hairless (*Hr*) is a TH receptor co-repressor known to be positively regulated by TH (122). In newborn *Mct8KO* mice, *Hr* mRNA expression was increased by 1.8-fold (**Fig. 10A**). This contrasts with results from adult *Mct8KO* mice in which *Hr* expression was decreased by 0.5-fold when compared to Wt mice (**Fig. 10B**), confirming the local hypothyroidism indicated by the TH content. This data suggested an apparent hyperthyroid state in the cortex of P0-day *Mct8KO* mice. To confirm this result, we analyzed gene expression of other T₃-dependent genes. Four gene markers of TH action were used, namely, Sonic hedgehog (*Shh*), Semaphorin 7a (*Sema7a*), Kruppel-like factor 9 (*Klf9*) and Aldehyde dehydrogenase 1a3 (*Aldh1a3*). These genes were recently identified to be regulated by TH, the former three positively and the latter one negatively (unpublished data from Dr. Juan Bernal's laboratory). The mRNA expression of the positive genes *Klf9*, *Shh*, *Sema7a*, was 2.1, 2.0, and 1.8 times higher, respectively, in the cortex of *Mct8KO* mice than in Wt mice (**Fig. 11A, B, C**). On the contrary, mRNA expression of *Aldh1a3* was not significantly reduced in *Mct8KO* mice than in Wt mice, even if the trend was toward a lesser expression (**Fig 11D**).

Finally we evaluated the mRNA of several TH transporters expressed in cortex. While *Lat2*, *Oatp1c1* and *Oatp3a1* are expressed at higher level in *Mct8KO* mice, no significant differences have been seen between the two genotypes for *Lat1* (**Fig. 12**).

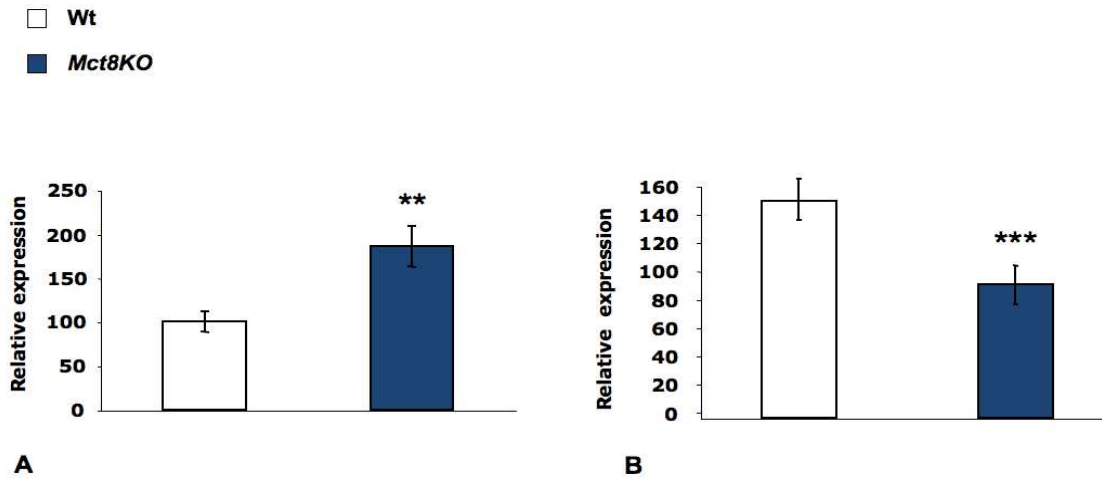


Figure 10. A, *Hairless* mRNA expression in cortex of *Mct8KO* and Wt mice at P0 day; B, *Hairless* mRNA expression in brain of adult *Mct8KO* and Wt mice. ***, $P < 0.001$

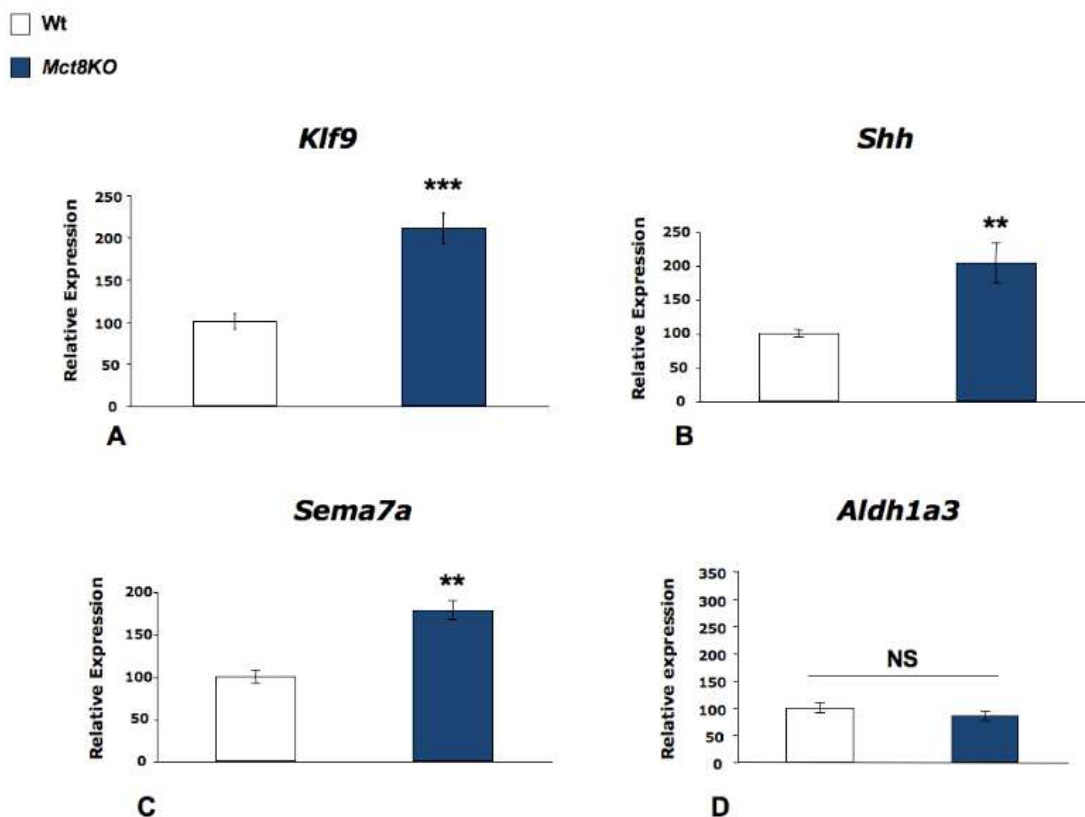


Figure 11. T_3 -dependent gene expression in cortex of *Mct8KO* and Wt mice: A, *Klf9*; B, *Shh*; C, *Sema7a*; D, *Aldh1a3*; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

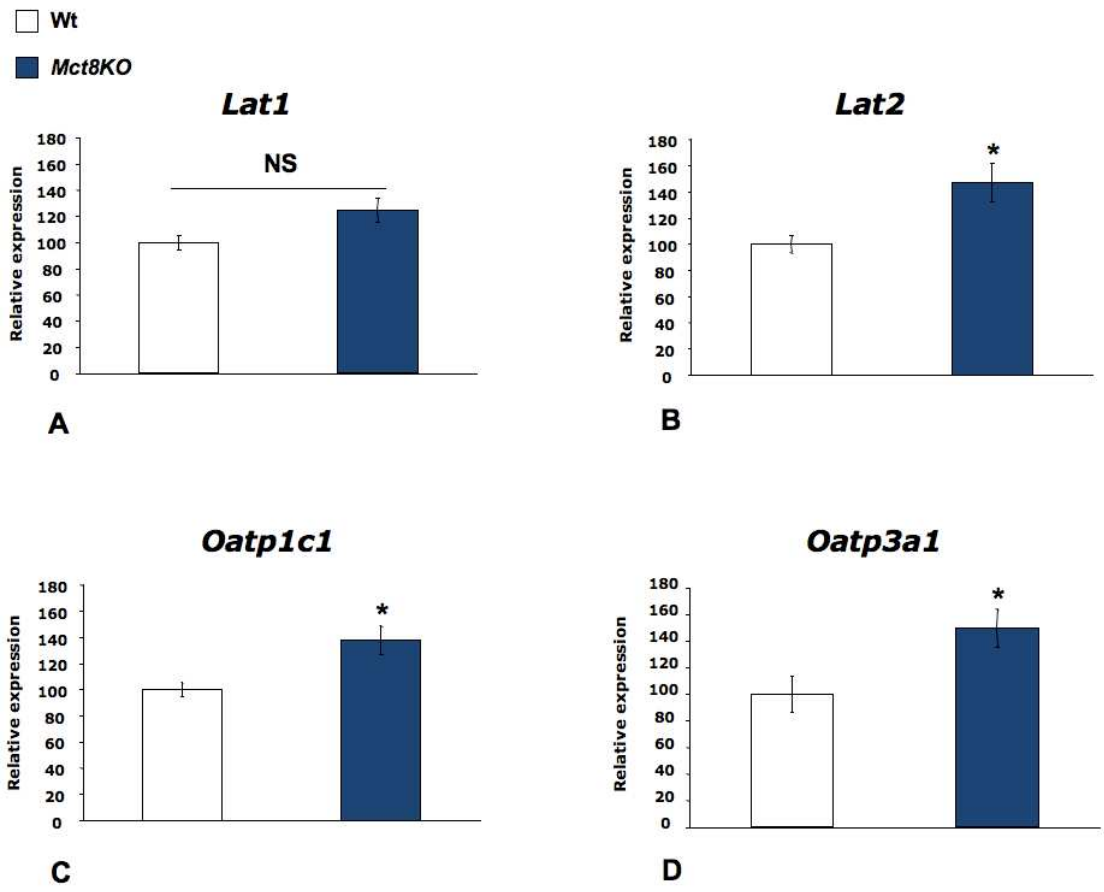


Figure 12. TH transporters mRNA in cortex of *Mct8KO* and Wt mice. A. *Lat1*; B. *Lat2*; C. *Oatp1c1*; D. *Oatp3a1*. *, $P < 0.02$; NS not significant.

Effect on liver.

Because P0-old mice were not perfused, the T₃ content in liver was not measured, as the contribution of blood T₃ is significant in this vascular organ.

No D1 enzymatic activity was identified at P0 (data not shown). *Dio1* and Nuclear receptor subfamily 4, group A, member 1 (*Nr4a1*) mRNA expression is high in *Mct8KO* adult mice compared to Wt mice (**Fig. 13**).

No difference in *Mct10* mRNA expression was seen between *Mct8KO* and Wt mice. Instead, *Lat2* mRNA expression was higher in *Mct8KO* mice (**Fig. 14**).

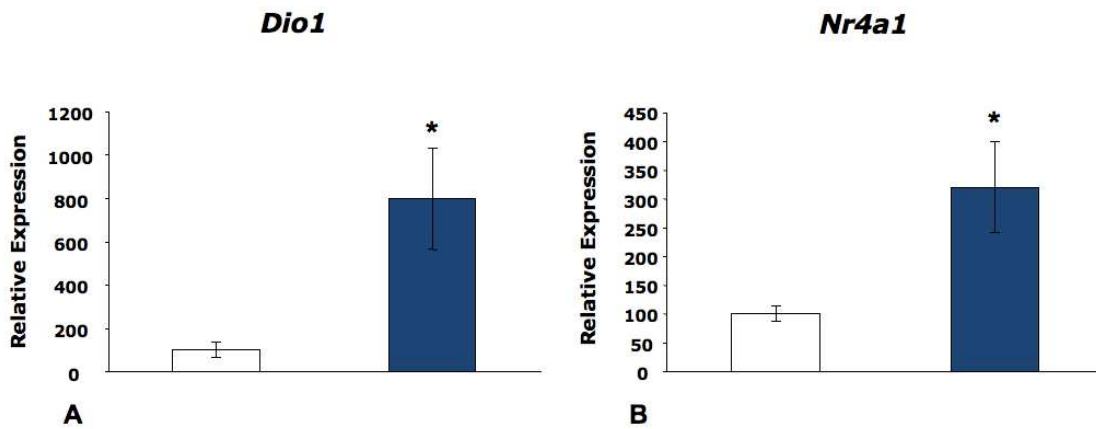
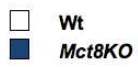


Figure 13. Hepatic gene expression in *Mct8KO* mice and Wt mice. A, *Dio1*; B, *Nr4a1*. * $P < 0,05$

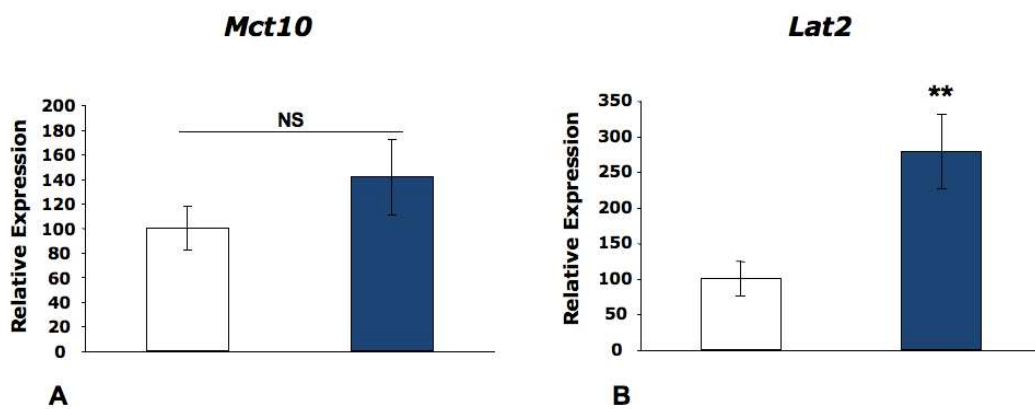
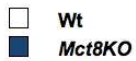


Figure 14. Gene expression of hepatic transporters in *Mct8KO* mice and Wt mice at P0. A, *Mct10* expression; B, *Lat2* expression. **, $P < 0,01$; NS, not significant.

Effects of L-T₄ treatment on serum TSH and T₄ levels

The suppression of maternal endogenous TH production during the last 9 days of pregnancy (from day E10 of pregnancy) with Lol diet and water containing MMI/CIO₄ (subsequently abridged as MMI), increased the serum TSH levels to the same extent in both *Mct8KO* and Wt newborns (**Fig. 15**) while serum T₄ dropped to levels less than 0.1 µg/dl in both genotypes (**Fig. 16**). The injection to pregnant mice of L-T₄ (2µg/100 mg body weight), from day E12 to delivery together with MMI treatment, did not suppress TSH levels in *Mct8KO* newborns (**Fig. 15**) despite the high levels of T₄ (**Fig. 16**). In Wt mice, the L-T₄ dose was not sufficient to bring the serum T₄ levels to those of untreated Wt mice (**Fig. 16**) and, consequently, TSH was not suppressed, though lower than that of *Mct8KO* mice (**Fig. 15**).

All results are summarized in **table 5**.

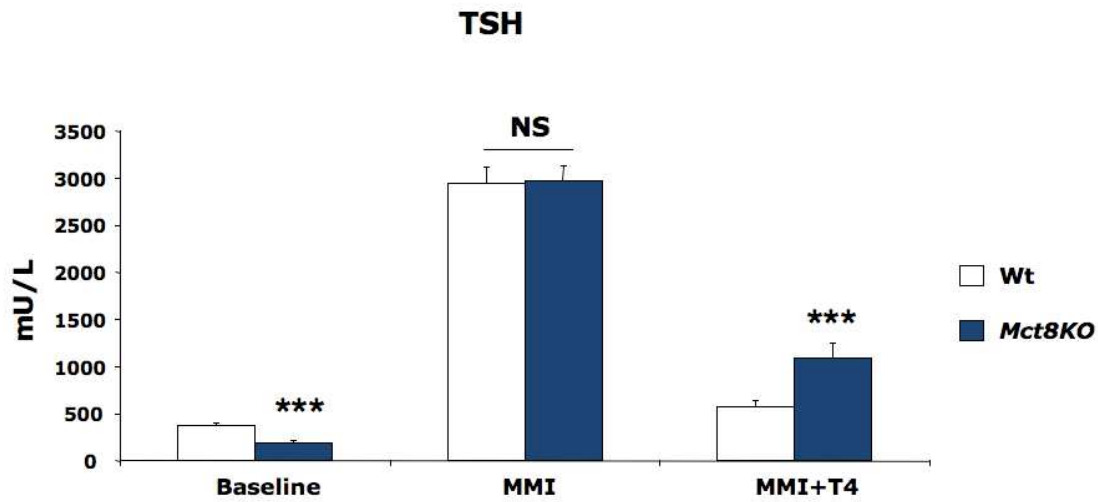


Figure 15. TSH serum levels in *Mct8KO* and Wt mice at P0. Values are at baseline, after MMI treatment, and after injection of 2 μ g/100 mg body weight of L-T₄ in mother treated with MMI/NaClO₄; ****P* < 0.001; NS, not significant.

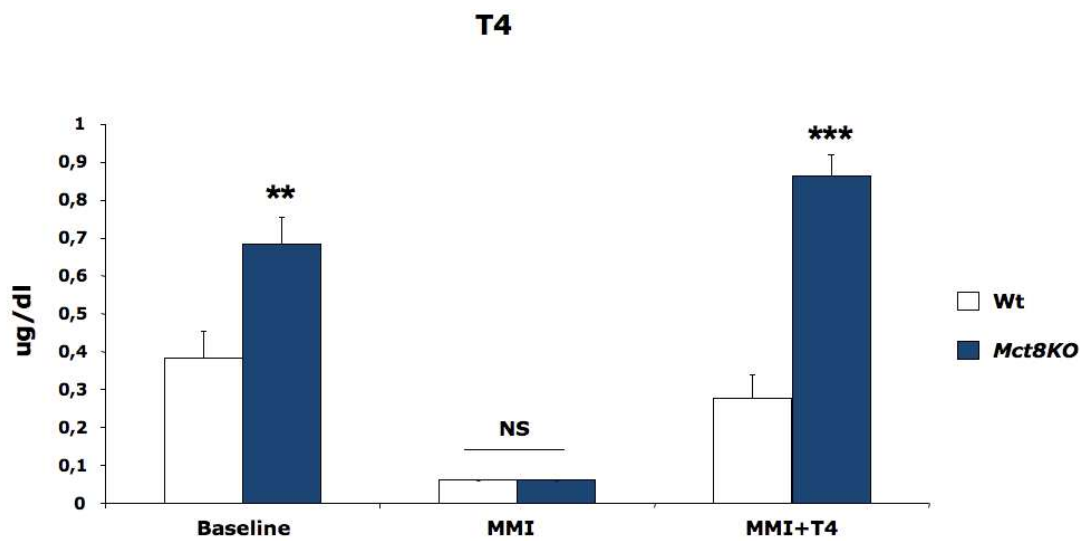


Figure 16. T₄ serum levels in *Mct8KO* and Wt mice at P0. Values are at baseline, after MMI treatment, and after injection of 2 μ g/100 g body weight of L-T₄ to mothers treated with MMI/NaClO₄; **, *P* < 0.01; ****P* < 0.001; NS, not significant.

Table 5. Comparison of *Mct8KO* to Wt mice as neonate and adult

		<i>Mct8KO</i> versus Wt	
		P0	Adult
TH levels			
<i>Serum</i>	T ₄	↑	↓
	T ₃	↔	↑
	rT ₃	↓	↓
	TSH	↓	↑
<i>Tissue</i>	Cortex T ₃	↔	↓ (B)
	Liver T ₃	N.K.	↑
TH action			
<i>Cortex</i>	D2 activity	↔	↑ (B)
	D2 mRNA	↔	↑ (B)
	D3 mRNA	↔	↓ (B)
	Hr	↑	↓ (B)
	Shh	↑	N.K.
	Sema7a	↑	N.K.
	Klf9	↑	N.K.
<i>Liver</i>	D1 mRNA	↑	↑↑
	Nr4a1	↑	N.K.
Transporters			
<i>Cortex</i>	Lat1	↔	N.K.
	Lat2	↑	N.K.
	Oatp1c1	↑	N.K.
	Oatp3a1	↑	N.K.
<i>Liver</i>	Mct10	↔	N.K.
	Lat2	↑	N.K.

