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BRIEF REPORT

Functional Analysis of Monocarboxylate Transporter 8 Mutations Identified in Patients with X-Linked Psychomotor Retardation and Elevated Serum Triiodothyronine

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Context: T_3 action in neurons is essential for brain development. Recent evidence indicates that monocarboxylate transporter 8 (MCT8) is important for neuronal T_3 uptake. Hemizygous mutations have been identified in the X-linked *MCT8* gene in boys with severe psychomotor retardation and elevated serum T_3 levels.

Objective: The objective of this study was to determine the functional consequences of *MCT8* mutations regarding transport of T_3.

Design: MCT8 function was studied in wild-type or mutant MCT8-transfected JEG3 cells by analyzing: 1) T_3 uptake, 2) T_3 metabolism in cells cotransfected with human type 3 deiodinase, 3) immunoblotting, and 4) immunocytochemistry.

Results: The mutations identified in *MCT8* comprise four deletions (24.5 kb, 2.4 kb, 14 bp, and 3 bp), three missense mutations (Ala224Val, Arg271His, and Leu471Pro), a nonsense mutation (Arg245stop), and a splice site mutation (94 amino acid deletion). All tested mutants were inactive in uptake and metabolism assays, except MCT8 Arg271His, which showed approximately 20% activity *vs.* wild-type MCT8.

Conclusion: These findings support the hypothesis that the severe psychomotor retardation and elevated serum T_3 levels in these patients are caused by inactivation of the MCT8 transporter, preventing action and metabolism of T_3 in central neurons. **(*J Clin Endocrinol Metab* 92: 2378–2381, 2007)**

THYROID HORMONE IS essential for the development of the brain (1, 2). The bioavailability of T_3 in the brain is locally regulated through the interplay of two types of cells, astrocytes and neurons. Astrocytes express the type II deiodinase that converts the prohormone T_4 to T_3, which is subsequently transported to the neurons, the major target cells for thyroid hormone in the brain (3, 4). In addition to nuclear T_3 receptors, neurons express the type III deiodinase (D3), which catalyzes termination of T_3 activity. Multiple transporters are involved in cellular iodothyronine uptake and efflux in different tissues (5, 6). Recent evidence suggests that monocarboxylate transporter 8 (MCT8) is important for T_3 uptake into central neurons (7, 8).

The *MCT8* gene is located on chromosome Xq13.2; depending on which of the two possible translation start sites is used, it codes for a protein of 613 or 539 amino acids, containing 12 putative transmembrane domains (TMDs). MCT8 is expressed in numerous human tissues, including brain, heart, placenta, lung, kidney, skeletal muscle, and liver. We and others have reported on patients with mutations in the *MCT8* gene (9–13). These patients, all male, show a distinct phenotype of severe psychomotor retardation in combination with elevated serum levels of T_3.

In the present study, we provide functional characteristics

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Abbreviations: D3, Type III deiodinase; ex, exon; IB, immunoblotting; ICC, immunocytochemistry; MCT8, monocarboxylate transporter 8; TMD, transmembrane domain.

of six *MCT8* mutations. The functional consequences of mutations in MCT8 on cellular uptake and metabolism of T_3 were determined in JEG3 cells transfected with wild-type or mutant MCT8 alone or in cells cotransfected with MCT8 and D3.

Patients and Methods

Serum and DNA analyses

Approval for these studies was obtained from the medical ethical committee of the Erasmus Medical Center. Informed consent was obtained from the parents of all patients. Serum T_4, free T_4, T_3, and TSH were measured by Vitros ECI technology (Immunodiagnostic System; Ortho-Clinical Diagnostics, Beerse, Belgium). The coding sequence of *MCT8* was analyzed in patient DNA using intronic primers flanking the six exons.

Cloning and site-directed mutagenesis of human MCT8

Construction of a human MCT8 cDNA-containing pcDNA3 expression vector was described previously (14). The point mutations identified in patients (Table 1) were introduced in MCT8 cDNA using the QuickChange Site-Directed Mutagenesis protocol (Stratagene, Amsterdam, The Netherlands) and confirmed by sequencing.