↑, ↔, ↓: higher, similar; lower than Wt, respectively. B, brain; N.K. not known.

DISCUSSION

The aim of this study was to gain further insight into the pathophysiology of the complex phenotype of *Mct8* deficiency, in particular to understand if the thyroid status of *Mct8KO* mice changes during life. To this purpose, we studied thyroid status and TH action during the early postnatal days of *Mct8KO* mice, with major emphasis given to P0-old day *Mct8KO* mice compared to *Wt* mice of exactly the same age (littermates). The main findings are summarized in **Table 5**.

Findings

Important findings of these studies are the dynamic changes in thyroid status of *Mct8KO* mice during postnatal life, both in terms of circulating hormone levels and in terms of TH action at tissue level, as it was demonstrated for cortex.

Serum

At P0, TSH levels were lower in *Mct8KO* than in *Wt* mice, they were similar from P3 to P11, but by P14 the TSH levels became significantly higher. T_4 levels were significantly higher in *Mct8KO* than in *Wt* at birth, similar to the *Wt* mice between P3 and P10, and lower from P11 and throughout adulthood. Finally, serum T_3 levels in *Mct8KO* mice were similar to those of *Wt* animals from birth to P14. However, they increased subsequently and remained significantly higher than those in *Wt* mice. rT_3 levels were lower than those in *Wt* mice at birth and remained so throughout life (**Table 5; Fig 7**). It is evident

that there are important differences in TFTs between P0-day old and adult *Mct8KO* mice (**Table 5**). The most important and unexpected finding is the hyperthyroxinemia that manifests at birth and its origin is a question to be addressed.

Hypotheses

It is unlikely that the high levels of T_4 are originated from the newborn thyroid gland. It is known that mouse thyroid starts the secretion of TH around birth (after E16.5) (123). Indeed, at birth T_4 levels in Wt mice are at the lowest levels, the maximum level being at P14 (**Fig. 7**). Moreover, since *Mct8* is also responsible of TH secretion from thyroid gland (57, 86) and since it is known that *Mct8* deficiency in the thyroid appears to interfere with TH efflux, one would not expect significant T_4 secretion from thyroid of *Mct8KO* newborn mice and certainly not in excess of that in Wt. Finally, it has been reported an enhanced uptake of T_4 into the kidneys of *Mct8KO* mice that may represent one possible pathway by which T_4 decreases in adult *Mct8KO* mice (85). It may be that at birth the renal filtration of T_4 could be not so efficient thus resulting in high levels of T_4 or it is possible that the high levels of T_4 in *Mct8KO* newborn mice override the loss of TH in the urinary tract. The latter phenomenon has been described in *Mct8KO* adult mice (85). These possible mechanisms are unlikely to play a role in the observed high serum T_4 levels in newborn *Mct8KO* mice. Indeed, when pregnant dams of mother/embryo units deprived of TH were injected with L- T_4 , we observed again that serum T_4 in *Mct8KO* mice at birth were higher than those in Wt newborn (**Fig. 15**). This observation establishes the maternal origin

of T_4 at P0. Interestingly, TSH was not suppressed in those mice probably because of the variation in serum T_4 levels caused by the intermittent effect of L- T_4 injections on local D2 activity in the hypothalamus. The fact that the same dose of L- T_4 had different effects on the two genotypes raises the possibility that the placenta may handle differently the delivery of TH to *Mct8KO* and Wt littermates. Further studies assessing the expression of TH transporters and the D3 activity in placenta could clarify the contribution of placenta to the TH economy in the *Mct8KO* mice at birth.

It is not straightforward to understand the serum finding of high T_4 , normal T_3 and low rT_3 in *Mct8KO* mice and their dynamic changes with age. Serum and tissue TH levels are controlled at various levels: synthesis, secretion, transmembrane transport, and metabolism. In this respect, the ontogenic profile of the deiodinases may play an important role by modulating TH-bioavailability, since deiodinases are expressed and active in a time- and region-specific manner. It is known that the main sources of plasma T_3 is T_4 , through deiodination by D1 and D2 (5). In rodents, D1 activity in liver, and D2 activity in brain are present at very low level by the last days of gestation and increase successively during the neonatal period (5). In newborn *Mct8KO* mice, D1 and D2 activities are not significantly different than that in Wt mice and both are lower than those in adult mice (Arturo Hernandez personal communication). Therefore, the normal T_3 concentration in the *Mct8KO* mice could be explained by the higher T_4 concentration that permits normal T_3 production in the

presence of reduced fractional conversion of T_4 to T_3 . Another possibility is that normal T_3 levels are due to T_3 transport from the mother through the placenta.

rT_3 levels remain low in *Mct8* deficient mice throughout life, being low even at birth when T_4 levels are actually high in *Mct8KO* mice. rT_3 is the product of 5-deiodination by D3 and it is the preferred substrate for metabolism by D1. As liver D1 activity in P0-day old *Mct8KO* mice is low and similar to that of Wt littermates the role it plays in these low rT_3 levels at birth is uncertain. However, D3 is highly expressed in the placenta, uterus and in the fetal brain. In adult, the decreased access of T_4 to D3 expressing tissues in the context of *Mct8* deficiency likely contributes to the decreased generation of rT_3 . The low levels of rT_3 at birth might be due in part to the same mechanism. Another possibility is a potential increased excretion of rT_3 through kidneys and/or intestine.

Brain T_3 content and TH action

As a result of the high circulating serum levels of T_4 and normal levels of T_3 , the T_3 concentration in cortex was not low in newborn *Mct8* deficient mice, opposite to that in adult mice (**Table 5**). Moreover, an unexpected finding is the apparent dissociation between T_3 content and T_3 action in *Mct8KO* mice cortex. Thus, whereas the brain T_3 content was similar to that in Wt mice, the expression levels of TH-responsive neuronal genes studied were increased, suggesting T_3 excess, compared to Wt mice. However, there are some caveats to be considered: 1) as only the expression levels of four TH-responsive genes were determined, it cannot be assumed that others would be similarly affected;

2) there is the possibility that the T₃ content may vary within specific regions, as already described (116), affecting cells that respond to local generation of T₃.

Hypothesis

A puzzling question has arisen from the finding of normal cortical T₃ content in *Mct8* deficient mice. Possible compensatory mechanisms are involved, such as increase of D2 activity in specific cortical cell populations providing specific regions with high levels of T₃, even though overall D2 activity was similar in newborn *Mct8KO* mice compared to Wt littermates. In addition, the accumulation of T₃ might be the result of decreased T₃ entry into D3-expressing cells, which in turn leads to decreased T₃ clearance and increased local retention of T₃ with a prolonged effect on TH-dependent gene expression.

Indeed *Hr*, *Shh*, *Sema7a* and *Klf9*, which are positively regulated by T₃, have a relative higher expression in *Mct8KO* than in Wt newborn littermates. Of note the high circulating levels of T₄ might contribute to an increased T₄ content in cortex, especially considering the finding of high *Oatp1c1* expression, a T₄ specific transporter in the cortex of newborn *Mct8KO* mice. Conceivably, in these circumstances, T₄ may bind directly to TH receptors and mitigate some of the effects of *Mct8* deficiency. This assumes that T₄ is more than a prohormone, and indeed an earlier study suggests that T₄ does have intrinsic activity. In cultures of tadpole red blood cells, which do not express either D1 or D2, the T₃ receptor number was increased by T₄ as well as T₃, both at near physiological concentrations (124). In addition, the possibility that the T₄ available in cortex may afford some protection for brain development by means of putative non-

genomic actions cannot be excluded (125, 126). Unfortunately our assay is not sensitive enough for the measure the small amount of T₄ present in brain.

Another mechanism contributing to the normal level of T₃ content in cortex in the setting of Mct8 deficiency, is the compensatory effect of other TH transporters. In the last decade, a number of TH transporters have been identified. Of these, we found high expression of *Lat2* and *Oatp3a1* transporters along with *Oatp1c1* over-expression. *Lat2* was recently described both in microglia and in neurons and it has been postulated to compensate for the lack of Mct8 (116). In vitro experiments have shown that *Lat2* is expressed in astrocytes and transports T₃. Genetic inactivation of *Lat2* is sufficient to reduce T₃ uptake (73). However, adult mice deficient *Lat2*, did not show an abnormal thyroid phenotype nor abnormal expression of T₃ dependent genes (72). Finally, *Oatp3a1* transporter has been recently isolated in rat cortex (62) and kidney (61) and its high expression in *Mct8KO* pups might further compensate for Mct8 deficiency.

Hepatic TH action

Intriguingly, like the adult mice, *Mct8KO* newborn mice show a hyperthyroid state in liver as indicated by the high expression of the TH-responsive genes *Dio1* and *Nr4a1* (**Table 5**). The true liver T₃ content could not be measured, as newborn pups were not perfused resulting in a significant contribution of serum T₃ in this vascular organ.

Hypothesis.

Similar to adult mice (90), in newborn mice multiple TH transporters expressed in liver could allow entry of serum T₃ producing hepatic thyrotoxicosis. Indeed *Lat2* transporter expression at P0 is higher in *Mct8KO* than in Wt mice (**Table 5**).

Implications for Mct8 deficient mice

The unexpected TFTs in *Mct8KO* newborn mice could contribute to the apparent normal development of the *Mct8KO* mice and the lack of gross neurological phenotype. The present work demonstrates that the TH-dependent genes *Klf9*, *Sema7a*, *Shh* and *Hr* have increased expression in cortex of *Mct8KO* newborns. It could be that the over-expression of those genes represents a compensatory mechanism for the brain of *Mct8KO* mice to develop normally. Indeed, it has been shown that the above-mentioned genes are involved in the maturation of the developing brain (127-133) and that their altered expression is detrimental for both the developing and mature brain (130-135). It is also possible that the normal T₃ levels in *Mct8KO* newborn cortex allow for the expression of specific genes that are transcriptionally responsive to TH only during select developmental time windows (136, 137), and their expression is fundamental for brain development.

The finding of neonatal hyperthyroxinemia in *Mct8KO* mice is most surprising and it is important to understand whether and for how long *Mct8KO* mice are hyperthyroxinemic during the fetal life, since it is documented that

prolonged fetal hypothyroidism and hyperthyroidism affect survival and development. Indeed, rats born to mothers rendered hypothyroxinemic during pregnancy by low-iodine diet have selective defects in prenatal neurodevelopmental events, such as migration during histogenesis and the cytoarchitectural organization of the cerebral cortex, showing that any situation resulting in a decreased availability of T₄ to the fetal brain has adverse potential for neurodevelopment (138).

For monitoring TH effects during the early postnatal neurodevelopment the *Pax8*KO mouse represents a suitable animal model (139). *Pax8* is an essential transcription factor for the development of thyroid structure and the expression of thyroid-specific genes. *Pax8*KO mice are, at birth, athyreotic and THs can be detected neither in tissues nor in the serum. *Pax8*KO mice are strongly retarded, deaf and exhibit an ataxic phenotype indicating that particularly the maturation of the cerebellum is impaired. However, since *in utero* *Pax8*KO mice are provided mostly with maternal TH, this animal model is not suited to assess the consequences of TH deficiency for prenatal neurodevelopmental events (140).

On the other hand, D3 deficient mice (D3KO) are a suitable model for assessing the consequences of prolonged fetal hyperthyroidism. Since *Dio3* is expressed from very early stages of embryonic life, its deficiency is associated with exposure of the fetus to high TH levels. Both male and female D3KO mice show impaired fertility, significant perinatal mortality, and growth impairment (31). In addition, the developmental programming of the hypothalamus-pituitary-

thyroid (HPT) axis is perturbed in the D3KO mice, presumably due to the overexposure of the animal to excessive levels of TH *in utero* and during the first weeks of perinatal life. Intriguingly, the abnormalities of the HPT in D3KO mice resemble those observed in children born to mothers affected by hyperthyroidism during pregnancy (32, 33).

In light of these two examples and considering that adult *Mct8KO* mice develop apparently normal and are fertile, it suggests that during fetal life *Mct8KO* mice are not profoundly hypothyroid nor are they exposed to high TH levels. Additional studies to evaluate the thyroid status in *Mct8KO* fetal life are required to address this point.

Implication for human MCT8

Finally, how do these findings modify our understanding of the pathophysiology of MCT8 deficiency in humans? The most striking difference between the patients with MCT8 deficiency and the mouse model is the absence of a gross neurological phenotype in mice, even though subtle behavioral changes have been recently reported in (116). There might be several explanations to account for this difference: 1) in humans, MCT8 may transport other, yet unidentified, substances, or 2) the degree of TH deficiency in mouse is less severe than that in humans, with the *Mct8* deficiency being compensated by other TH transporters, *i.e.* *Oatp1c1* and *Lat2* which are both expressed in mouse brain, at higher levels than in humans (51, 116), finally 3) it is possible that mouse brain may not represent a good model for the human

brain. Human neurons might be much more sensitive to minor deviations from normal T₃ supply than mouse neurons. Higher cortical functions like speech are simply not present in mice and cannot be studied in mouse models.

When comparing TH action in human and rodent developing brains, one has to keep in mind that the mouse brain at birth approximates the developing human brain at 4 months of gestation whereas the human brain at birth exhibits a stage of differentiation that is similar to a mouse brain at postnatal day P10 (a detailed comparison of the developmental timing between rodents and humans is displayed at <http://www.translatingtime.net>). Therefore, the timing at which *Mct8KO* mice show hyperthyroxinemia corresponds still to gestation in humans, a time when TH is required for important brain developmental events, such as cell migration, layer formation, neuronal and glial cell differentiation and synaptogenesis (141).

Considering the findings from *Mct8KO* newborns reported herein, there are two possibilities: 1) patients with MCT8 deficiency do not manifest at all the hyperthyroxinemia and the resulting increase in cortical TH action shown by *Mct8KO* newborns and, instead, have a relative local hypothyroid state. This hypothesis could be supported by the fact that the clinical presentation of AHDS is similar to that caused by gestational maternal hypothyroidism, due to severe iodine deficiency and characterized by mental retardation, deaf-mutism, spastic diplegia, and normal stature (142); 2) it is possible that patients with MCT8 deficiency have a similar stage of hyperthyroxinemia at some point during the intrauterine life, corresponding to the newborn mice. Of note, similarly to the

developmental damage caused by fetal hypothyroidism, fetal hyperthyroidism has severe consequences on intrauterine development. Indeed, thyrotoxic fetuses may develop goiter, tachycardia, hydrops fetalis associated with heart failure, growth retardation, craniosynostosis, increased fetal motility and accelerated bone maturation (143). Moreover, it has been reported the association between neonatal hyperthyroidism and mental retardation (144), delayed cerebral development manifesting as ventriculomegaly, increased space in the interhemispheric fissure, and an exaggerated gyral pattern on cranial computed tomographic scans (145) and between fetal hyperthyroidism and paroxysmal dyskinesia (146-149). As hyperthyroid symptoms such as failure to thrive, cranyosynostosis and paroxysmal dyskinesia have been reported in MCT8 deficient patients (92, 96), one can speculate that MCT8 deficient fetuses could have a local brain hyperthyroidism and that this hyperthyroid status is detrimental for the brain development. The possibility that a hyperthyroid status could in part be the cause for the neurological damage in MCT8 deficient patients has been recently hypothesized (103). In this report, two patients showed high intracellular T_3 content, fact that made the authors conclude that, in MCT8 deficiency, a cell thyrotoxic state could be responsible of the neurological phenotype; 3) since the clinical presentation of AHDS is a mixture of fetal hyper- and hypothyroidism, it is conceivable that in patients with AHDS the neurological failure is due to a double-phase event: a hyperthyroid state during the early fetal life, similar to the hyperthyroxinemia seen in *Mct8KO* newborns, followed by a local hypothyroidism from the late fetal life to postnatal

life. The latter condition is confirmed by the fact that at birth, some MCT8 deficient patients present with low T₄, as showed at neonatal screening for congenital hypothyroidism (102, 103, 150)

For a complete understanding of the pathophysiology of MCT8 deficiency in humans It would be important to know what is the thyroid status of MCT8 patients during fetal life. A comparison with our finding in *Mct8KO* mice at P0 might be helpful in understanding the mechanisms that lead to brain defects and to work out a possible therapeutic intervention. So far the only study in this direction has monitored *MCT8* heterozygous women during pregnancy (151). In this study, the authors suggested that in heterozygous *MCT8* women TFTs should be monitored closely from the 1st month of pregnancy to delivery for requirement of L-T₄ therapy to prevent fetal and neonatal hypothyroidism.

CONCLUSIONS

These present studies give challenging insights into how the thyroid status and TH actions modify the evolution of the phenotype of *Mct8* deficient mice. They provide evidence for dynamic changes throughout their early life. Indeed, an initial hyperthyroxinemia at birth, with low TSH and rT_3 and normal T_3 levels, is followed, in the first few days of life, by a pattern of TFTs similar to that of *Wt* mice with the exception of rT_3 , which continues to remain low. Finally the pathognomonic TFTs of *Mct8* deficiency characterized by high T_3 , low T_4 and rT_3 , and slightly elevated TSH serum levels appear before weaning and persist throughout adulthood. Cortical expression of TH-dependent genes reflects these modifications.

Far from being completely understood, the effect of the initial hyperthyroxinemia might be protective for the developing brain against the known effects of *Mct8* deficiency. Indeed by P7, when the brain is completely structured (152-163), serum TH levels in *Mct8KO* mice are still normal.

Considering that *Mct8KO* mice replicate the thyroid phenotype observed in humans with MCT8 deficiency, the results presented in the present work should spur the investigation of early, fetal, thyroid status of AHDS patients, being the most critical time of intrauterine development for MCT8 deficiency to exert its detrimental effects. The findings might open new therapeutic possibilities for MCT8 deficient patients, as an early treatment could be beneficial with respect to “the sooner, the better” motto. Encouraging data come from the beneficial effects of TH analogs in adult *Mct8KO* mice (164).

ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my Italian advisor Prof. Paolo E. Macchia for the continuous support of my study and research from medical school to Ph.D. program, for his patience, advice and guidance.

I am heartily thankful to my American supervisor, Dr. Samuel Refetoff, whose encouragement, supervision and support from the preliminary to the concluding level enabled me to develop my thesis. His guidance and immense knowledge helped me in all the time of research and writing of this thesis.

I also would like to make a special reference to Dr Alexandra M. Dumitrescu for the suggestions and help she gave me about my thesis.

I thank my labmates at The University of Chicago: Xiao-Hui Liao and Pilar Gil for the help they gave me in the planning and execution of the experiments and for the stimulating discussions.

Last but not the least, I would like to thank my family for supporting me spiritually throughout my life and Zora for bearing with me.

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Appendix: original publications

Attached manuscript #2

A somatic gain-of-function mutation in the thyrotropin receptor gene producing a toxic adenoma in an infant.

Kohn B, Grasberger H, Lam LL, Ferrara AM, Refetoff S
Thyroid. 2009 Feb;19(2):187-91

A Somatic Gain-of-Function Mutation in the Thyrotropin Receptor Gene Producing a Toxic Adenoma in an Infant

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Background: Activating mutations of the thyroid stimulating hormone receptor gene (*TSHR*) are rare in the neonate and in the pediatric population. They are usually present in the germline, and are either inherited or occur *de novo*. Somatic mutations in *TSHR* are unusual in the pediatric population.

Methods: We describe a nine-month-old infant with thyrotoxicosis who harbored an activating somatic mutation in *TSHR* that was not present in the germline.

Results: As genomic DNA analysis failed to show a *TSHR* gene mutation, a radioiodide scan was performed to reveal a unilateral localization of uptake suppressing the remaining thyroid tissue. Genomic and complementary DNA analyses of the active thyroid tissue, removed surgically, identified a missense mutation (D633Y) located in the sixth transmembrane domain of the *TSHR*. The absence of this *TSHR* mutation in circulating mononuclear cells and in unaffected thyroid tissue confirmed the somatic nature of this genetic alteration.

Conclusions: To the authors' knowledge, this is the youngest patient to receive definitive treatment for hyperthyroidism due to an activating mutation of *TSHR*.

Introduction

ACTIVATING MUTATIONS in the thyrotropin receptor gene (*TSHR*) have been shown to be a major cause of non-autoimmune hyperthyroidism and hyperfunctioning thyroid adenomas of the adult (1). The reported prevalence of *TSHR* mutations in toxic adenomas varies widely (reviewed by Gozu *et al.* [2]) but may be as high as 80% of patients with toxic nodules (3,4). Since the first description in 1993 of a *TSHR* mutation in a hyperfunctioning thyroid adenoma (5), approximately 50 different activating *TSHR* mutations have been reported (6) (see <http://gris.ulb.ac.be/>). Of these, more than one half are somatic mutations (7). Many are located within the sixth transmembrane domain and the third intracellular loop of the *TSHR* where the receptor interacts with G proteins. In contrast, the presence of a hyperfunctioning somatic mutation of *TSHR* is highly unusual in the neonate and infant and has been so far reported in a single case (8).

Patients and Methods

Case report and subjects

The patient was a boy born at 37 weeks, by cesarean section, to non-consanguineous African American parents. The

mother, a non-smoker and on no medications, followed a regular diet and had an uneventful pregnancy. Delivery was complicated by meconium with a transient decrease in fetal heart rate. Birth weight was 7 pounds 6 ounces (3.35 kg) and length 21 inches (53.3 cm). The infant was nursed with adequate weight gain until 3 months of age, at which time he developed eczema and recurrent episodes of wheezing. At 6 months, a pediatrician evaluated the patient for emesis, feeding difficulties, and a decrease in weight. Further evaluation by a gastroenterologist and an allergist lead to the institution of food supplementation in the form of a hypercaloric diet. This produced a catch-up weight gain by 9 months of age. Thyroid function tests performed at that visit revealed a TSH of <0.01 mU/L (normal range: 0.70–6.40 mU/L) and a free thyroxine (T₄) level of 5.1 ng/dL (normal range: 0.8–2.2). The patient was referred to the endocrine service for further evaluation. Family history is notable for autoimmune thyroid disease in the paternal grandmother who is on L-thyroxine. The parents and two paternal half-siblings are healthy.

Physical examination at 9 months revealed a head circumference of 48.5 cm (above the 95th percentile) with anterior fontanel barely palpable, length 75 cm (95th percentile), weight 8.0 kg (10th percentile). The patient was hyperkinetic

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with a pulse of 100 beats per minute and blood pressure of 90/60 mm Hg. Skin showed diffuse eczematous lesions. Eyes showed lid retraction and stare but full extraocular movements. The thyroid gland was not enlarged and no palpable masses were appreciated. The remainder of the exam was unremarkable with appropriate for age neurological development. TSH was <0.01 mU/L; free T_4 , 3.8–5.1 ng/dL; and total triiodothyronine (T_3), 377–609 ng/dL, on several determinations; and total T_4 , 17.0 μ g/dL. Thyroid stimulating IgG was 82% (normal 0–129%), and thyroperoxidase (TPO) and thyroglobulin (Tg) antibodies were negative. Bone age was 4 years at a chronologic age of 9 months. Skeletal survey showed increased ossification of the proximal and distal femoral and proximal tibial ossification centers for age but there was no craniosynostosis. There was normal sinus rhythm on electrocardiogram and normal pulmonary arterial pressures by echocardiography with normal left ventricular size and function. Thyroid ultrasound with color Doppler showed a focal, $3.1 \times 1.5 \times 1.6$ cm, hypoechoic and hypervascular area replacing the right lobe with normally appearing glandular tissue in the upper pole. The left lobe was normal in size and echotexture, measuring $1.7 \times 0.6 \times 0.7$ cm. Thyroidal ^{123}I scan revealed markedly increased uptake within the right lobe with suppression of the remainder of the gland (Fig. 1A).

The patient was referred for partial thyroidectomy with removal of the hyperfunctioning nodule. Thionamide therapy and supersaturated potassium iodide (SSKI) were instituted in preparation for surgery. At surgery, the right lobe of the thyroid gland appeared to be larger than the left and more vascular. The left lobe appeared normal. A right thyroid lobectomy was performed. Several small biopsies were taken from the contralateral lobe for histology and *TSHR* sequencing. Histological examination showed thyroid hyperplasia demarcated from the surrounding normal thyroid tissue by a thin rim of fibrous tissue (Fig. 1B and 1C). Subsequent to the surgery, the patient remains clinically and biochemically euthyroid on treatment with 37.5 μ g daily levothyroxine (LT_4). Head circumference, growth, and development are normal.

Studies were approved by the Institutional Review Board and informed consents were obtained to perform thyroid and

genetic evaluations of the patient and all available immediate family members.

Thyroid function tests

Total T_4 and T_3 were measured using commercial automated chemiluminescent immunometric methods and TSH by a third generation chemiluminescence assay (Elecsys 2010, Roche, Indianapolis, IN). 3,3',5'-L-triiodothyronine, or reverse T_3 (rT_3), was measured by radioimmunoassay (Adaltis, Italy) and serum Tg by an in-house assay as previously reported (9). The free T_4 index (FT_4I) was calculated as the product of the serum total T_4 and the normalized resin T_4 uptake ratio. TPO and T_G antibodies were measured by passive hemagglutination (Fujirebio, Inc., Tokyo, Japan).

DNA and RNA isolation, amplification, and sequencing

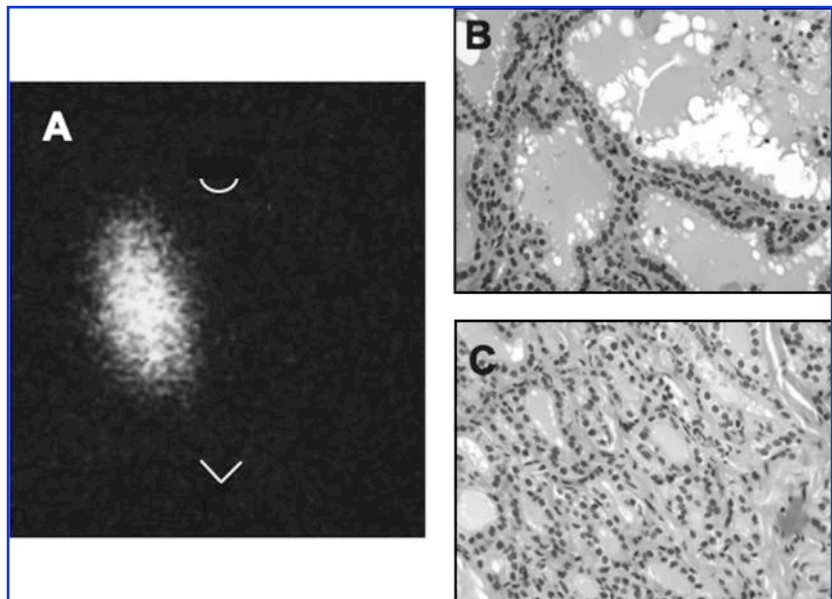
Genomic DNA was extracted from circulating mononuclear cells and all *TSHR* exons were amplified by the polymerase chain reaction (PCR) as described (10), and sequenced. PCR conditions will be provided upon request.

Thyroid tissue samples (hyperplastic and normal) excised at surgery were placed in a monophasic solution of phenol and guanidine isothiocyanate (TRIZOL[®] Reagent, Invitrogen Life Technologies, Carlsbad, CA) and shipped by FedEx from New York City to Chicago. Total RNA was extracted and the first strand cDNA was synthesized using the SuperScript[™] III First-Strand Synthesis System for reverse transcriptase (RT)-PCR protocol (Invitrogen Life Technologies). *TSHR* cDNA was then amplified by PCR using specific primer pairs (11) and sequenced.

Results

Thyroid function tests confirmed the diagnosis of hyperthyroidism in the proband. In addition to high T_4 and T_3 concentrations, rT_3 level was also high. The endogenous source of iodothyronines, accompanied by suppressed TSH, was confirmed by the presence of high serum Tg (III-1, Fig. 2). Thyroid function tests of the parents and paternal grand-

FIG. 1. Anatomical and histological findings. (A) Radioiodide scintillation scan showing increased uptake in the upper pole of the right lobe of the thyroid with suppression of the remainder of the gland. The locations of the chin (∨) and suprasternal notch (∨) are indicated. (B) Photomicrograph of affected thyroid tissue from the right lobe, showing hyperplasia. Epithelial cells lining the colloid are columnar with acidophilic cytoplasm and hyperchromatic basally located nuclei. The colloid is pale with prominent scalloping where it abuts the epithelium. (C) Photomicrograph of a biopsy from the left lobe of the thyroid gland, showing smaller follicles of fairly uniform size. The lining epithelium is cuboidal with pale acidophilic cytoplasm and uniform nuclei. The colloid is deeply eosinophilic.



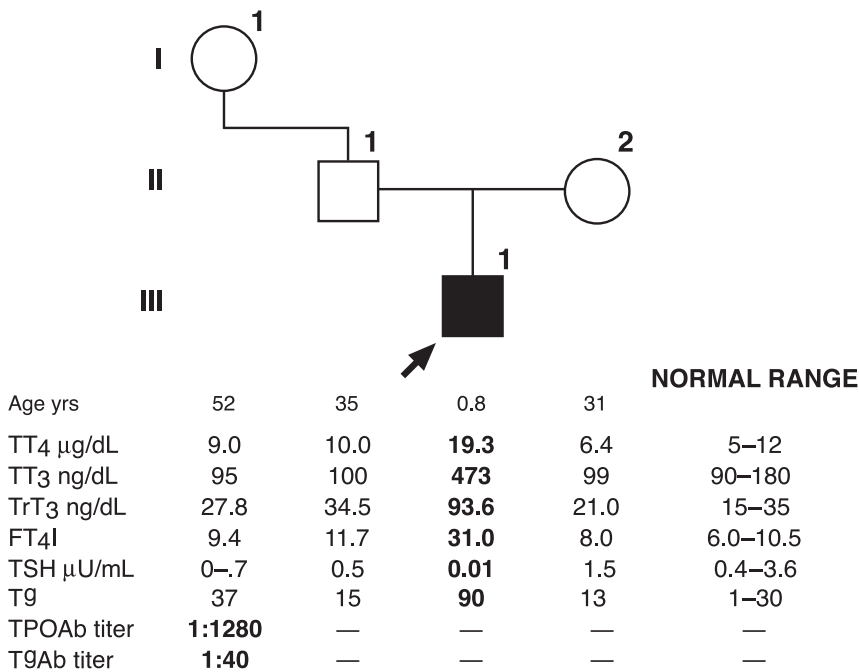


FIG. 2. Family pedigree and results of thyroid function tests. Results are aligned with the individual's symbol and abnormal values are shown in bold numbers. Note that the proband (arrow) has increased serum concentration of all three iodothyronines and thyroglobulin but a suppressed thyrotropin. TT₄, total thyroxine; TT₃, total triiodothyronine; TrT₃, total 3,3',5'-L-triiodothyronine; FT₄I, free T₄ index; FT₄, free thyroxine; TSH, thyrotropin; Tg, thyroglobulin; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody.

mother were within the range of normal, except for the presence of TPO and Tg antibodies in the latter (I-1, Fig. 2), confirming the diagnosis of autoimmune thyroid disease for which she was taking LT₄.

No mutation was found in genomic DNA from circulating mononuclear cells, or from genomic DNA or complementary DNA obtained from normal thyroid tissue (left lobe of the thyroid gland). On the other hand, both genomic and complementary DNAs obtained from two samples of the hyperplastic thyroid tissue from the right lobe revealed the same mutation in one of the two alleles of *TSHR*. A point mutation replacing the normal guanosine with thymidine at codon 633 (GAC→TAC) was detected, resulting in the replacement of the normal aspartic acid with a tyrosine (D633Y) located in the sixth transmembrane domain of the *TSHR*.

Discussion

Hyperthyroidism in the neonate and young infant is often described in the context of maternal autoimmune hyperthyroidism, irrespective of whether the mother is toxic during pregnancy. Approximately 1% of children born to women with a history of autoimmune thyrotoxicosis will develop neonatal thyrotoxicosis. Resolution usually occurs by 3–4 months of age, as the immunoglobulin levels of maternal origin fall. Non-autoimmune hyperthyroidism in neonates and infants is rare. In McCune–Albright syndrome, hyperthyroidism is caused by an activating mutation of the gene that encodes the α -subunit of stimulatory G proteins (1).

Constitutive activation of the *TSHR* resulting from germline mutations is responsible for most hereditary (familial) forms of non-autoimmune hyperthyroidism (cases reviewed by Chester *et al.* [12]). First reported in 1982 (13) and confirmed in 1994 (14), 35 families harboring 25 different activating germline mutation have been identified to date (6) (see <http://gris.ulb.ac.be/index.html?home.html>); 23 were familial and 12 sporadic. This autosomal dominant disorder is characterized by high variability in the phenotypic expression of

the disease with intrafamilial heterogeneity in age of onset and severity of disease among several generations (15–19). In these patients, thyrotoxicosis, in the presence or absence of goiter, may first become manifest at any time from birth to adulthood. The patients' course is prolonged with relapses occurring even two decades following subtotal thyroidectomy (19). Epigenetic factors modulating the impact of this heterozygote mutation and differences in iodine intake may alter the phenotypic expression of this inherited disorder.

Family studies were initially performed on our patient with the possibility of an inherited *TSHR* germline mutation. Sequencing of the entire coding region of *TSHR* and intron/exon junctions, using DNA extracted from circulating mononuclear cells, failed to show any abnormality. This finding prompted us to obtain a radioiodide scan, which indicated the presence of a hyperfunctioning nodule in the right thyroid lobe. Upon surgical excision, the lobe showed hyperplasia on histological examination (Fig. 1B). Genomic and complementary DNA analysis of this tissue revealed a missense *TSHR* mutation D633Y located in the sixth transmembrane domain of the *TSHR*. The absence of this *TSHR* mutation in circulating mononuclear cells and in the contralateral thyroid tissue, confirmed the somatic nature of this genetic alteration. An identical mutation has been reported previously in six hyperfunctioning adenomas of adults (20–23). In addition, the same codon has been found to undergo three different mutations producing hyperfunctioning adenomas in adult subjects: six D633E (3,5,20,24–26), two D633H (5,27), and one D633A (5).

The youngest patient with an activating somatic *TSHR* mutation reported to date demonstrated evidence of hyperthyroidism *in utero* as documented by fetal tachycardia at week 35 of gestation (8). However, definite treatment, by surgical removal of the hyperfunctioning thyroid adenoma, followed by molecular diagnosis, did not take place until the age of 2 years. Sequencing of *TSHR* revealed a heterozygous missense mutation, S281I, in the extracellular domain, which was restricted to the adenomatous tissue. Compared to the

wild-type TSHR, the mutant had an eightfold increase in the basal cAMP activity and a more modest increase in the affinity for TSH. This is comparable to 7.4-fold increase in the basal cAMP activity of the mutant TSHR D633Y reported herein (22).

We date the onset of clinically significant thyrotoxicosis in our patient sometime between 3 and 6 months of age. The earliest signs suggestive of thyrotoxicosis, in subsequently proven activating *TSHR* mutations, are in premature infants born at 32 and 33 weeks of fetal life (28–31). However, intrauterine hyperthyroidism, manifesting as fetal tachycardia was observed in only two sporadic cases (8,29). As expression of *TSHR* in humans does not occur prior to the latter half of gestation (32), the presence of an activating *TSHR* mutation would theoretically not become apparent *in utero* prior to this time. Review of the prenatal data for our patient revealed no evidence for fetal tachycardia. Furthermore, blood obtained on the third day for neonatal screening of hypothyroidism had a T_4 of 19 $\mu\text{g}/\text{dL}$ (normal range 10–28 $\mu\text{g}/\text{dL}$) and a TSH <20 mU/L. Nevertheless, growth of the monoclonal cells expressing the mutant TSHR was sufficient to produce excess thyroid hormone before the age of 6 months, suggesting that the mutation in a single cell must have occurred *in utero*.

Acknowledgments

We thank Dr. Cristine Hajdu from the Department of Pathology at New York University Medical Center for the histological analysis of the thyroid tissue and preparation of the photomicrographs. Thanks are also due to Dr. William Spivak from the Department of Pediatrics at Albert Einstein College of Medicine, for referral of the patient. This study was supported in part by grants DK15070 and RR04999 from the National Institutes of Health.

Disclosure Statement

The authors report no conflicts of interest.

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Attached manuscript #3

Mutations in TAZ/WWTR1, a co-activator of NKX2.1 and PAX8 are not a frequent cause of thyroid dysgenesis

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J Endocrinol Invest, 2009 Mar; 32(3): 238-41

Mutations in TAZ/WWTR1, a co-activator of NKX2.1 and PAX8 are not a frequent cause of thyroid dysgenesis

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ABSTRACT. *Aim:* In 80-85% of cases, congenital hypothyroidism is associated with thyroid dysgenesis (TD), but only in a small percentage of cases mutations in thyroid transcription factors (NKX2.1, PAX8, FOXE1, and NKX2.5) have been associated with the disease. Several studies demonstrated that the activity of the transcription factors can be modulated by the interaction with other proteins, such as co-activators and co-repressors, and TAZ (transcriptional co-activator with PDZ-binding motif or WWTR1) is a co-activator interacting with both NKX2.1 and PAX8. In the present study we investigate the role of TAZ in the pathogenesis of TD.

Material and methods: By Single Stranded Conformational Polymorphism, we screened the entire TAZ coding sequence for mutations in 96 patients with TD and in 96 normal controls. *Results:* No mutations were found in patients and controls, but we found several polymorphisms in both groups. No significant differences could be demonstrated in the prevalence of the mutations between patients and controls. *Conclusions:* Our data indicate that TAZ mutations are not a cause of TD in the series of patients studied.

(J. Endocrinol. Invest. 32: 238-241, 2009)

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INTRODUCTION

Primary congenital hypothyroidism (CH) is the most frequent endocrine-metabolic disease in infancy, with an incidence of about 1/3-4000 newborns. In most cases (80-85%), primary permanent CH is caused by alterations in thyroid gland morphogenesis, with a gland that can either be absent (athyreosis), ectopically located and/or severely reduced in size (hypoplasia) (1). All these entities are grouped under the term "thyroid dysgenesis" (TD) (OMIM #218700).

Mutations in thyroid transcription factors have been reported in patients with TD; however a genetic cause of the disease has been demonstrated only in a small percentage of patients with TD (1, 2).

Recently, increasing interest has been expressed in the role of transcriptional co-regulators (co-activators, co-repressors, adaptors) as a new putative cause of human diseases (3) and several diseases have been linked to transcriptional co-activators including insulin resistance, associated to peroxisome proliferator-activated receptor-gamma coactivators (PGC-1 α and PGC-1 β) mutations (4, 5), Rubinstein-Taybi syndrome linked to Creb Binding Protein (CBP) mutations (6), and mutations in Rb1 found in several tumors (7).

TAZ (transcriptional co-activator with a PDZ-binding motif) is a recently described transcriptional co-activator (8). The human gene, also named *WWTR1*, consists in 6 ex-

ons, maps to chromosome 3q24 and encodes for a 400 amino acids protein with a molecular mass of 45 kD. The protein contains a putative 14-3-3 protein-binding motif (Ser) in the highly conserved N-terminus, a central WW domain, and a putative 2-stranded coiled-coil and a PDZ-binding motif in its C terminus. TAZ mRNA is ubiquitously expressed, with the exception of thymus and peripheral blood leukocytes (8). Very recently, Di Palma et al. demonstrated that TAZ is also present in thyroid tissue and in differentiated thyroid cell lines, and that in thyroid cells TAZ is detected primarily in the nuclear compartment (9).

PAX8 and NKX2.1 are required for the expression of thyroid-specific genes, but their mechanism of action as transcription factors remains poorly understood. TAZ acts as a potent co-activator of both PAX8 (9) and NKX2.1 (10), and it may be physically associated with both NKX2.1 and PAX8. Evidence suggests that PAX8 and NKX2.1 are part of a multiprotein complex whose members are still largely unknown (11, 12). In this model TAZ can act as a "bridge" between NKX2.1, and PAX8. Recently, it has been demonstrated that PAX8 biochemically interacts with NKX2.1, and the physical interaction between the two factors leads to a synergistic effect on the transcriptional activation of thyroid-specific gene promoter (13). The presence of TAZ strongly potentiates the synergism of NKX2.1 and PAX8 on the thyroglobulin promoter. In addition, PAX8, NKX2.1 and TAZ are all present in a fraction of about 44 kDa obtained by size exclusion chromatography (9). Finally, Di Palma and coworkers studied the expression of TAZ during thyroid embryogenesis and demonstrated that TAZ temporal distribution perfectly overlaps with that of the thyroglobulin (*Tg*) gene, suggesting that TAZ is one of the potential factors required for thyroglobulin expression and for determination of the thyroid differentiated phenotype.