Transfection of JEG3 cells

JEG3 cells were grown at 37 C in DMEM-F12 medium containing 9% fetal bovine serum and 100 nM sodium selenite and transfected at 70–80% confluency using 0.3 μl FuGENE 6 transfection reagent (Roche Diagnostics, Almere, The Netherlands) per 100 ng DNA. For T_3 uptake studies and immunoblotting (IB), cells were grown in six-well plates and transfected with 500 ng empty pcDNA3 or pcDNA3 containing wild-type or mutated MCT8 cDNA. Renilla luciferase vector (50 ng; pRL-SV40; Promega, Leiden, The Netherlands) was cotransfected to enable correction for transfection efficiency. For intact-cell T_3 metabolism assays, cells were cultured in 24-well plates and cotransfected with 100 ng pcDNA3 containing wild-type or mutant MCT8 and 100 ng pCI-Neo containing D3 cDNA (14). For analysis of D3 activity in cell lysates, JEG3 were cotransfected for 48 h with 500 ng wild-type or mutant MCT8 and 500 ng human D3 cDNA in six-well plates. For immunocytochemistry (ICC), cells were cultured on 15-mm coverslips and transfected with 100 ng cDNA.

T_3 uptake and metabolism assays, IB, and ICC

Two days after transfection, cells were rinsed with DMEM-F12 medium plus 0.1% BSA. For the T_3 uptake assay, the cells were incubated for 5 min at 37 C in 1.5 ml DMEM-F12/0.1% BSA containing 1 or 100 nM (2×10^5 cpm) [^{125}I]T_3 (Amersham Biosciences, Roosendaal, The Netherlands). Incubation was stopped by removing the medium and washing once with DMEM-F12/0.1% BSA. Cells were lysed with 0.1 M NaOH, and the lysates were counted in a γ counter. Renilla luciferase activity was measured in parallel wells according to the protocol of the manufacturer.

For the intact-cell T_3 metabolism assay, MCT8 and D3 (co)transfected cells were incubated for 4 h at 37 C in 0.5 ml DMEM-F12/0.1% BSA containing 1 nM (1×10^6 cpm) [^{125}I]T_3. After incubation, medium was harvested and analyzed by HPLC as described previously (14). For analysis of D3 activity in cell lysates, cells were harvested in PE buffer [0.1 M phosphate (pH 7.2), 2 mM EDTA] containing 1 mM dithiothreitol and sonicated. Appropriate dilutions of the sonicates were incubated for 1 h at 37 C with 1 nM (2×10^5 cpm) [^{125}I]T_3 in 0.1 ml PE buffer containing 10 mM dithiothreitol. Incubations were stopped, and samples were analyzed by HPLC. IB of transfected cell homogenates was performed as described previously (14). For ICC, cells were fixed and permeabilized with 4% paraformaldehyde and 0.2% Triton X-100 and stained with MCT8-specific polyclonal antibody 1306. The plasma membrane was stained using zona occludens 1 antibody (Invitrogen, Breda, The Netherlands). Alexa fluor 488 and 633 (Invitrogen) were used as detection antibodies; analyses were performed on a Zeiss Axiovert 100 confocal microscope (Zeiss, Sliedrecht, The Netherlands) using Zeiss LSM software.

Results

We report here on nine boys with severe psychomotor retardation, five of which have been presented previously (Table 1). The clinical phenotypes and serum thyroid parameters of the additional four patients are similar to those of patients with *MCT8* mutations described by us and others. All of our patients have been identified with severe psychomotor retardation, characterized by axial hypotonia, spastic or flaccid quadriplegia, dystonic movements, and absence of speech. Mean serum T_4 and free T_4 are decreased, TSH is mildly increased, and serum T_3 is markedly elevated. Patients are between 2.5 and 18 yr old and come from various ethnic backgrounds.

All nine patients were found to have different hemizygous mutations in *MCT8* (Table 1). Patient 1 has a 24,527 bp deletion, stretching from 15 kb upstream to 9 kb downstream of exon 1. Patient 2 has a 671C→T missense mutation in exon 2, resulting in an Ala224Val substitution located in the second putative TMD (9, 15). Patient 3 has a 1412T→C mutation in exon 5, resulting in a Leu471Pro substitution located in the ninth TMD (9). Patient 4 has a 2374 bp deletion, which comprises a large part of exon 3, entire intron 3 and exon 4, and part of intron 4. Patient 5 has a nonsense 733C→T mutation in exon 2, resulting in premature translation termination (Arg245stop) (9). Patient 6 has a missense 812G→A mutation in exon 3, resulting in an Arg271His substitution located in the second extracellular loop. Patient 7 has a 14-bp deletion (nucleotides 631–644), resulting in a frame shift and truncation of the protein at amino acid residue 235. Patient 8 has a G→C mutation in the acceptor splice site of exon 3, *i.e.* ACCT instead of AGCT. RT-PCR analysis of mRNA isolated from fibroblasts of his affected brother indicated the loss of 282 nucleotides from exon 3 and, thus, of 94 amino acids, including TMDs 4–6. Patient 9 has a 3-bp (TCT) deletion in exon 2, leading to deletion of Phe230.