Key-words: Cofactor, congenital hypothyroidism, thyroid development, thyroid dysgenesis, transcription factor.

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Accepted September 10, 2008.

The aim of the present study was to investigate the role of TAZ in the pathogenesis of TD. To this aim, we have screened for mutations the entire TAZ coding sequence in a group of 96 patients with TD and in 96 normal controls.

MATERIALS AND METHODS

Patient recruitment

Ninety-six patients with permanent CH (30 with athyreosis, 35 with ectopy, 6 with hemiagenesis, 18 with hypoplasia, and 7 with diagnosis of CH without goiter but not further clinically characterized) were included in the study. Ninety-six healthy subjects with no thyroid-related disorders were enrolled as controls. The study was approved by the Ethics Committees of the participating Institutes and the procedures followed were in accordance with the Helsinki Declaration. All individuals, or parents of minors, gave informed consent.

Single stranded conformational polymorphism

The entire TAZ coding region was screened for mutations on genomic DNA extracted from peripheral blood lymphocytes. Oligonucleotide primers (sequences available upon request) were designed based on reported human TAZ cDNA (NM_015472) and genomic sequences. PCR reactions were carried out in 25 μ l reaction volume at the specific annealing temperature, as indicated in Figure 1. Samples were denatured 1:1 in denaturing solution (99% formamide, 1% xylene cyanol solution, 0.1% bromophenol blue) at 95 C for 5 min and thereafter

directly placed on ice to prevent reannealing of the single stranded product. Six μ l of each sample were applied to a non-denaturing gel of acrylamide (GeneGel™ Excel 12.5/24 Kit, GE Healthcare Bio-Sciences AB, Sweden) and run on a GenePhor™ Electrophoresis Unit (GE Healthcare Bio-Sciences AB, Sweden). The running conditions were the following: plate temperature: 15 C, 600 V, 25 mA, 15 W. Following separation, we stained nucleic acids in acrylamide gel using the PlusOne™ DNA Silver Staining Kit (GE Healthcare Bio-Sciences AB, Sweden), following the producer's protocol.

Direct sequencing

PCR products of the samples showing an abnormal single stranded conformational polymorphism (SSCP) migration were purified with Antarctic Phosphatase-Exonuclease I (New England BioLabs, USA) and directly bi-directionally sequenced.

Statistical analysis

Comparison in the prevalence of the observed polymorphisms was performed using the chi-square test.

RESULTS

The SSCP method was used as first approach to screen the TAZ coding sequence for mutations in patients with TD using specific primer-pairs. Figure 1 shows some of the SSCP and the results are summarized in Table 1. Briefly, no mutations were identified, while we found two

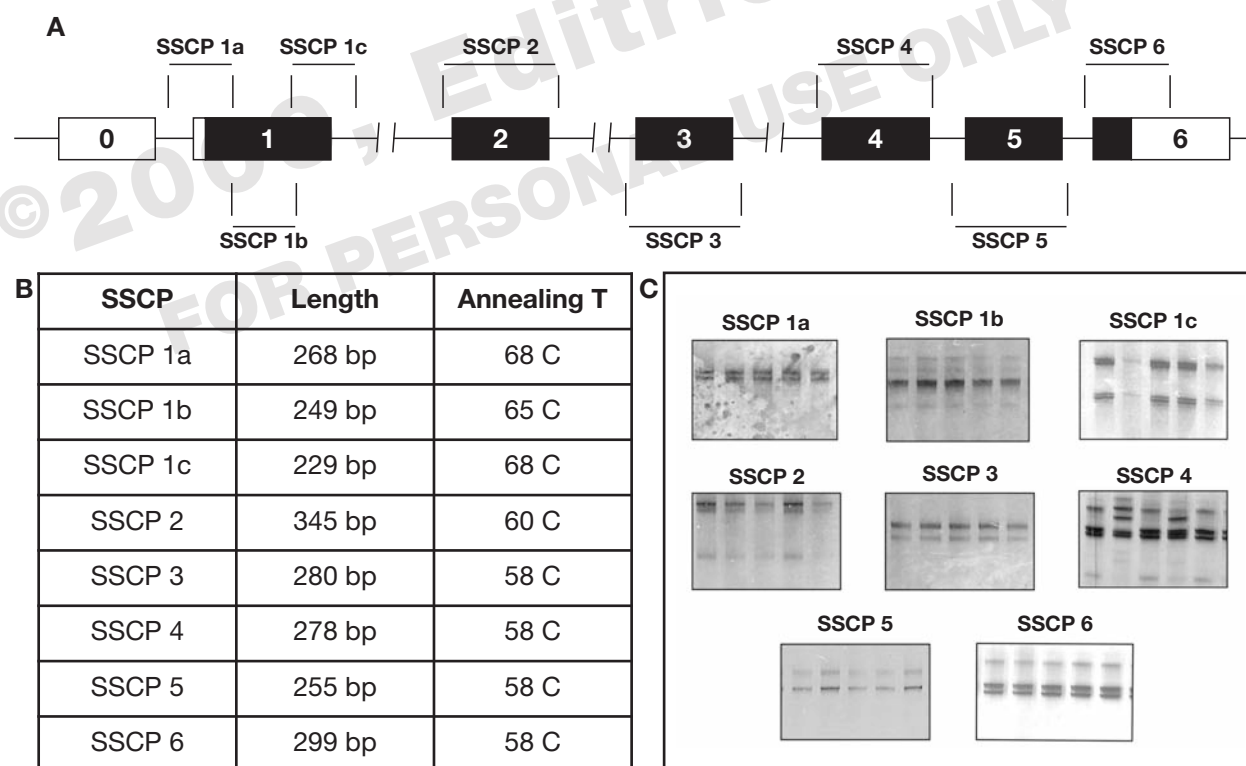


Fig. 1 - Schematic representation of the transcriptional co-activator with a PDZ-binding motif (TAZ) gene (A) showing the relative position of the single stranded conformational polymorphism (SSCP). In the figure the size of PCR with the annealing temperatures (T) used for the reactions (B) and some samples of the SSCP (C) are also reported. The multiple bands in SSCP4 correspond to the T285S polymorphism.

polymorphisms responsible for amino acid change and two silent polymorphisms.

In exon one, the triplet encoding for the proline at position 74 of TAZ (CCG) was changed to CAG, encoding for glutamine. The P74Q change, already reported in the GeneCards database (<http://www.genecards.org/>) was identified in heterozygous state in 8 patients (3 with agenesis, 3 with ectopy, and 2 with hypoplasia) and in 13 controls. Similarly, in exon four the triplet encoding for threonine at position 285 (ACC) was changed to TCC [encoding for amino acid serine (T285S)] in one control in heterozygous state. Moreover, we found a heterozygous AAC to AAT change in exon six in the triplet encoding for asparagine at position 391 in one patient with athyreosis, a CTG to CTT change in the triplet encoding for leucine at position 400 in 18 patients in heterozygous state and in one patient with thyroid ectopy in homozygous state, as well as in 21 controls in heterozygous state and in one in homozygous state.

The chi-square test demonstrates no significant differences in the prevalence of any of the observed polymorphisms between patients and controls.

DISCUSSION

TD accounts for about 80-85% of the cases of congenital hypothyroidism, and mutations in genes involved in thyroid development are responsible for TD in animal models as well as in patients. An important role in the pathogenesis of TD has been demonstrated for thyroid specific transcription factors including NKX2.1, FOXE2, PAX8, and NKX2.5; however mutations have been found only in a small percentage of patients.

Tissue-specific transcriptional regulation is achieved by the combined action of transcription factors, co-regulators and components of the basal transcriptional machinery. TAZ was recently demonstrated to be expressed in the thyroid gland during morphogenesis and to be functionally associated and to directly interact with both NKX2.1 (10) and PAX8 (9). These observations suggest a possible role of TAZ in the pathogenesis of TD.

Very recently, *Taz* knock out mice have been produced (14, 15): mice are viable and about 50% of the pups reach adult age. No data are available on the thyroïdal status of the animals. Interestingly, the knock out mice are smaller compared to wild type (WT). To this regard, it is worth noting that hypothyroid animals grow less than the cor-

responding WT (16). Moreover, in agreement with this hypothesis, the *Taz*^{-/-} mice also have minor skeletal abnormalities. In addition, 35-50% of the pups die by the age of weaning due to unknown causes. A similar phenotype has been observed in *Pax8*^{-/-} mice, in which the injection of thyroid hormone completely rescues the animals (17).

Several experiments have demonstrated that TAZ plays an important role in the human embryogenesis (18). TAZ modulates mesenchymal stem cells differentiation, by stimulating bone development and blocking fat cell differentiation (19), it associates TEF transcription factors in muscle differentiation (20), and it also associates with TBX5, a T-box transcription factor which plays an essential role in cardiac and limb development (21).

TAZ is expressed in type II epithelial cells of the murine fetal lung where it interacts with NKX2.1 to regulate NKX2.1-mediated activation of the gene encoding for the Surfactant Protein-C in respiratory epithelial cells (10). In the thyroid and in thyroid follicular cell lines TAZ is present and physically interacts with NKX2.1 and PAX8, and potentiates the transactivation properties of both factors alone or together. In addition, TAZ expression completely overlaps in temporal terms with thyroglobulin expression, suggesting that this cofactor may be necessary to determine the differentiated thyroid phenotype. All these findings indicate that TAZ is an important co-activator in the thyroid environment and that its combinatorial interactions with thyroid transcription factors are likely to play a relevant role in the regulation of thyroid differentiation. This study was carried out to evaluate whether TAZ/*WWTR1* gene mutations were present in patients with CH and if these mutations could play a role in the pathogenesis of TD. We were not able to demonstrate mutations in the TAZ/*WWTR1* gene in a large group of patients with thyroid dysgenesis. This can be the result of a more generic role of TAZ as co-activator in several organs and tissues. In addition, no data are available on the thyroïdal status of TAZ knock out mice, and it would be very useful to investigate these aspects. In fact, the complex phenotype observed in *Taz*^{-/-} mice could be the direct consequence of the absence of this co-activator or the effect of the hypothyroidism postulated in these animals. Finally, this study does not exclude that TD could be due to mutations in other genes necessary for TAZ activity. Moreover, the present study did not investigate for mutation(s) in the TAZ promoter as well as in intronic regions of the gene.

Table 1 - Distribution and allelic frequencies of the polymorphisms in the transcriptional co-activator with a PDZ-binding motif (TAZ) gene in the studied population (no. of alleles).

		Athyreosis	Thyroid ectopy	Thyroid hypoplasia	Hemiagenesis	CH with no goiter	Normal controls	Allelic frequency in patients	Allelic frequency in controls
CACCC/AGGGG	P74Q	3	3	2	0	0	13	C: 95.83 A: 4.17	C: 93.23 A: 6.77
ATGA/TCCCCA	T285S	0	0	0	0	0	1	A: 100.0 T: 0.0	A: 99.48 T: 0.52
CTGAAC/TAAA	Asn391	1	0	0	0	0	0	C: 99.48 T: 0.52	C: 100.0 T: 0.0
TGGCTG/TTAA	Leu400	7	5	1	1	4	22	G: 90.10 T: 9.89	G: 88.54 T: 11.46

CH: congenital hypopituitarism.

In conclusion, we did not find mutations in the coding region of the TAZ gene in patients with TD. Our results could be explained by the essential role of this factor in embryonic development. In fact, since TAZ is expressed ubiquitously and interacts with different proteins involved in the development of different tissues, its mutations may be responsible for perinatal lethality or for a more severe phenotype, where TD is only a minor problem.

ACKNOWLEDGMENTS

This work was supported by a grant of the Italian Ministry of education, University and Research (PRIN 2007) to PEM.

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Attached manuscript #4

The DREAM protein is associated with thyroid enlargement and nodular development.

Rivas M, Mellström B, Torres B, Cali G, Ferrara AM, Terracciano D, Zannini M, Morreale de Escobar G, Naranjo JR.

Mol Endocrinol. 2009 Jun;23(6):862-70. Epub 2009 Mar 19

The DREAM Protein Is Associated with Thyroid Enlargement and Nodular Development

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G protein-coupled receptors (GPCRs) are involved in the pathophysiology of a wide range of diseases and constitute an attractive therapeutic target. In the thyroid gland, TSH receptor (TSHR), a member of the GPCR family, is a major regulator of thyroid differentiation and function. Alterations in TSHR activity are often involved in the development of pathologies such as thyroid cancer and thyroid enlargement (goiter). Here we show that DREAM (downstream regulatory element antagonist modulator) modulates TSHR activity through a direct protein-protein interaction that promotes coupling between the receptor and $G\alpha_s$. In transgenic mice, DREAM overexpression provokes a marked enlargement of the thyroid gland. Increased levels of DREAM protein were observed in human multinodular goiters, suggesting a novel etiopathogenic mechanism in nodular development in humans. Taken together, these findings identify a mechanism for the control of TSHR activity and provide a new approach for the study and treatment of thyroid pathologies associated with impaired TSHR function. (*Molecular Endocrinology* 23: 862–870, 2009)

G protein-coupled receptors (GPCRs) are membrane proteins located on the cell surface from where they control cell physiology through the activation of various signaling cascades. Impaired function of specific GPCRs is strongly implicated in the pathophysiology of a wide range of conditions, including among others, pain, inflammation, obesity, hypertension, cardiovascular disease, or generalized anxiety disorder (1–7). Because of this and their accessibility to drugs, GPCRs are indisputably an attractive class of therapeutic targets. In the thyroid gland, TSH is the major regulator of thyroid growth and differentiation. TSH binds to the TSH receptor (TSHR) and induces coupling to G proteins (8, 9), of which heterotrimeric G_s protein mediates most of the TSHR signaling and activates a cAMP cascade that controls the expression of key genes in thyroid function (10).

TSHR is associated with many thyroid diseases (11). Inactivating mutations of TSHR are responsible for asymptomatic resistance to TSH and overt congenital hypothyroidism. Acti-

vating mutations of TSHR are found in autonomously functioning thyroid nodules, thyroid hyperplasia, and congenital hyperthyroidism (12–15). Development of TSHR autoantibodies that bind to and stimulate TSHR activity causes Graves' disease, which is one of the most prevalent human autoimmune diseases (16, 17). Furthermore, it has been shown that TSHR maintains a relatively high activity in the absence of TSH (18, 19), yet the molecular mechanism and the physiological relevance for this are unknown (20).

DREAM (downstream regulatory element antagonist modulator) is a neuronal calcium sensor preferentially expressed in the central nervous system and the thyroid gland (21). DREAM, also named KCHIP-3 (K^+ channel interacting protein-3), belongs to a group of structurally and functionally related proteins (KCHIP-1 to -4) that interact with Kv4 potassium channels to regulate their membrane expression and gating (21–23). In the nucleus, DREAM is a calcium-, cAMP-, and phosphatidylinositol 3-kinase-sensitive transcriptional repressor that regulates

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.

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doi: 10.1210/me.2008-0466 Received December 19, 2008. Accepted March 13, 2009.

First Published Online March 19, 2009

Abbreviations: CREB, cAMP response element-binding protein; Ct, C-terminal; DREAM, downstream regulatory element antagonist modulator; GPCR, G protein-coupled receptor; HA, hemagglutinin; KCHIP-3, K^+ channel interacting protein-3; NIS, sodium-iodide symporter; NP40, Nonidet P-40; Nt, N-terminal; Tg, thyroglobulin; TPO, thyroid peroxidase; TSHR, TSH receptor.

transcription by binding to specific sites in target genes (21, 24–27) or through the interaction with other nucleoproteins (28, 29). In neurons, DREAM represses basal expression of the prodynorphin gene, and DREAM knockout mice display a hypoalgesic phenotype, suggesting a critical role for DREAM in pain modulation (30). In thyroid follicular cells, DREAM modulates the transcriptional activity of thyroid transcription factor-1 (TTF-1) and represses thyroglobulin (Tg) gene expression (29).

In the present work, we show that DREAM functions as an intracellular TSHR ligand that controls receptor protein levels and promotes its coupling to G α s protein. Analysis of transgenic mice and human patients support an etiopathological role of elevated levels of DREAM in human multinodular goiters.

Results

DREAM regulates thyroid growth and differentiation

DREAM regulates Tg expression in follicular cells (29). To further investigate DREAM function in the thyroid gland, we generated two independent lines of transgenic mice, LeuBTA7 and LeuBTA15, which express different levels of a dominant active DREAM mutant insensitive to both calcium and cAMP/protein kinase A (21, 25) (Fig. 1A). Histological analysis of 2- to 3-month-old mice revealed a nonnodular, diffuse, and homogeneous hyperplasia of the gland with small follicles lined by columnar epithelium in both transgenic lines (Fig. 1, B–E). Quantitative RT-PCR analysis of the glands confirmed this observation and showed an increase in the proliferation marker proliferating cell nuclear antigen (Fig. 1F). Analysis of thyroid glands from 9- to 12-month-old mice showed a marked enlargement of the transgenic gland [12.4 ± 2.1 mg (LeuBTA7) and 13.84 ± 1.6 mg (LeuBTA15)] compared with wild-type (4.4 ± 0.3 mg) that could be either diffuse, resembling a colloid goiter, or nodular, resembling human multinodular goiters (Fig. 2A). In the colloid goiter phenotype, many follicles showed a large lumen, irregular shape, colloid accumulation, and low cuboidal or flattened epithelium, which is a sign of hypoactivity (Fig. 2, compare B and E with C and F). Heterogeneity prevailed, and the glands showed also hyperplastic areas with follicles surrounded by a tall, active epithelium forming papillary infoldings protruding into the lumen (Fig. 2, D and G) or with small follicles lined with a tall epithelium resembling follicular adenomas (Fig. 2H), nodules (Fig. 2I), and highly hyperproliferative foci without follicular structure. Nodularity is the typical evolution of colloid goiters and was observed also in old mice. Some heterogeneity, however, was found between animals and within each thyroid gland, which is a common finding in thyroid pathologies and agrees with the variable phenotypes of the thyroid in response to the same stimulus (15, 31).

Analysis of several differentiation markers in 2- to 3-month-old mice showed a significant down-regulation of Tg mRNA and up-regulation of genes involved in thyroid hormone synthesis, such as the sodium-iodide symporter (NIS), and thyroid peroxidase (TPO) (Fig. 3). Similar results, although less pronounced, were observed in older mice (Fig. 3).

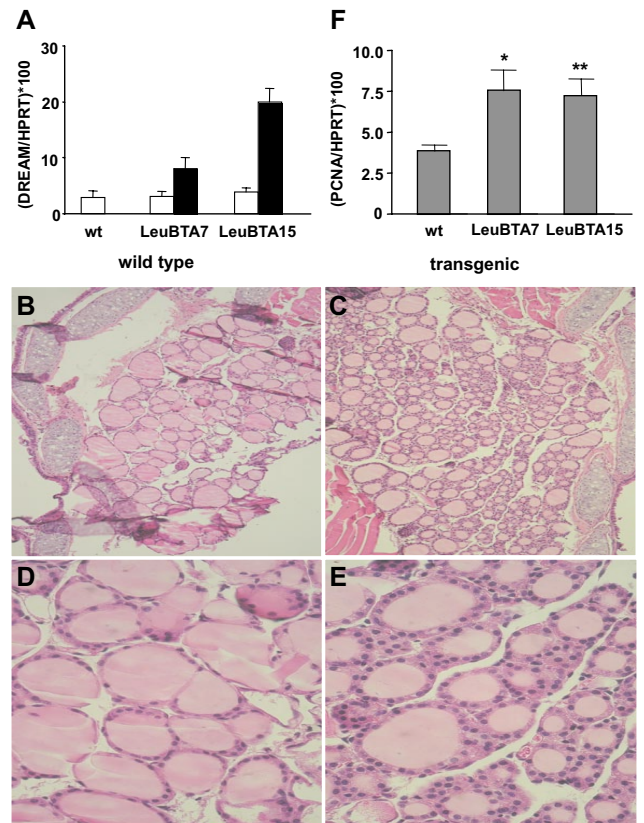


FIG. 1. Early morphological and proliferative changes in young transgenic mice. A, Quantitative real-time PCR showing HPRT mRNA-normalized expression levels (mean \pm SEM) of endogenous (white bars) and mutant DREAM (black bars) in the thyroid of 2- to 3-month-old wild-type (wt) and LeuBTA7 and LeuBTA15 DREAM transgenic mice. B–E, Hematoxylin and eosin staining of paraffin sections from 2- to 3-month-old wild-type (B–D) and transgenic (C–E) mice. A representative example of the hyperproliferative phenotype observed in both transgenic lines is shown. Magnification, $\times 100$ (B and C) and $\times 600$ (D and E). F, Quantitative real-time PCR showing HPRT mRNA-normalized expression level (mean \pm SEM) of proliferating cell nuclear antigen in wild-type (n = 9), LeuBTA7 (n = 8), and LeuBTA15 (n = 11) mice. Significant differences from wild-type mice by unpaired Student's *t* test are indicated: *, *P* = 0.0252; **, *P* = 0.0027.