TABLE 1. Nine patients with mutations in MCT8

Patient no.	1	2	3	4	5	6	7	8	9
Year of birth	1997	1999	2001	1998	2002	1987	1995	1999	2002
Mutation	del ex1	671C→T	1412T→C	del ex3,4	733C→T	812G→A	del 631–644	ex3 −1G→C	del 689–691
Protein	Absent	A224V	L471P	Truncated	R245X	R271H	Truncated	del 267–370	del F230
T_3 (N, 1.40–2.55 nmol/liter)	6.10	5.44	4.51	5.20	7.28	3.95	3.45	3.17	5.46
T_4 (N, 54–127 nmol/liter)	58	57	56	58	35	44	36	42	88
Free T_4 (N, 11–25 pmol/liter)	9.0	9.3	8.2	11.2	5.7	10.6	9.6	8.8	15.1
TSH (N, 0.10–4.0 mU/liter)	8.79	3.76	1.81	3.99	4.72	1.66	1.73	2.97	0.20
In vitro function (% of wild-type)	0[a]	0	0	0[a]	0	10–20	0[a]	0	0

N, Normal range.
[a] Synthesis of functional protein impossible.

Figure 1A shows the uptake of T_3 by JEG3 cells transfected with wild-type or mutant MCT8 cDNA after 5 min of incubation at 37 C. Significant T_3 uptake was observed in empty pcDNA3-transfected cells. JEG3 cells do not show endogenous expression of MCT8 (14). Therefore, this background uptake is likely facilitated by other, as yet unidentified, transporter(s). Transfection with wild-type MCT8 increased T_3 uptake 2.8-fold. Transfection of cells with MCT8 mutants Ala224Val, Leu471Pro, Arg245stop, splice site mutant ex3 −1G→C, and delPhe230 did not increase T_3 uptake over control cells. However, transfection with the MCT8 Arg271His mutant induced a modest but significant 1.4-fold increase in T_3 uptake ($P < 0.05$). Uptake experiments using 100 nM T_3 produced similar results (data not shown).

JEG3 cells transfected with control plasmid or MCT8 cDNA alone did not show significant metabolism of T_3, indicating very low deiodination capacity of these cells (data not shown). Figure 1B shows that transfection with D3 cDNA resulted in 11% metabolism of T_3 after 4 h. Cotransfection of cells with wild-type MCT8 and D3 greatly increased T_3 metabolism to approximately 60%. Cotransfection of cells with the MCT8 Ala224Val, Leu471Pro, Arg245stop, or delPhe230 mutants and D3 did not increase T_3 metabolism compared with D3 transfection alone. However, cotransfection with the MCT8 Arg271His mutant and D3 showed 20% metabolism, again indicating that some T_3 transport is preserved in this mutant.

D3 activity in lysates of JEG3 cells cotransfected with the different MCT8 mutants amounted to 85–123% of that in cells cotransfected with wild-type MCT8 (Fig. 1D), indicating that the impaired T_3 metabolism in intact cells cotransfected with mutant *vs.* wild-type MCT8 is indeed attributable to inhibited T_3 uptake rather than decreased D3 expression. IB (Fig. 1C) showed marked expression of wild-type MCT8 and of mutants Ala224Val and Arg271His, little expression of Leu471Pro and delPhe230, and no expression of splice site mutant ex3 −1G→C and Arg245stop. Control cells transfected with pcDNA3 also showed no expression of MCT8. ICC demonstrated marked plasma membrane expression of wild-type MCT8 and mutant Arg271His, whereas Ala224Val was mainly localized in the cytoplasm. Leu471Pro, ex3 −1G→C, and delPhe230 showed very little expression of protein (data not shown).