As a result, the euthyroid state was maintained and no significant changes in T₃, T₄, and TSH serum levels were observed at any age in DREAM transgenic mice (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>).

Increased cAMP signaling in the thyroid gland of DREAM transgenic mice

To investigate the mechanism underlying the observed phenotype, we focused on modifications in the cAMP cascade, which is known to be involved in the pathogenesis of several thyroid diseases. We found that transgenic thyroid glands contained significantly higher levels of TSHR and cAMP than wild-type glands (Fig. 4, A and B). As a consequence, levels of phosphorylated cAMP response element-binding protein (CREB) were also increased in transgenic glands, without change in total CREB protein (Fig. 4B and supplemental Fig. 2). Noteworthy, increased TSHR protein did not correlate with increased TSHR mRNA (supplemental Fig. 3), indicating that the increase in TSHR levels is not due to transcriptional activation of the TSHR gene.

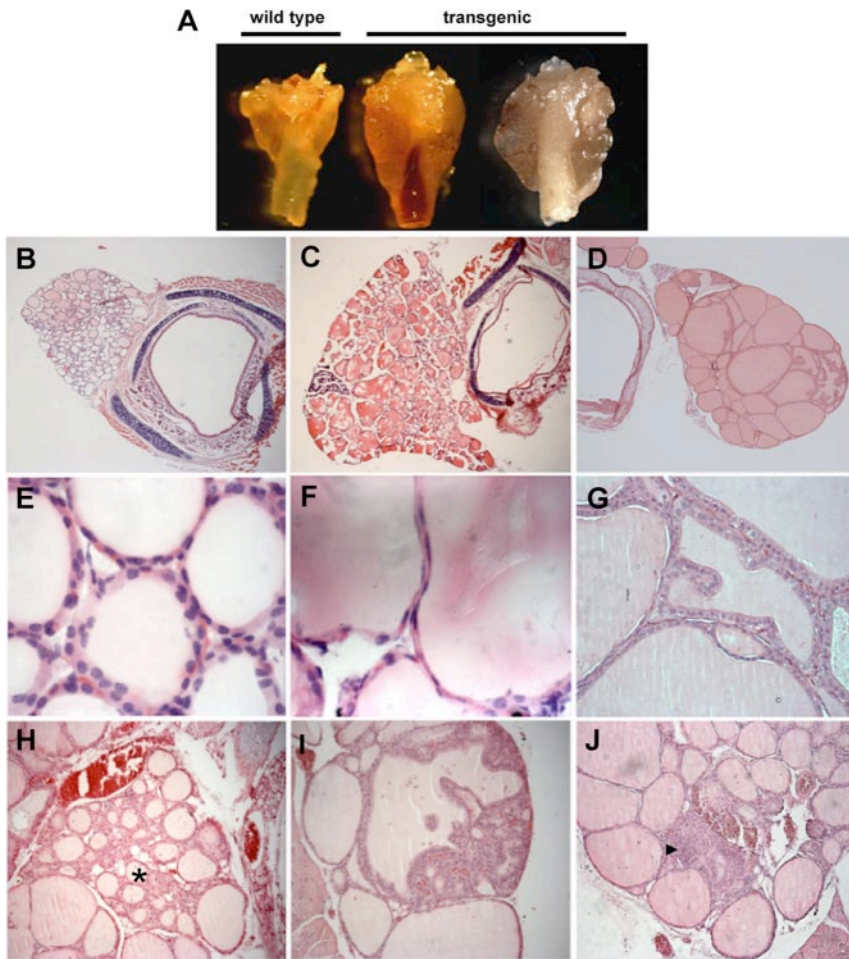


FIG. 2. Thyroid histopathology in 9- to 12-month-old mice. A, Gross appearance of thyroid glands from wild-type (*left*) and transgenic mice with diffuse goiter (*middle*) or multinodular goiter (*right*). B–J, Hematoxylin and eosin staining of paraffin sections from wild-type (B and E) and transgenic (C, D, F–J) mice. C and F, Transgenic glands showing follicles with large lumens surrounded by flat epithelium. D and G, Hyperplastic areas with follicles surrounded by tall epithelium forming papillary infoldings protruding into the lumen. H, Adenomatous hyperproliferation (*asterisk*). Nodules with disorganized follicular structure (I) and nonfollicular hyperproliferative foci (*arrowhead* in J). Similar heterogeneity and pathological phenotypes were observed in both transgenic line LeuBTA7 (D, G, H, and J) and LeuBTA15 (A, C, F, and I). Magnification, $\times 50$ (B–D), $\times 1000$ (E and F), $\times 400$ (G), and $\times 200$ (H, I, and J).

To analyze the relation between DREAM overexpression and increased TSHR/cAMP signaling, we generated stable clones of thyroid PC Cl3 cells that overexpress wild-type DREAM, mutant DREAM insensitive to calcium (EF-mDREAM), or mutant DREAM insensitive to cAMP (Leu-mDREAM) (21, 28). These mutations correspond to those expressed simultaneously in DREAM transgenic mice. Unexpectedly, overexpression of wild-type DREAM induced an increase in intracellular TSHR, cAMP, and phospho-CREB similar to that observed with either DREAM mutant (supplemental Fig. 3, B–D). These results indicate that overexpression of DREAM protein, regardless of whether it is mutated or not, is responsible for the induction of the cAMP cascade observed in transgenic mice. The fact that DREAM mutations are not needed for TSHR/cAMP activation suggests a role for endogenous DREAM in tonic activation of TSHR in basal conditions.

DREAM-induced activation of the cAMP signaling pathway is mediated by TSHR

To link the effect of DREAM on cAMP signaling to TSHR, we transfected DREAM into TSHR-10,000 cells, a CHO-derived cell line, that stably overexpress TSHR (19) or into the original CHO-K1 cells. In basal conditions, TSHR-10,000 cells contain higher levels of cAMP and phospho-CREB (Fig. 4, C and D) than CHO-K1 cells due to the constitutive activity of TSHR in the absence of TSH (19). Expression of DREAM resulted in a significant increase in cAMP and phospho-CREB levels in TSHR-10,000 cells and no changes in CHO-K1 cells (Fig. 4, C and D). The effects of DREAM on TSHR and cAMP signaling were not reproduced by expression of KChIP-2 (Fig. 4C and supplemental Fig. 4), a closely related member of the DREAM/KChIP family that is expressed in the thyroid gland (29). These data indicate that activation of cAMP signaling is specific of DREAM and is mediated by TSHR.

DREAM interacts with TSHR

DREAM has been reported to interact with proteins in the nucleus and in the cytoplasm (32). Therefore, we next investigated whether DREAM is able to interact with TSHR. Using thyroid glands from wild-type and transgenic mice, we show that TSHR coimmunoprecipitates with DREAM (Fig. 5A). The interaction was confirmed using cell extracts from TSHR-10,000 cells transfected with DREAM-hemagglutinin (HA) (Fig. 5B).

To further characterize the interaction between DREAM and TSHR, we used Myc-tagged TSHR fragments (Fig. 5C) transfected together with DREAM-HA in CHO-K1 cells. Immunoprecipitation showed that DREAM specifically interacts with the C-terminal (Ct) intracellular region of TSHR (Fig. 5D). Conversely, experiments using Myc-tagged DREAM fragments (Fig. 6A) revealed that the N-terminal (Nt) region of DREAM containing the first 90 amino acids (DREAM Δ 1-90) is needed for the interaction with the receptor (Fig. 6B). Coimmunoprecipitation experiments using TSHR Ct-Myc and DREAM Δ 1-90-HA constructs confirmed that the Ct region (amino acids 696-763) of the receptor is able to interact directly with the 1-90 fragment of DREAM (Fig. 6C).

DREAM-derived peptides activate cAMP signaling

Previous work by others has shown that calreticulin interacts with and stabilizes TSHR (33). Overexpression of calreticulin in TSHR-10,000 cells, however, did not increase cAMP levels

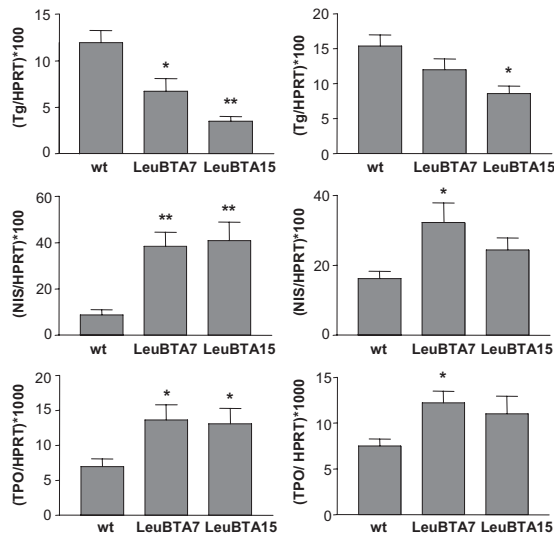


FIG. 3. Thyroid differentiation markers in DREAM transgenic mice. Tg, NIS, and TPO mRNA levels were quantified by real-time quantitative PCR. *Left panels* correspond to young mice (2–3 months) from wild-type ($n = 7$), LeuBTA7 ($n = 10$), and LeuBTA15 ($n = 10$) transgenic lines. *Right panels* correspond to old mice (12 months) from wild-type ($n = 6$), LeuBTA7 ($n = 6$), and LeuBTA15 ($n = 6$) transgenic lines. Values are normalized by the content of HPRT mRNA. Data are mean \pm SEM. Significant differences from wild-type mice by unpaired Student's *t* test are indicated. *, $P < 0.05$; **, $P < 0.01$.

(supplemental Fig. 5), indicating that receptor stabilization and increased cAMP signaling are independent events. To further investigate the molecular basis for this effect, we checked

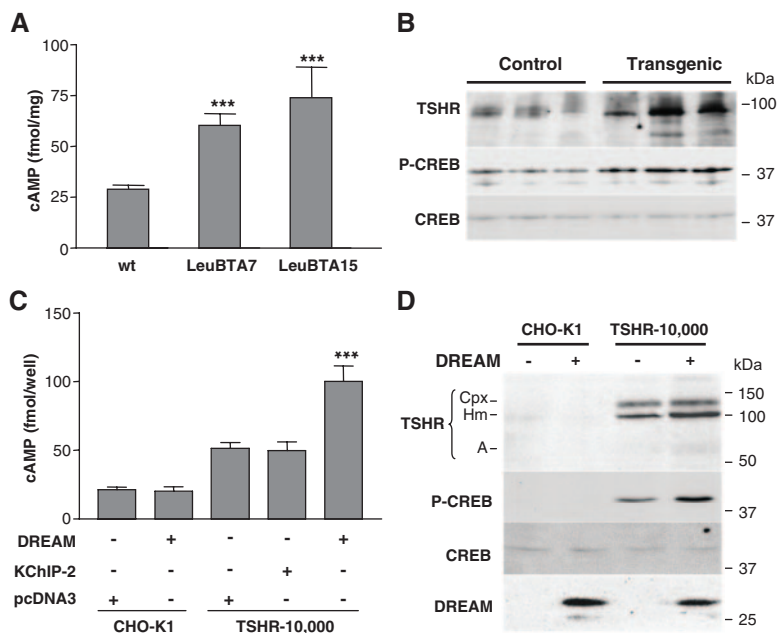


FIG. 4. DREAM overexpression activates the cAMP signaling pathway through TSHR. **A**, The levels of cAMP in thyroid glands from 9-month-old wild-type (Wt) ($n = 6$) or DREAM transgenic LeuBTA7 ($n = 5$) and LeuBTA15 ($n = 4$) mice were determined. Data are mean \pm SEM. Significant differences from wild-type mice by unpaired Student's *t* test are indicated: ***, $P < 0.001$. **B**, Representative Western blot using whole-cell extracts from 9-month-old wild-type and transgenic thyroid glands (line LeuBTA15 is shown). Blots were incubated with specific antibodies against TSHR, phospho-CREB (P-CREB), and total CREB. **C**, Analysis of intracellular cAMP levels after transfection with DREAM, KChIP-2, or pcDNA3 in CHO-K1 or TSHR-10,000 cells as indicated. Significant differences from control (pcDNA3 in TSHR-10,000 cells) by unpaired Student's *t* test are indicated: ***, $P < 0.001$. **D**, Western blot using whole-cell extracts from CHO-K1 or TSHR-10,000 cells after transfection with DREAM. Blots were incubated with specific antibodies against DREAM, TSHR, phospho-CREB, and total CREB. TSHR immunoreactive bands correspond to TSHR holoreceptor-bearing complex carbohydrate (Cpx) or high-mannose carbohydrate (Hm) and the A subunit (A).

whether DREAM fragments can directly activate the receptor as intracellular agonists. Expression in TSHR-10,000 cells showed that amino acids 43–90 of DREAM are involved in the activation of TSHR (Fig. 7A). To narrow down the residues directly responsible, we analyzed the effect of three overlapping peptides, P1, P2, and P3, that cover this region (Fig. 7A). Exposure of TSHR-10,000 cells to P1 specifically activated the cAMP cascade and increased phospho-CREB levels without increasing TSHR protein levels (Fig. 7, B and C). Similar results were observed in PC Cl3 thyroid cells (data not shown). P1 also increased cAMP levels in the human ML-1 line of poorly differentiated thyroid carcinoma cells (Fig. 7D) that contain low levels of TSHR (34) but not in NPA cells (Fig. 7E), a cell line originally described as papillary thyroid cancer (35), which does not express detectable levels of TSHR (36). Using TSHR-10,000 cells, coimmunoprecipitation showed that both DREAM and P1, like TSH (10), enhanced the coupling between TSHR and G α s (Fig. 7F). These data indicate that the P1 peptide, which neither stabilizes nor increases TSHR levels is still able to induce the cAMP cascade, strongly supporting DREAM as an intracellular TSHR ligand.

Endogenous DREAM regulates TSHR activity

To further substantiate a physiological role of endogenous DREAM in TSHR function, we transiently knocked down DREAM expression in PC Cl3 thyroid cells using a previously characterized antisense vector (37). Reduction by 50–60% of the endogenous DREAM levels resulted in decreased TSHR, cAMP, and phospho-CREB levels, without change in total CREB (Fig. 8, A and B). This effect was specific for DREAM knock-down because it was not observed using an antisense vector for KChIP-2, in keeping with the lack of effect of KChIP-2 on the receptor (see Fig. 4C and supplemental Fig. 4). Analysis of thyroid glands from DREAM knockout mice (30) confirmed the antisense knock-down data and showed reduced levels of TSHR and cAMP (Fig. 8, C and D). Consistently, thyroid glands from DREAM knockout mice showed unstructured follicular pattern and cellular hypoactivity (supplemental Fig. 7). Complete characterization of the thyroidal phenotype in DREAM knockout mice is currently under investigation (Zannini, M. S., personal communication). Taken together, these data support a physiological role for endogenous DREAM in the control of TSHR activity in thyroid cells.

Analysis of DREAM and TSHR in human multinodular goiters

To investigate the pathophysiological relevance of the DREAM-TSHR interaction, we carried out Western blot analysis of nodules from human multinodular goiters using the surrounding normal tissue as control (Fig. 9). We found a higher than 2-fold increase in DREAM protein levels in nodular

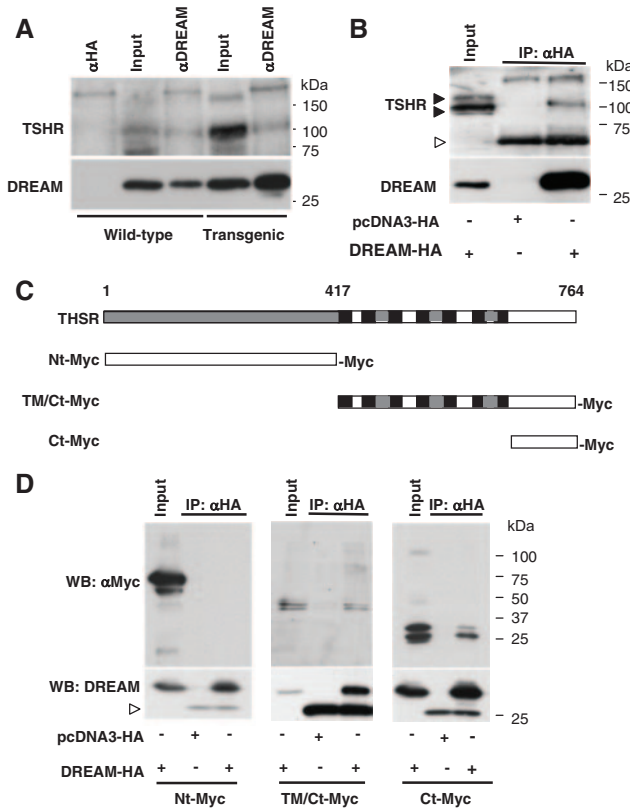


FIG. 5. DREAM interacts with the Ct region of TSHR. **A**, Coimmunoprecipitation of DREAM and TSHR with a monoclonal antibody for DREAM in whole-cell extracts of thyroid glands from wild-type and transgenic mice (LeuBTA15). A nonrelated antibody (anti-HA) was used as a negative control. TSHR and DREAM were detected by Western blot. Note that transgenic mice contain higher levels of TSHR and total DREAM proteins, as expected. Equal amounts of total protein extracts were used in both cases. **B**, Coimmunoprecipitation of DREAM-HA and TSHR with an anti-HA antibody in whole-cell extracts from TSHR-10,000 cells transfected with the indicated plasmids. TSHR and DREAM were detected by Western blot. *White arrowhead* indicates Ig chain. **C**, Schematic representation of the TSHR protein and deletion fragments containing the indicated Nt, intracellular (TM/Ct), and Ct regions tagged with Myc. Extracellular domains (gray), transmembrane domains (black), and intracellular domains (white) are indicated. **D**, Coimmunoprecipitation of DREAM-HA and the Myc-tagged TSHR deletion fragments with an anti-HA antibody in whole-cell extracts from CHO-K1 cells transfected with the indicated plasmids. Western blots were developed with anti-Myc and with anti-DREAM as a control. Input corresponds to 5% of total protein. *White arrowhead* indicates Ig chain.

samples in 10 of 16 multinodular goiters. Representative examples (patients 1, 2, 4, 6, 7, and 8) are shown in Fig. 9A (for complete patient data set, see supplemental Table 1). Changes in DREAM levels were paralleled by TSHR, and statistical analysis showed a positive correlation between DREAM and TSHR proteins, supporting the notion that DREAM is able to modulate TSHR levels in the human thyroid gland (Fig. 9A). The increase in TSHR protein was not associated with an increase in TSHR mRNA (data not shown). A positive correlation was also observed between DREAM and phospho-CREB, suggesting that DREAM affects the cAMP signaling pathway. By contrast, a negative correlation was found between DREAM and Tg (Fig. 9C), which agrees with the transcriptional repression of the Tg promoter by DREAM *in vitro* (29) and in transgenic mice (see Fig. 3). No statistically significant correlations between DREAM and ERK-2, β-actin, and CREB were found in the same samples (Fig. 9A and data not shown). Together, these data

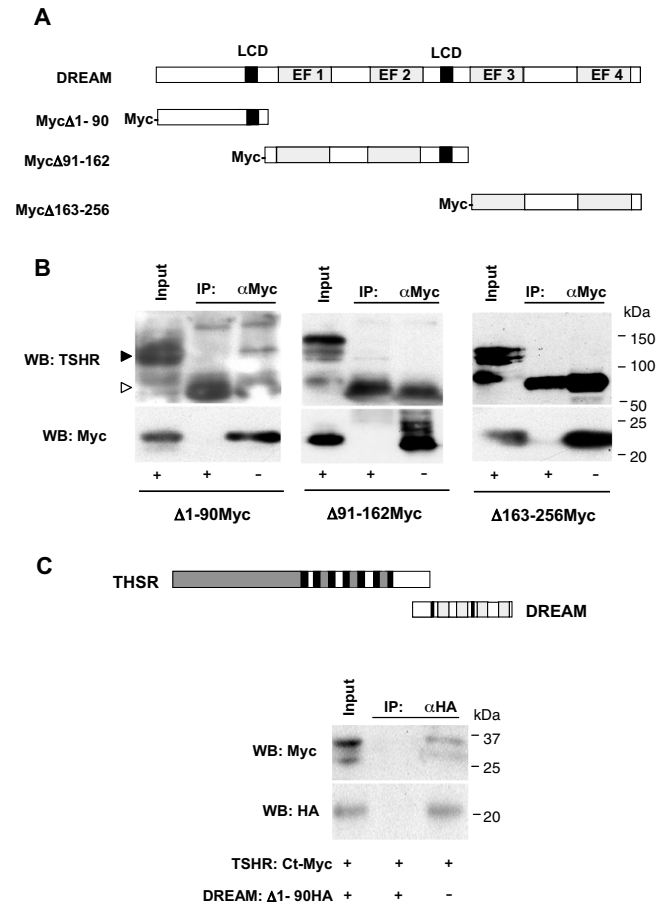


FIG. 6. The Nt region of DREAM interacts with TSHR. **A**, Schematic representation of DREAM protein and fragments containing the Nt (Δ1-90), middle (Δ91-162), and Ct (Δ163-256) regions tagged with Myc. **B**, Coimmunoprecipitation of DREAM fragments with anti-Myc antibody in whole-cell extracts from TSHR-10,000 cells transfected with indicated plasmids. Endogenous TSHR was detected by immunoblot. Input corresponds to 5% of total protein. *White arrowhead* indicates Ig chain. **C**, Coimmunoprecipitation of the Myc-tagged Ct fragment of TSHR, amino acids 696-763, and the HA-tagged 1-90 fragment of DREAM transfected in CHO-K1 cells as indicated. Schematic representation of the interaction between the TSHR intracellular Ct region and DREAM Nt region is shown. TSHR domains are as indicated in Fig. 5C; DREAM functional domains are indicated in A.

suggest that up-regulation of endogenous DREAM may contribute to the development of thyroid nodules.

Discussion

Binding of TSH to its receptor controls thyroid function mainly through activation of the cAMP signaling pathway (10, 38, 39). Changes in TSHR activity due to point mutations or in response to auto-antibodies modify the cAMP cascade and have clinical consequences (11). In addition, TSHR displays a relatively high basal activity in the absence of TSH. The physiological significance and the existence of endogenous mediators of such spontaneous activity are not well understood (18, 19). In the present work, we show that DREAM is an endogenous intracellular effector of TSHR function that activates cAMP signaling.

DREAM transgenic thyroid glands showed increased cAMP and phospho-CREB levels, induction of the cAMP-regulated

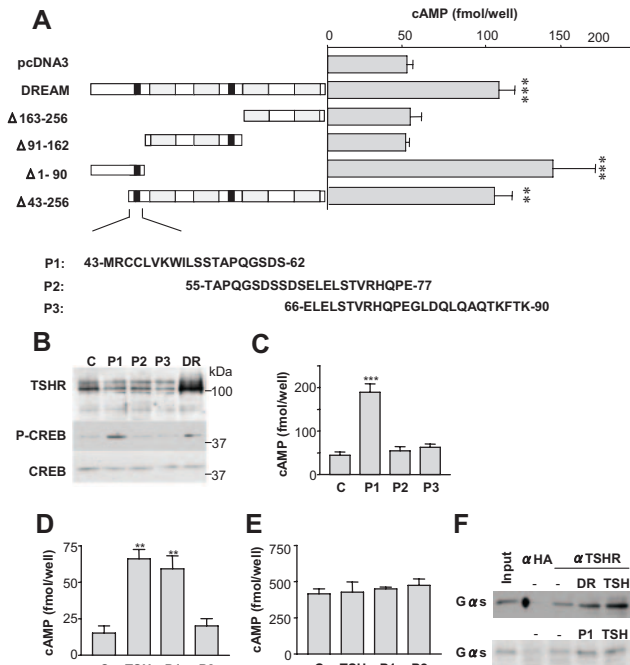


FIG. 7. DREAM-derived peptides enhance TSHR activity. **A**, Increased cAMP levels in TSHR-10,000 cells after transfection with DREAM or DREAM fragments, schematically represented next to each bar. Data are mean \pm SEM. Significant differences from control (pcDNA3) by unpaired Student's *t* test are indicated: **, $P < 0.01$; ***, $P < 0.001$. The sequence of three overlapping peptides (P1, P2, and P3) that cover amino acids 43–90 in DREAM is also shown. **B**, TSHR-10,000 cells were incubated with DREAM-derived peptides P1, P2, and P3 or a nonrelated peptide as negative control. Cells transfected with DREAM (DR) were included as positive control. Western blot with TSHR, phospho-CREB (P-CREB), and total CREB is shown. **C–E**, Effect on cAMP levels in TSHR-10,000 cells (**C**), thyroid follicular carcinoma ML-1 cells (**D**), and papillary carcinoma NPA cells (**E**) after incubation with DREAM-derived peptides (2 μ M), a control nonrelated peptide (2 μ M) or TSH (0.5 nM/ml, 10 min) used as a positive control. Data are mean \pm SEM. Significant differences from control by unpaired Student's *t* test are indicated: ***, $P < 0.001$. **F**, Coimmunoprecipitation of TSHR and G α s with an anti-TSHR antibody using whole-cell extracts from TSHR-10,000 cells transfected with DREAM (DR, upper panel), the P1 peptide (lower panel), and the corresponding controls, empty vector, or nonrelated peptide, respectively. Coimmunoprecipitation with a nonrelated antibody (anti-HA) was used as a negative control. Treatment with TSH (0.5 nM/ml, 10 min) was used as a control. Input corresponds to 1% of total extracts.

genes NIS and TPO, hyperproliferation, and goiter development. DREAM transgenic mice, however, do not show significant changes in T₃, T₄, or TSH levels as described in other models in which cAMP is constitutively activated (40). Maintenance of the euthyroid state could be related to the repression of Tg observed in DREAM transgenic glands (29), although the exact mechanism has not been established.

Calcium-binding proteins of the neuronal calcium sensor superfamily have been shown to regulate the activity of several cytosolic and membrane proteins. Thus, visinin-like protein-1 modulates the activity of guanylyl cyclase B as well as the surface expression and sensitivity for agonists of nicotinic receptors (22, 41–43). Ca-binding protein 4 regulates calcium influx in photoreceptor synaptic terminals through its interaction with the Cav1.4 channel (44). Likewise, DREAM has been associated with trafficking of Kv4 potassium channels to the plasma membrane and regulation of channel gating (22, 45), and like visinin-like protein-1 and other neuronal calcium sensor family mem-

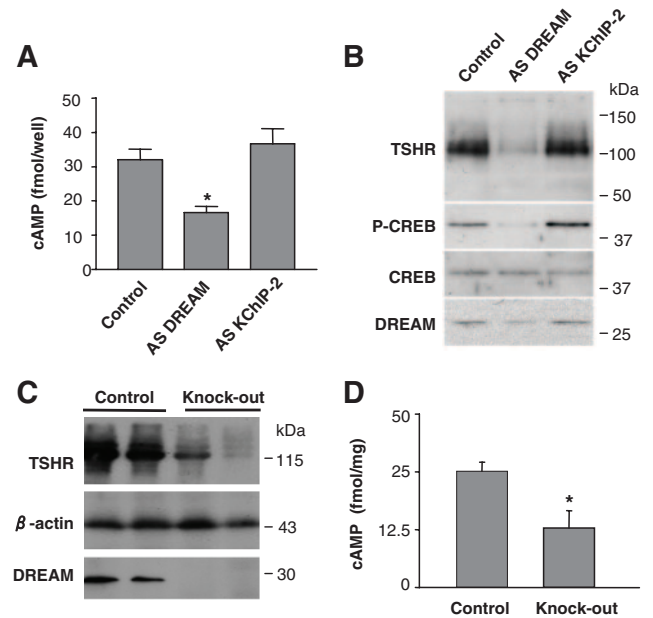


FIG. 8. Endogenous DREAM controls TSHR signaling. **A**, Determination of cAMP levels in PC Cl3 thyroid cells 4 d after transfection with specific antisense (AS) vectors for DREAM, KChIP-2, or control pcDNA3. Data are mean \pm SEM of two independent experiments carried out in triplicate. Significant difference from control by unpaired Student's *t* test is indicated: *, $P = 0.0122$. **B**, Western blot analysis of PC Cl3 thyroid cells 4 d after transfection as in **A**. The immunoblot was sequentially probed with antibodies for TSHR, phospho-CREB (P-CREB), total CREB, and DREAM. **C**, Western blot using whole-cell extracts from DREAM knock-out and control thyroid glands. Two different knockout and control mice are shown. Blots were incubated with specific antibodies against DREAM, TSHR, and β -actin. **D**, *In vivo* analysis of cAMP levels in thyroid glands from DREAM knock-out ($n = 7$) and control mice ($n = 6$). Significant difference from control by unpaired Student's *t* test is indicated: *, $P = 0.035$.

bers (46), DREAM regulates the membrane binding and kinase activity of G protein-coupled receptor kinase (47). The effect of DREAM on TSHR activity is specific and is mediated through the Nt TSHR-interacting region of DREAM. This domain is not present in KChIP2, otherwise a highly conserved member of the DREAM/KChIP family. Thus, KChIP2 does not regulate TSHR activity, and its expression in the thyroid gland could be related to transcriptional control in combination with DREAM (29). Previous work has shown that, like DREAM, calreticulin interacts and stabilizes TSHR. Calreticulin, however, did not induce the cAMP cascade, indicating that protein stabilization is not enough to explain the TSHR activation elicited by DREAM. Likewise, the DREAM-derived P1 peptide directly activates TSHR, promotes its coupling to G α s and increases cAMP levels without stabilizing the receptor. Therefore, the activation elicited by DREAM is an independent effect not related to TSHR levels.

TSHR signaling is modulated by a number of posttranslational modifications, including phosphorylation by GRKs and Nt glycosylation (48, 49) changes that determine receptor desensitization and proper folding and membrane expression, respectively. In addition, TSHR function is regulated by oligomerization (50) and interaction with the membrane-associated PDZ protein hScrib (51), which determine intracellular trafficking and promotes receptor recycling blocking endocytosis, respectively. Whether DREAM interferes at these levels is presently unknown, but a Ca²⁺-dependent interaction between DREAM and the PDZ-containing pro-

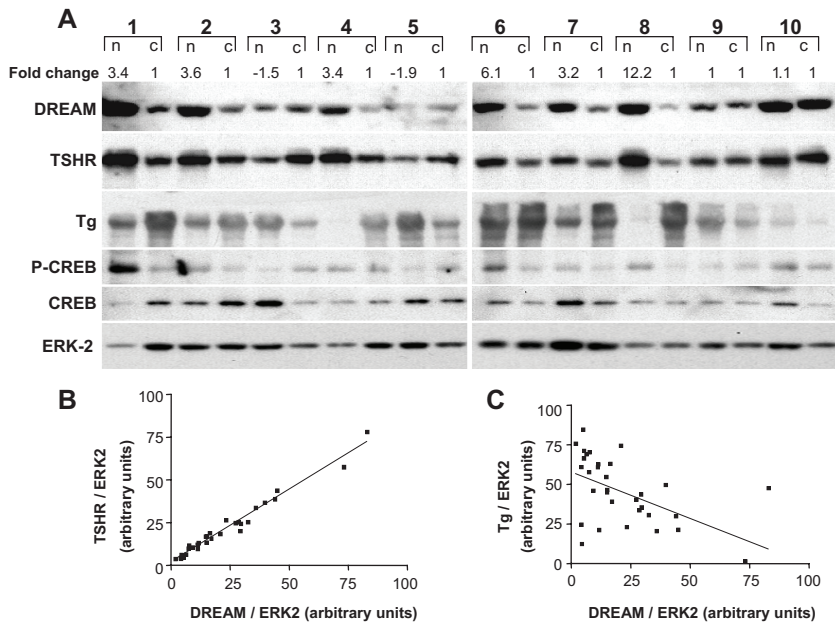


FIG. 9. Increased levels of DREAM are associated with human multinodular goiters. **A**, Western blot analysis of human multinodular goiter (n) and control (c) tissue. Fold change for the DREAM protein is indicated. Immunodetection of DREAM, TSHR, Tg, ERK-2, phospho-CREB (P-CREB), and total CREB is shown. Only the 115-kDa band of TSHR, corresponding to the uncleaved form containing high-mannose carbohydrate, was clearly detected. **B**, Quantification of immunoreactive bands shows a positive correlation (***, $P < 0.0001$; Pearson $r^2 = 0.974$) between DREAM and TSHR, whereas a negative correlation (**, $P = 0.002$; Pearson $r^2 = 0.2858$) was found between DREAM and Tg (see panel C).

tein PSD-95 has been observed in neurons (Naranjo, J. R., unpublished observations).

Our results show that DREAM interacts with the Ct cytosolic domain of TSHR. This region shares an approximately 70% homology with the Ct region of other glycoprotein-hormone receptors such as the FSH receptor and LH/chorionic gonadotropin receptor (10). Low molecular weight agonists or antagonists for these receptors have the potential to become oral therapeutics for infertility or contraception treatment, respectively (52). These studies have given rise to the discovery of molecules that bind to the TSHR transmembrane region and display partial agonist or antagonist activity although too moderate to be clinically useful (52, 53). Further experiments will be required to investigate the therapeutic value of DREAM and DREAM-derived peptides in pathologies associated with impaired TSHR, FSH receptor, or LH/chorionic gonadotropin receptor function. Activation or blockade of GPCRs by cell-penetrating peptides has been previously described (54) and was found to be of potential therapeutic value (55, 56). These peptides (named pepducins) derive from the intracellular transmembrane loops of GPCRs and, like DREAM, require the Ct of the receptor to activate G proteins.

Taken together, our data from cultured follicular cell lines, thyroid glands from transgenic mice, and human multinodular goiters reveal a new molecular mechanism that links deregulated DREAM expression with thyroid enlargement and nodular development.

Materials and Methods

Plasmids

Plasmids for wild-type DREAM, DREAM mutants, and expression vectors for antisense DREAM and KChIP-2 have been previously described

(25, 37). TSHR and DREAM deletion fragments were cloned in the pCS2+Myc expression vector using the *Clal* and the *NcoI-XhoI* sites, respectively. Calreticulin cDNA (a gift from Dr. M. Michalak, Canada) was subcloned in a pcDNA3-HA vector. Sequences of PCR primers for the DREAM and TSHR fragments are given in supplemental information. The plasmids were verified by sequencing on both strands.

Animals

The proximal bovine Tg promoter (40, 57) was used to target the dominant active DREAM mutant insensitive to calcium and cAMP (LeuEFmDREAM) to the thyroid gland. The transgenic cassette was microinjected into the pronuclei of one-cell embryos (C57BL/6 \times CBA F1) using standard techniques. Transgenic progeny were identified by qualitative PCR of tail DNA using specific primers (supplemental information). Founder males were backcrossed to C57BL/6 females to generate lines that were maintained as heterozygotes. DREAM knockout mice (30) were kindly provided by J. M. Penninger. Female mice were analyzed in all experiments using wild-type littermates as controls. Experiments were approved and conducted according to institutional review board guidelines.

Cells

CHO-K1 cells stably transfected with human TSHR (TSHR-10,000 cells) (19) and the original CHO-K1 cell line were kindly provided by Dr. B. Rapoport. The cells were maintained in Ham's F12 medium supplemented with Glutamax, fetal bovine serum (5%), and penicillin/streptomycin. Rat thyroid follicular PC Cl3 cells were cultured in DMEM/F12 (1:1) medium with Glutamax and supplemented with 5% calf serum and a six-growth-factor complement including TSH (0.5 mU/ml), insulin (10 μ g/ml), somatostatin (10 ng/ml), hydrocortisone (10 nM), transferrin (5 μ g/ml), and glycyl-histidyl-lysine (10 ng/ml). When cells were incubated in the absence of TSH, the calf serum concentration was reduced to 0.2%. Transfections were carried out with the JetPEI transfection reagent. For stable transfection, PC Cl3 cells received 5 μ g plasmid DNA expressing either wild-type DREAM, EFmDREAM, EFLmDREAM, or empty pcDNA3 vector. Cells were selected after 3 wk with 300 μ g/ml G418. ML1 and NPA cell lines, kindly provided by Dr. K. Törnquist, were cultured as described (58).

Peptide delivery

Peptides were delivered using the PULSin protein delivery reagent (Polyplus transfection) using the manufacturer's protocol. For cAMP assays, peptides were added to 12-well plates (final concentration 2 μ M), and for Western blots, peptides were added to 35-mm dishes (final concentration 2 or 3 μ M). Peptide penetration into cells was analyzed by immunofluorescence using rhodamine-labeled peptides and showed that more than 90% of cells incorporated the peptide after 4 h incubation (not shown). Therefore, cells were analyzed after 4 h incubation with peptides.

cAMP assay

cAMP concentration in cells and thyroid gland extracts was measured using the Biotrak enzyme immunoassay system (GE Healthcare, Piscataway, NJ). Cells were plated in 12-well plates (1.7×10^5 PC Cl3 cells or 1.25×10^5 TSHR-10,000 cells per dish) and collected after 24 h (PC Cl3 stable clones) or 24 h after transient transfection (TSHR-10,000 cells). Proliferation profiles were not significantly different among control and DREAM-transfected cultures, and therefore cAMP values were normalized per well. In the experiments with DREAM-derived peptide, cells were collected after 4 h incubation with the peptide.

Histology

Thyroid glands were fixed in 4% paraformaldehyde in PBS overnight at 4 C, dehydrated through ethanol series, cleared in xylene, embedded in paraffin, and sectioned at 5 μ m. For histological examinations, serial sections from transgenic and wild-type mice were stained with Harry's hematoxylin and eosin.

Quantitative real-time RT-PCR

Total RNA from thyroid glands was prepared using Trizol. After reverse transcription, quantitative PCR was performed using specific primers and TaqMan probes. The sequences of primers and probes are given in supplemental information.

Coimmunoprecipitation and Western blot

For coimmunoprecipitation experiments, whole-cell extracts were prepared from thyroid glands, PC Cl3 cells, or transfected cells by incubation in Nonidet P-40 (NP40) lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP40, and protease inhibitor cocktail]. Extracts were precleared with protein G-Sepharose for 1 h and incubated overnight at 4 C with a monoclonal antibody against DREAM (28) or with anti-HA (sc-7392; Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were captured for 2 h with protein G-Sepharose in lysis buffer containing 5% BSA, and beads were washed three times in lysis buffer. The same protocol using protein A-Sepharose was used for immunoprecipitation of TSHR and Myc-fusion proteins with goat and rabbit polyclonal antibodies, respectively. Protein complexes were eluted with SDS sample buffer and analyzed by immunoblot. For membrane protein preparations cells were lysed in buffer [20 mM Tris (pH 7.5), 0.32 M sucrose, 0.2 mM EDTA, and 0.5 mM EGTA and protease inhibitor cocktail]. After sonication, cells were centrifuged at 50,000 rpm and pellets resuspended in GTED buffer [20% glycerol, 10 mM Tris (pH 7.5), 1 mM EDTA, and 1 mM dithiothreitol]. Cell extracts were resolved in SDS-PAGE and transferred to polyvinylidene difluoride membranes. Rabbit polyclonal anti-DREAM has been described (37). The polyclonal antibody for TSHR (sc-7816; Santa Cruz) is directed against the N-terminal region and recognizes the full-length and the A subunit of the receptor. Antibodies against ERK-2 (sc-153), Gas (sc-823), and c-Src (sc-19) were from Santa Cruz; phospho-CREB (9191S) and total CREB (9192) from Cell Signaling (Beverly, MA); Myc (ab9106-100) from Abcam (Cambridge, MA); and β -actin (A-5441) from Sigma Chemical Co. (St. Louis, MO). Blots were developed by enhanced chemiluminescence and quantified using the NIH Image software.

Human sample analysis

Human samples were obtained after informed consent and formally approved by the Ethical Committee at the Federico II University in Naples, Italy. All clinical investigation has been conducted according to the principles expressed in the Declaration of Helsinki. After surgical removal, samples were immediately frozen in liquid nitrogen. Samples were lysed in NP40 lysis buffer and analyzed by Western blot as indicated above.

Statistics

The correlation analysis and Student's *t* test applied for two group comparisons were done using the Prism statistical software. Values with *P* < 0.05 were considered significant. For cAMP assays, the number of independent experiments were at least three (*n* \geq 3) carried out in triplicate.

Acknowledgments

We acknowledge Dr. B. Pintado and Dr. A. Gutiérrez-Adán for preparation of transgenic mice. We thank P. González and Dr. E. Lopez for assistance and Dr. L. Nitch and C. Mark for critical reading of the manuscript.

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This work was supported by grants from Ministerio de Ciencia e Innovación and Comunidad Autónoma de Madrid (to J.R.N. and B.M.) and from Fondo de Investigaciones de la Seguridad (to M.R. and B.M.)

Disclosure Summary: The authors have nothing to disclose.

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Attached manuscript #5

Benign hereditary chorea: clinical and neuroimaging features in an Italian family.

Salvatore E, Di Maio L, Filla A, Ferrara AM, Rinaldi C, Saccà F, Peluso S, Macchia PE, Pappatà S, De Michele G.
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Benign Hereditary Chorea: Clinical and Neuroimaging Features in an Italian Family

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Video



Abstract: Benign hereditary chorea is an autosomal dominant disorder characterized by early onset nonprogressive chorea, caused by mutations of the *thyroid transcription factor-1 (TITF-1)* gene. Clinical heterogeneity has been reported and thyroid and respiratory abnormalities may be present. We describe 3 patients of an Italian family carrying the S145X mutation in the *TITF-1* gene with mild motor delay, childhood onset dyskinesias, and subtle cognitive impairment. A child in the third generation presented with congenital hypothyroidism and neonatal respiratory distress. Imaging studies in 2 patients showed mild ventricular enlargement and empty sella at magnetic resonance imaging and hypometabolism of basal ganglia and cortex at 18-Fluoro-2-deoxy-glucose positron emission tomography. © 2010 Movement Disorder Society

Key words: benign hereditary chorea; *thyroid transcription factor-1*; congenital hypothyroidism; MRI; FDG-PET

Benign hereditary chorea (BHC) is an autosomal dominant disorder characterized by childhood onset chorea with little or no progression into adult life. Mental deterioration does not occur, but slightly lower I.Q. scores have been reported. Mutations in the *thyroid transcription factor-1 (TITF-1)* gene on chromo-

Additional Supporting Information may be found in the online version of this article.

Elena Salvatore and Luigi Di Maio contributed equally to the study, and both should be considered as first authors.

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Potential conflict of interest: Nothing to report.

Received 23 September 2009; Revised 28 December 2009; Accepted 28 January 2010

Published online 11 June 2010 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/mds.23065

some 14q have been identified as causative in several families, most of them recently reviewed.^{1,2} A second locus (8q21) for BHC has been recently mapped in two Japanese families with adult onset chorea.³

The *TITF-1* gene is a homeodomain-containing transcription factor essential for the organogenesis of lung, thyroid, and basal ganglia.⁴ Thus, it is not surprising that the clinical spectrum in families carrying *TITF-1* mutations includes thyroid and lung disorders, such as congenital hypothyroidism and respiratory distress. The putative mechanism of disease results from gene haploinsufficiency and reduced protein product.

We previously described molecular and functional data of the novel *TITF-1* S145X mutation in an Italian pedigree.⁵ Here, we report in detail the clinical features and the neuroimaging data of the family.

CASE REPORTS

This three-generation family shows three affected individuals, one for each generation, all carrying a previously unreported mutation of the *TITF-1* gene. The genetic defect and the molecular mechanisms have been described in the previous article.⁵ The index patient was Patient 1, who had been referred for exclusion of Huntington's disease (HD). The information that her father (Pt. 2) had abnormal movements and that her son (Pt. 3) had congenital hypothyroidism led to the clinical suspicion of BHC and to the molecular analysis of the *TITF-1* gene.

Patient 1, 26 years old, began walking at the age of 18 months, but she was clumsy and fell repeatedly. Her gait much improved around puberty. Mild generalized choreic movements appeared at the age of 7 years and remained stable thereafter. No mental or behavioral abnormalities were present, she did not encounter difficulties at school, and she was graduated at a Hotel school. During puerperium, when she was 19 years old, her chorea worsened and she presented with a postpartum psychosis, characterized by depression and aggressiveness toward the newborn and successfully treated with risperidone and lamotrigine.

At the age of 23 years she was admitted to our hospital. Neurological examination showed generalized choreic movements (video) and was otherwise normal. Neuropsychological evaluation demonstrated long term verbal memory deficit and low-normal score at Raven Matrices test.

Molecular analysis of HD gene and laboratory testing were normal, a part from elevation of thyroid-stimulating hormone.⁵ Thyroid hormone replacement was started. Brain magnetic resonance imaging (MRI)

revealed ventricular dilatation, more marked in the posterior part of lateral ventricles (Fig. 1a–c) and partial empty sella (Fig. 1d). Brain 18-Fluoro-2-Deoxy-Glucose Positron Emission Tomography (FDG-PET) showed slight relative hypometabolism of the caudate nuclei and of the medial frontal and temporo-parietal cortices (Fig. 2). The patient was treated with tetrabenazine up to 75 mg daily, with mild improvement. At the age of 26 years she withdrew the therapy abruptly and presented marked worsening of chorea, irritability, emotional lability, poor sleep, inappropriate dress, and behavior. She was admitted to our hospital again, treated with quetiapine, 75 mg/daily, and discharged improved after a week.

Patient 2, the proband's father, 56 years old, had meningitis at the age of 6 months. Subsequent motor development was delayed with walking starting at the age of 5 years and normal language skills. His school performances were poor. Since childhood, slight, sporadic, hyperkinesias were present, which mainly involved the abdomen and had been stable over time. He did not report improvement by alcohol. He worked as a school-caretaker and had a normal social life. At examination jerky abdominal movements were evident; mild and rare choreic movements were present in other body regions (video). Neuropsychological evaluation showed short term verbal and spatial memory deficit, slight attentional deficit, and constructive apraxia.

Thyroid hormone screening showed primary hypothyroidism with increased TSH and mildly reduced FT3. Brain MRI evidenced slight, asymmetrical ventricular dilatation, more marked in the right side and in the posterior part of lateral ventricles (Fig. 1e–g), and complete empty sella (Fig. 1h). FDG-PET demonstrated relative hypometabolism of basal ganglia, more prominent in the caudate nuclei, and a slight relative hypometabolism of the left temporo-parieto-occipital cortex (Fig. 2). Tetrabenazine, up to 50 mg daily, was prescribed, but the drug was withdrawn for insomnia and nervousness.

Patient 3, the 5 years old proband's son, born at term by cesarean delivery because of transverse position, received continuous positive airway pressure therapy for neonatal respiratory distress. The infant presented with multiple congenital anomalies: severe bilateral vesicoureteral reflux with pyelectasis and megabladder, patent foramen ovale, and congenital hypothyroidism for which thyroid replacement treatment was started.

Psychomotor development was delayed: sitting at 10 months, walking at 26 months, first words at 26 months, at present only few words in vocabulary and lack of sphincter control. His I.Q. was 76 at the age of

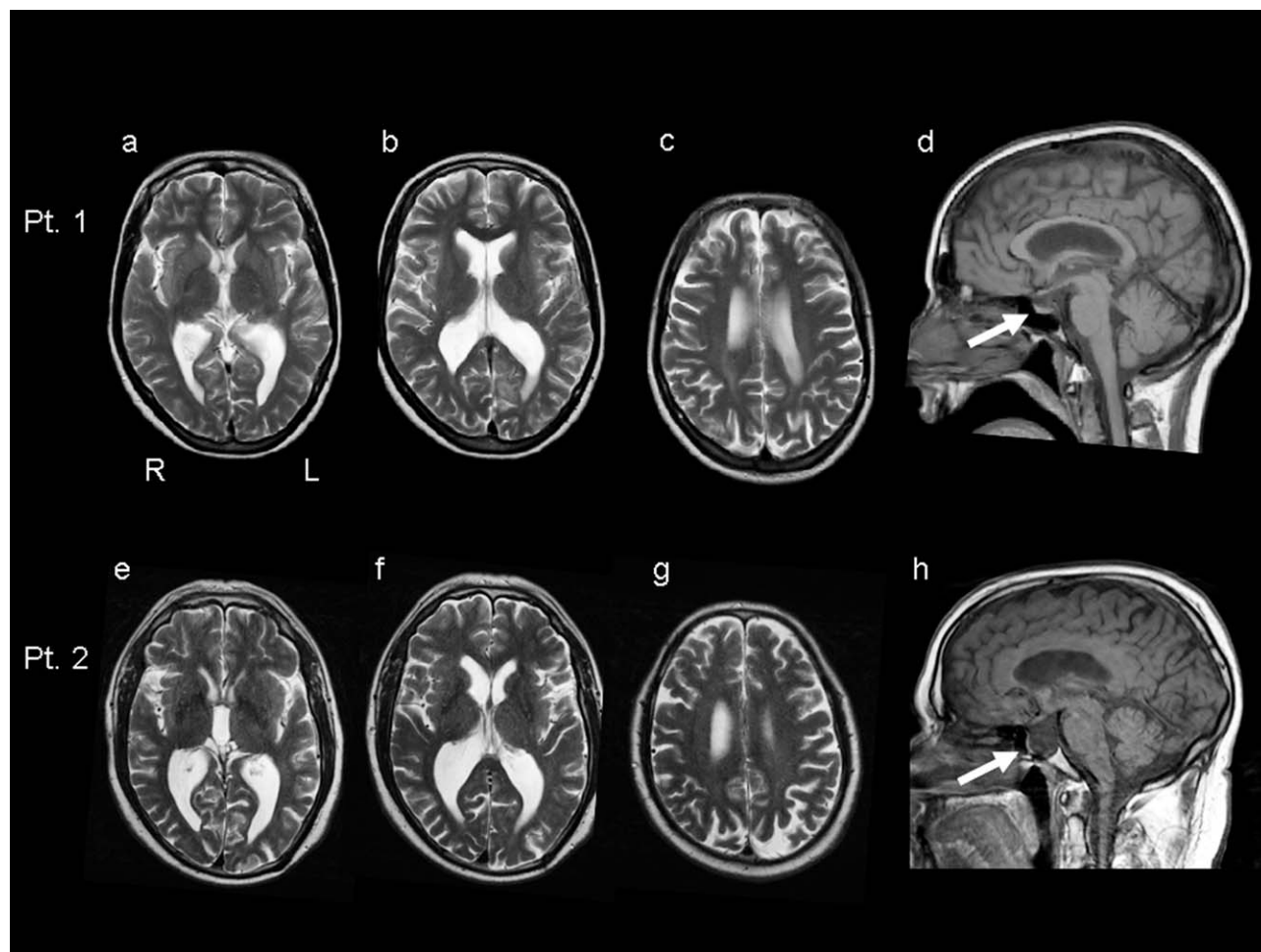


FIG. 1. Axial T2-weighted (Pt. 1 a–c; Pt. 2 e–g) and sagittal T1-weighted (Pt. 1 d; Pt. 2 h) MR images. Dilatation of supratentorial ventricular system is evident in both patients. Enlargement of the third ventricle is also evident in Patient 2. In both patients there is evidence of empty sella, partial in Patient 1, complete and prominent in Patient 2.

4 years. He is a pleasant boy, has no behavioral problem and developed normal social relationships. At the age of 4 years he developed slight, generalized choreic movements (video).

Molecular analysis: Direct sequencing of the *TITF-1* gene showed, in all the 3 patients, the new heterozygous mutation C609A in exon 2, resulting in a substitution of serine at codon 145 for a stop codon (S145X). The mutation predicted a truncated protein of about 14.5 kDa that lacks the entire homeodomain and the carboxy-terminus portion.⁵

DISCUSSION

BHC shows heterogeneity of the clinical presentation within and among the families. In the present family the neurologic presentation, characterized by mild

motor delay, early-onset dyskinesias, and slightly lower intelligence, was quite similar in the 3 patients, although the abnormal movements are somewhat different among individuals. Although chorea is the movement disorder characteristic of BHC, dystonia, myoclonic jerks, and ataxia have been also described.⁶ The distinction among chorea, myoclonus, and jerky dystonia may be difficult. The diagnosis of chorea, which is characterized by a random flow of rapid, unpredictable abnormal movements,⁷ better applies to Patients 1 and 3, whereas the sudden, more predictable and repetitive abdominal jerks in Patient 2 seem to be more consistent with myoclonus. As described in other patients with BHC,⁶ dyskinesias, contrary to myoclonus-dystonia, were not worsened by action nor improved by alcohol.

Concerning extra-neurologic features subclinical hypothyroidism was present in Patients 1 and 2, whereas

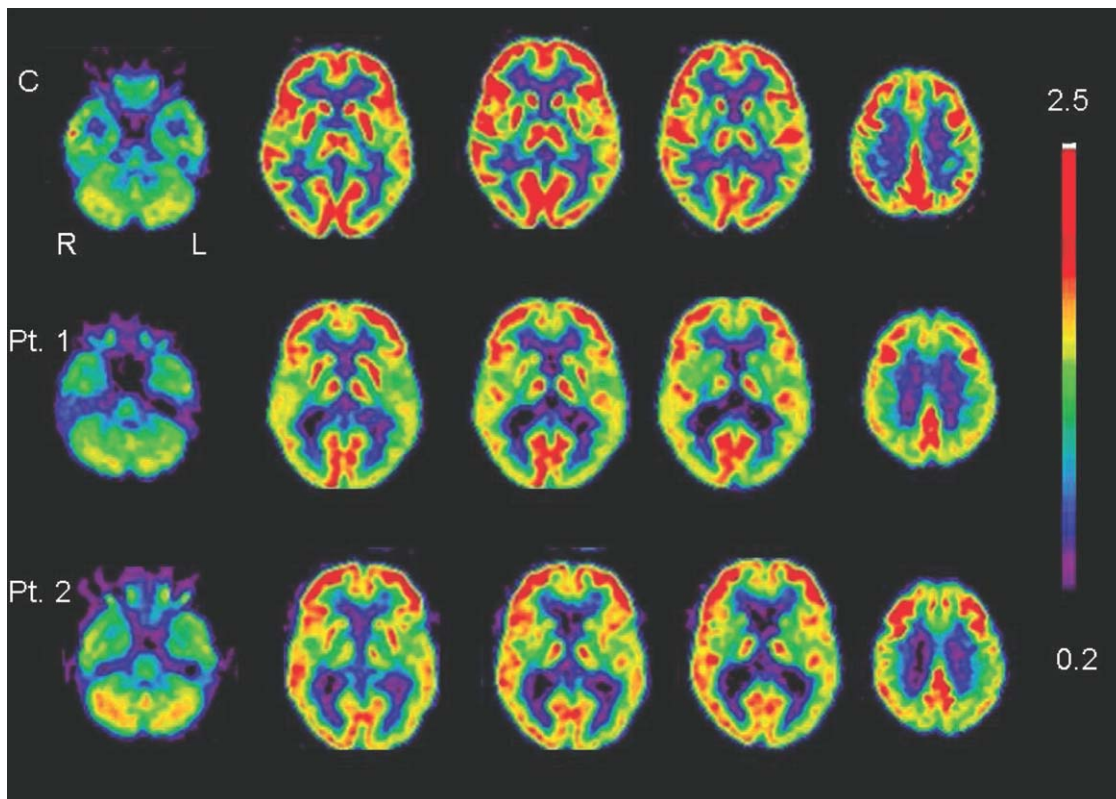


FIG. 2. Axial images of brain ^{18}F -deoxy-glucose uptake obtained with PET in a 39 years control, in Patient 1 and in Patient 2. The images were spatially normalized into the Montreal Neurological Institute (MNI) space and normalized to globals. The scale shows values of highest uptake in red and lowest uptake in blue. In Patient 1 a mild reduction of tracer uptake is present in the caudate nuclei and in the medial frontal and temporo-parietal cortex, bilaterally. The basal ganglia hypometabolism is more marked in Patient 2, involving more the caudate than the putamen regions. In Patient 2 there is also a mild temporo-parietal metabolism reduction on the left side. L left, R right.

Patient 3 had congenital hypothyroidism and neonatal respiratory distress. Anticipation and more severe phenotype in subsequent generations have been suggested,⁸ but not demonstrated in BHC. Environmental factors and genetic background might also influence the clinical expression. A review of the reported cases^{1,2,6,9-16} reveals 11 cases of congenital hypothyroidism due to *TITF-1* mutations in patients with de novo mutations or with no information about parental phenotype or genotype, 11 (including the present one) with maternal inheritance of the allele carrying the mutation, and one with paternal inheritance.¹⁶ However, there are also reports of maternal inheritance without congenital hypothyroidism. The predominance of maternal inheritance of congenital hypothyroidism in BHC may be due to chance or may be related to imprinting or maternal environment.

It remains unclear if some peculiar features of our patients, as postpartum psychosis in Patient 1 and urinary tract malformations in Patient 3, are related to the mutation. Psychosis occurred in two previously reported patients^{16,17} and hypospadias has been described before

recognition of the molecular defect.^{18,19} We are not aware of a role of *TITF-1* in urinary tracts organogenesis, although the gene is expressed in small cell carcinoma of the urinary bladder.²⁰ We suggest special attention to urinary tract malformations in patients with BHC.

Imaging data also appear to be heterogeneous in BHC. CT/MRI findings are usually normal, but ventricular dilatation and other abnormalities have been also reported.^{10,21,22} A cystic mass in the posterior part of the sella turcica has been described in two cases.²² In the 2 patients investigated by us, MRI showed ventricular dilatation, more evident at trigone and occipital horn level, whereas in HD ventricular enlargement mostly affects the frontal horns.²³ Empty sella was present in both patients, more marked in Patient 2, which has the longest disease duration. Haploinsufficiency of the *TITF-1* gene could lead to congenital deficiency of the sellar diaphragm, which is a frequent cause of an enlarged sella. FDG-PET scan was reported to be normal in 4 patients with BHC,⁹ although a study performed when the molecular diag-

nosis was not available showed caudate hypometabolism.²⁴ More recently reduction of technetium 99 m ethyl cysteinate dimer uptake has been demonstrated in the basal ganglia of two children studied by SPECT.²⁵ Using FDG-PET we showed cortex and basal ganglia hypometabolism in both Patient 1 and Patient 2. These findings are consistent with the significant reduction of striatal and neocortical interneurons demonstrated by immunohistochemical staining in BHC²⁶ and with the patients' choreic syndrome and mild cognitive impairment. The pattern of metabolic changes is similar, but less severe than that found in HD,²³ consistently with the milder, non progressive BHC phenotype.

Legends to the Videos

Segment 1. Patient 1 examination shows generalized, moderate to marked, choreic movements involving the face, the neck, the trunk, the limbs, both proximally and distally. Finger-to-nose and walking do not worsen the abnormal movements. Mild unsteadiness is also evident.

Segment 2. Slightly staggering gait and mild limb choreic movements, not worsened by action, in Patient 2. Brisk abdominal wall contractions are evident.

Segment 3. In Patient 3 mild choreic movements involved the trunk and the four limbs, both proximally and distally, not worsened by action. Brisk myoclonic-like movements are also evident. Tottering was probably too marked for his age.

Acknowledgments: Financial Disclosures: Elena Salvatore, Luigi Di Maio, Alfonso M. Ferrara, Carlo Rinaldi, Francesco Saccà, Silvio Peluso, Paolo E. Macchia, and Sabina Pappatà: report no disclosures. Alessandro Filla: funded by research grants and serves as study coordinator of a project funded by the EUROSCA association. Giuseppe De Michele: receives research support from the EURO-HD Network.

Author Roles: Salvatore E.—Organization and execution of research project, writing of the first draft of the manuscript. Di Maio L.—Organization and execution of research project, writing of the first draft of the manuscript. Filla A.—Review and critique of manuscript. Ferrara AM.—Execution of research project. Rinaldi C.—Execution of research project and writing of the first draft of the manuscript. Saccà F.—Execution of research project. Peluso S.—Execution of research project. Macchia PE.—Organization of research project. Pappatà S.—Execution of research project and review and critique of manuscript. De Michele G.—Conception of research project and review and critique of manuscript.

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Long-Term Effect of Unilateral Pallidotomy on Levodopa-Induced Dyskinesia

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Abstract: Unilateral pallidotomy has been effectively used to treat parkinsonism and reduce levodopa induced dyskinesia (LID). We sought to determine the long-term effects of pallidotomy on LID in 10 patients who had initial benefit from pallidotomy but went on to require DBS surgery for symptom progression. The Dyskinesia Rating Scale (DRS) was used to rate and quantify LID in a blinded fashion. Though sample size was small, there was a trend towards a reduction in LID lasting up to 12 years suggesting that posteroventral pallidotomy may provide sustained benefit in reducing LID. © 2010 Movement Disorder Society

Key words: Parkinson's disease; pallidotomy; dyskinesia

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Potential conflict of interest: None reported.

Received 4 March 2009; Revised 27 May 2009; Accepted 8 October 2009

Published online 21 June 2010 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/mds.23155

In the era before DBS, as well as currently, in many countries around the world, unilateral postero-ventral pallidotomy as a treatment for Parkinson's disease (PD) has been the surgical alternative of choice. Pallidotomy ameliorates parkinsonism and is particularly effective in reducing levodopa-induced dyskinesia (LID) most prominently in the contralateral hemibody.¹ Despite initial control of disabling symptoms, parkinsonism generally worsens several years following pallidotomy and many patients have subsequently undergone STN DBS when their symptoms again became resistant to medical regimens.^{2–4}

No long-term follow-up studies have blindly evaluated the persistent effects of unilateral pallidotomy on LID. It has been our personal experience that the anti-dyskinetic effects may be evident many years after the original surgery and Hariz reported that these effects could last up to 13.5 years.⁵

We sought to determine the long-term effect of pallidotomy on dyskinesia in a selected sample of patients who had previously undergone pallidotomy and were undergoing preoperative evaluation for STN DBS due to symptom progression. Given the extensive preoperative assessment for DBS, ON/OFF evaluations were available for review in these patients. We evaluated efficacy of pallidotomy on dyskinesia by comparing contralateral and ipsilateral dyskinesia at the STN-DBS preoperative evaluation. We postulated that there would be a difference between sides due to lasting effects of pallidal lesioning with less severe dyskinesia contralateral to the previous surgery.

PATIENTS AND METHODS

Patient Population

Ten patients (8 male) with PD and prior pallidotomy on average 7.3 years (range 2–12 years) earlier were evaluated for consideration of STN DBS. All patients were felt to have obtained an initial good response to pallidotomy with respect to parkinsonism and particularly LID. Not all patients had received pallidotomy at our center; pre and postoperative LID scores were available in 6 of the 10. Before DBS patients were evaluated under the protocol of the Core Assessment Program for Intracerebral Transplantation⁶ (CAPSIT) before STN DBS surgery. Dyskinesia was assessed using the Dyskinesia Rating Scale (DRS) (maximum score for unilateral limbs = 8). The dosage of anti-parkinsonian medication required by the patient was recorded; levodopa equivalent doses (LED) were calculated in a manner described elsewhere.⁷ Evaluations

Attached manuscript #6

Screening for mutations in the ISL1 gene in patients with thyroid dysgenesis.
Ferrara AM, Rossi G, Zampella E, Di Candia S, Pagliara V, Nettore IC, Capalbo
D, De Sanctis L, Baserga M, Salerno MC, Fenzi G, Macchia PE.
J Endocrinol Invest. 2011 Jul-Aug;34(7):e149-52. Epub 2010 Nov 8

Screening for mutations in the *ISL1* gene in patients with thyroid dysgenesis

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ABSTRACT. Context: Congenital hypothyroidism (CH) is a common endocrine disorder with an incidence of 1:3000-4000 newborns. In 80-85% of cases, CH is caused by defects in thyroid organogenesis, resulting in absent, ectopically located, and/or severely reduced gland, all conditions indicated as "thyroid dysgenesis" (TD). A higher prevalence of congenital heart diseases has been documented in children with CH compared to the general population. This association suggests a possible pathogenic role of genes involved in both heart and thyroid development. Among these, it can be included *Isl1*, a transcription factor containing a LIM homeodomain that is expressed in both thyroid and heart during

morphogenesis. **Objective:** In the present study, we investigate the role of *ISL1* in the pathogenesis of TD. **Settings and patients:** By single stranded conformational polymorphism, we screened for mutations the entire *ISL1* coding sequence in 96 patients with TD and in 96 normal controls. **Results:** No mutations have been found in patients and controls. **Conclusion:** Our data indicate that, despite the relevant role of *ISL1* in thyroid and heart morphogenesis, mutations in its coding region are not associated with TD in our group of patients.

(J. Endocrinol. Invest. 34: e149-e152, 2011)
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INTRODUCTION

Primary congenital hypothyroidism (CH) is the most frequent endocrine metabolic disease in the infancy, with an incidence of 1/3000-4000 live birth. In about 15% of cases, the disease is caused by inborn errors in the mechanisms required for thyroid hormone biosynthesis, a condition known as dysmorphogenesis. In the remaining 85% of the cases, CH is caused by alterations occurring during the thyroid gland organogenesis, that results in a thyroid that is completely absent (athyreosis), severely reduced in size (hypoplasia) or located in abnormal position (ectopy). All these entities are grouped under the term of "thyroid dysgenesis" (TD) (1).

Several genes have been found mutated in patients with TD, including *NKX2.1*, *FOXE1*, *PAX8*, *TSHR*, and *NKX2.5*, suggesting that in a small percentage of cases TD is a genetic disorder (2).

Congenital malformations, mostly cardiac, have higher frequency in children with CH than in the general population (3-5), and we recently demonstrated that *NKX2.5*, a transcription factor that plays a crucial role in heart morphogenesis (6-8) and found altered in patients with congenital heart disease (9-12), can also be associated to TD

(13). These observations suggest that genes expressed during embryogenesis of both thyroid and heart can be good candidates in the pathogenesis of TD. One of these is the gene encoding for *ISL1*, a LIM homeodomain transcription factor originally proven to be necessary for the differentiation of both exocrine and endocrine pancreas (14). Pancreatic organogenesis requires *Isl1* activity in endoderm progenitors as well as in the mesenchymal tissues surrounding the dorsal pancreatic bud. A similar expression pattern of *Isl1* has been demonstrated in the anterior foregut and adjacent cardiogenic mesoderm in early steps of mouse (15) and *Drosophila* cardiogenesis (16). *Isl1* knock out mice show severe malformations of the heart and of the cardiac outflow tract that leads to embryonic lethality around E10.5 (15). Since thyroid progenitor cells assemble in this portion of the endoderm, it can be hypothesized that *Isl1* might also play a role in this process. This possibility is also suggested by recent findings demonstrating that *Isl1*-regulated cardiac morphogenesis is mediated via *Sonic hedgehog* (*Shh*) (17) and that *Shh* null mice exhibit thyroid malformations (18, 19). Finally, *Isl1* has previously reported to be expressed in the early thyroid placode in chicken (20) and in the adult rat thyroid gland, although only C-cells showed *Isl1* immunoreactivity in this study (21).

Very recently, it has been showed that, in mouse embryo, *Isl1* is widely expressed in the anterior foregut endoderm including the thyroid primordia. During further developmental steps, *Isl1* expression is maintained in all thyroid progenitor cells until the anlagen fuse at 13.5. Therefore *Isl1* expression seems to be restricted to the C-cells precursors, and it is down-regulated in the presumptive follicular cells. The adult thyroid contains only few *Isl1*-positive cells, but their number is in clear minority compared

Key-words: Congenital hypothyroidism, development, hearth, thyroid dysgenesis, transcription factor.

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Accepted May 24, 2010.

First published online November 8, 2010.

to the total number of C-cells. These data suggest that *Isl1* is a putative transcriptional regulator of both follicular and C-cell during thyroid organogenesis (22). Given the foregoing, we considered *ISL1* as a good candidate gene for TD. In the present work, *ISL1* mutational screening was carried out in a group of 96 patients with TD but no mutation has been identified.

MATERIALS AND METHODS

Patient recruitments

Ninety-six patients with permanent CH (35 with ectopy, 31 with hypoplasia, 30 with athyreosis), were included in the study. For all of them, together with blood sample, pediatricians provide a questionnaire with several clinical informations. With the exception of 3 cases, no congenital heart defects have been indicated in the questionnaire, suggesting that patients did not presented relevant cardiac alterations. In 3 patients minor heart congenital defects have been reported: 2 patients (1 with thyroid ectopy and the other 1 with thyroid hypoplasia) with patent foramen ovale at birth that resolved spontaneously and 1 (with thyroid ectopy) with minor pulmonary valve insufficiency. Screening for mutations in other genes associated to TD (*NKX2.1*, *FOXE1*, *PAX8*, *TSHR*) and in *NKX2.5* (associated with TD and congenital heart disease) was negative in all the patients. Also 96 healthy subjects with no thyroid-related disorders were also enrolled as controls. The study was approved by the Ethics Committees of the participating Institutes. All individuals, or parents of minors, gave their informed consent.

Single stranded conformational polymorphism

The entire *ISL1* coding region was screened for mutations on genomic DNA extracted from peripheral blood lymphocytes. Oligonucleotide primers (sequence available upon request) were designed on the human *ISL1* cDNA (NM_002202) and genomic sequences to produce PCR products suitable for single stranded conformational polymorphism (SSCP) analysis (Fig. 1). PCR

reactions were carried out in 10 µl reaction volume with the following cycling parameters: 94 C, 8 min; 94 C, 45 sec; the specific temperature for each exon, 30 sec; 72 C, 45 sec for 35 cycles; 72 C, 15 min. Samples were denatured 1:1 in denaturing solution (99% formamide, 1% xylene cyanol solution, 0.1% bromophenol blue) at 95 C for 5 min and thereafter directly placed on ice to prevent reannealing of the single stranded product. Six µl of each sample were applied to a non-denaturing gel of acrylamide (GeneGel™ Excel 12.5/24 Kit, GE Healthcare Bio-Sciences AB, Sweden) and run on a GenePhor™ Electrophoresis Unit (GE Healthcare Bio-Sciences AB, Sweden). The running conditions were the following: plate temperature: 15 C, 600 V, 25 mA, 15 W. The running was stopped when bromophenol blue reached the anode buffer strip (at least 80 min). Following separation, we stained nucleic acids in acrylamide gel using the PlusOne™ DNA Silver Staining Kit (GE Healthcare Bio-Sciences AB, Sweden), following producer's protocol.

Direct sequencing

PCR products of the samples showing an abnormal migration at the SSCP were purified with Antarctic Phosphatase-Exonuclease I (New England BioLabs, USA) at the following conditions: 37 C for 15 min and 80 C for 15 min, respectively for enzymes activation and inactivation, and then, directly sequenced bi-directionally with a 377 ABI sequencer.

Statistical analysis

Statistical analysis were performed using the 2x2 contingency table available on line at <http://graphpad.com/quickcalcs/contingency1.cfm>. *p* values were calculated using the Fisher's exact test.

RESULTS

SSCP method was used as first approach to screen for mutations the *ISL1* coding sequence in patients with TD using specific primer-pairs. Examples of the SSCP are

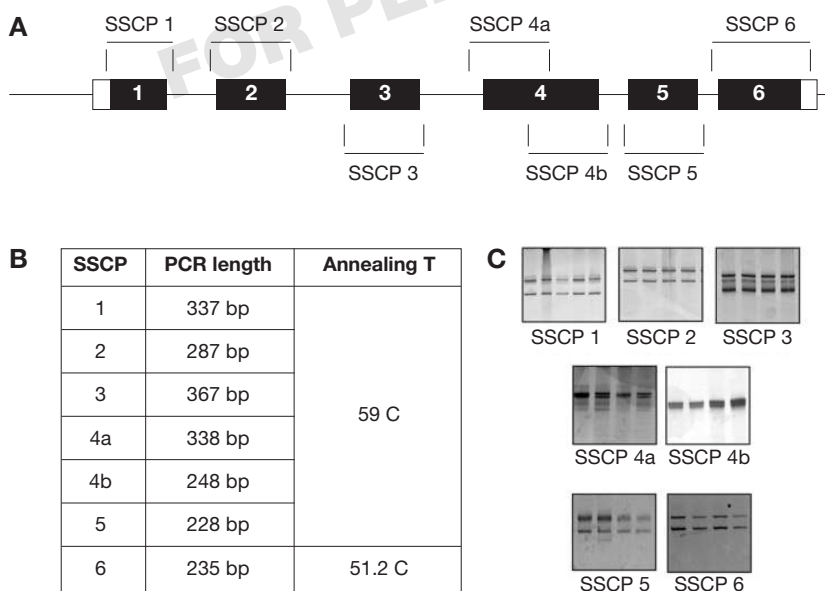


Fig. 1 - Panel A shows a schematic representation of the human *ISL1* gene and the location of the primers used for single stranded conformational polymorphism (SSCP) analysis. In panel B, the PCR product length and the annealing temperature of the respective PCR amplification are reported for each DNA exon. In panel C, typical results of SSCP analysis are shown. Please note in SSCP4a the different migration patterns of samples 1 and 3 compared to samples 2 and 4. They correspond to a heterozygous CCA to CCG change in the triplet encoding for proline at position 168 of *ISL1*.

shown in Figure 1. No mutations have been identified in DNA extracted from either patients or controls. We found a silent polymorphism corresponding in a CCA to CCG change in the triplet encoding for proline at position 168 in 8 patients (4 with ectopy and 4 with hypoplasia) in heterozygous state and in 10 patients (5 with athyreosis, 2 ectopy, 1 with thyroid ectopy and patent foramen ovale and 2 with CH not further characterized) in homozygous state. The same polymorphism was found in 28 controls (8 in heterozygous state and 20 in homozygous state). This polymorphism has been previously reported (<http://www.genecards.org>). Statistical analysis (Fisher's exact test) demonstrated no significant difference between patients and controls.

DISCUSSION

The genetic origin of TD is still widely discussed, despite several genetic factors have been demonstrated as cause of CH in animal models and in a small percentage of patients with TD.

Since TD is frequently associated to other congenital malformations, mostly cardiac (3-5), it is possible to hypothesize that genes involved in heart organogenesis might also participate to thyroid development. The ventral pharyngeal endoderm, from which the thyroid follicular cells originate, lies in close opposition to the heart mesoderm, and the role of the endoderm in the "specification" and differentiation of myocardial cells has been already defined (23). Conversely, the influence of the developing heart on thyroid organogenesis has not been demonstrated, although a recent work identify a critical role of developing arteries on thyroid development (18). The hypothesis of close relationship between developing heart and thyroid is also confirmed by the recent identification of mutation in the NKX2.5 gene, a transcription factor critical for the heart morphogenesis, in patients with TD (13). Based on these findings, we are investigating the possible role of genes involved in heart development for their role in thyroid organogenesis, and *ISL1* could be considered an attractive candidate in the pathogenesis of TD.

Cai et al. showed that *Isl1* null mice die at approximately E10.5 because of severe cardiovascular malformations. These are determined by the lack of *Isl1*-expressing cardiogenic progenitor cells in embryonic heart and as consequence of a decreased cell proliferation and increased apoptosis in the endoderm and in the adjacent splanchnic mesoderm in *Isl1* null mice embryos (15).

More recently Wendl et al. demonstrated a role for cardiac mesoderm in early thyroid development (24): thyroid progenitor cells were missing and the thyroid failed to develop in zebrafish mutants deficient of *Hand2*, a transcriptional factor involved in heart development. Since *Hand2* was suggested to function downstream of *Isl1* in the developing heart (25), it might be possible that the *Isl1*-dependent signals in the pharyngeal mesoderm might also influence the growth of the thyroid bud.

Westerlund et al. speculated that *Isl1* might be an interesting gene involved in the control of the early phases thyroid formation (thyroid specification), and demonstrated that *Nkx2.1*-positive thyroid progenitor cells express *Isl1* during evagination from the pharyngeal endo-

derm at E10.75 and when the thyroid is completely detached at E11.5 (22). Moreover, *Isl1* is expressed in the endoderm of the 4th pouches at E10.75 and in the completely detached E12.5 ultimobranchial bodies, from which calcitonin-producing cells (C-cells) originate. At E13, *Isl1* expression is present in the trachea, in the ultimobranchial bodies, in midline thyroid primordium, and in the mesenchyme surrounding the trachea, but, after the fusion of the cells deriving from the ultimobranchial bodies with the midline precursors of the follicular thyroid cells at E13.5, *Isl1* is present only in the thyroid, while its expression is markedly decreased in the midline mesoderm and trachea. *Isl1* expression disappears when the number of C-cells gradually increase between E15.5 and E17.5 (22). Of note, starting from E9.5 the thyroid placode is significantly smaller in *Isl1*^{-/-} mice when compared wild type embryos, and the thyroid follicular cells at this stage present a normal *Nkx2.1* and *Pax8* expression (22). Finally, in cardiac progenitors *Nkx2.5* is transcriptionally regulated by *Isl1* (26). Takeuchi and coworkers studied the upstream regulatory regions of the proximal *Nkx2-5* enhancer and identified a conserved *Isl1*-binding site, adjacent to the Gata site required for *NKX2.5* expression in the anterior heart field. Mutations in this *Isl1*-binding site abolish *Nkx2.5* expression in the right ventricle and in the outflow tract. We previously demonstrated the association between *NKX2.5* mutation and thyroid dysgenesis (13), and Takeuchi's data clarify the strong interaction between these two transcription factors, at least in the developing heart.

All these observations allow to hypothesize a possible role of *ISL1* also in the thyroid development. In the present study, the entire coding region of the *ISL1* gene was screened for mutations in patients with CH due to thyroid dysgenesis in order to evaluate the potential role of this gene in the pathogenesis of TD. We were not able to demonstrate mutations in the *ISL1* coding sequence in 96 patients with TD as well as in 96 healthy normal controls.

The absence of *ISL1* mutations in our patients well correlate with the complex phenotype observed in *Isl1* knock-out mice, were the severe cardiac malformations are responsible for early embryonic lethality (22), and it is possible to hypothesize that alterations in this gene are not compatible with life also in humans as well as in mice. Our results do not exclude the potential role of *ISL1* in the pathogenesis of TD, since we have not investigated other genes potentially necessary for *ISL1* activity, including cofactors or cell-specific co-activators. Moreover, mutation(s) in the *ISL1* promoter responsible for a decrease in the expression of the gene, would have been undetected in this study as well as mutations occurring in intronic regions of the gene that can produce inactive splice variants.

In conclusion, despite the fact that *ISL1* was a good candidate for TD, we did not find mutations in the coding region of this gene in patients with TD alone as well as in the 3 patients with TD and congenital heart disease. Our results could be explained by the essential role of this factor in embryonic heart development. As described in mouse models, it should be hypothesized that patients with *ISL1* mutations die before birth because of heart ma-

cor defects. In these patients TD might be not diagnosed and the detection of *ISL1* mutations lost.

ACKNOWLEDGMENTS

This work was supported by a grant of the Italian Ministry of Education, University and Research (PRIN 2007) to PEM.

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