Discussion

We present nine unrelated young males with severe X-linked psychomotor retardation and elevated serum T_3 levels

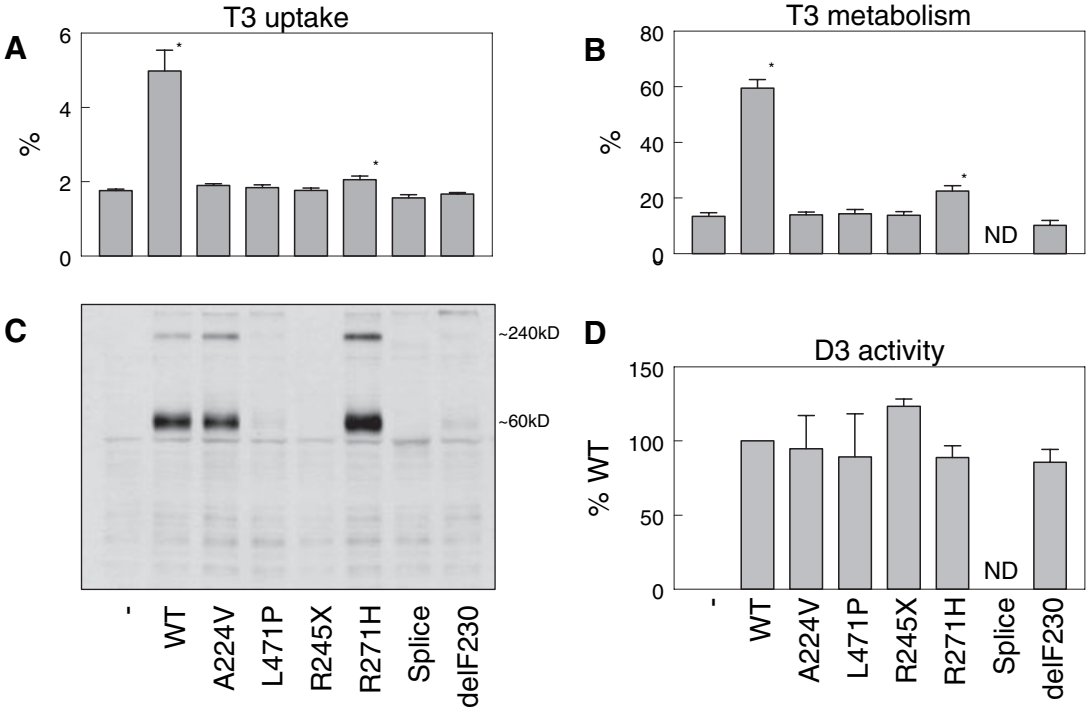

FIG. 1. A, T_3 uptake in wild-type or mutant MCT8-transfected JEG3 cells, shown as percentage of added T_3 after 5 min incubation. Transfection of wild-type MCT8 induces uptake approximately 2.8-fold compared with empty vector-transfected controls. Most mutants do not induce uptake, but Arg271His mutant induces a significant 1.4-fold increase. B, Metabolism of T_3 in intact JEG3 cells cotransfected with wild-type or mutant MCT8 and D3. Metabolism is shown as percentage of metabolites (3,3′-diiodothyronine and 3′-monoiodothyronine) in the medium after 4 h incubation. Wild-type MCT8 induces metabolism approximately 6-fold and Arg271His approximately 2-fold. The other mutants do not show induction of metabolism. C, Western blot of homogenates of JEG3 cells transfected with wild-type or mutant MCT8. Specific bands of approximately 60 and 240 kDa, representing monomeric MCT8 and a MCT8-containing protein complex, are detected clearly in wild-type and Ala224Val- and Arg271His-transfected cells. Less protein is detected for Leu471Pro and delPhe230; no expression is seen of splice site mutant ex3 −1G→C and Arg245stop. D, D3 activity in cell lysates, expressed as percentage of activity in cells cotransfected with wild-type MCT8 and D3. No significant differences in D3 activity are observed, indicating that reduced metabolism in intact cells transfected with mutant MCT8 is attributable to decreased intracellular availability of T_3 and not to differences in D3 activity. Uptake and metabolism data are presented as mean ± SE of four experiments. *, $P < 0.05$ *vs.* control. ND, Not determined; WT, wild type.

with mutations in the *MCT8* gene. We show that these mutations result in loss of function, demonstrated as reduced uptake and subsequent metabolism of T_3 *in vitro*. Several mechanisms may be involved in this loss of function, including reduced protein expression, impaired trafficking to the plasma membrane, or reduced substrate affinity. The mutant Ala224Val protein is clearly detectable by IB. However, ICC shows the protein to be mostly distributed in the cytoplasm, suggesting that this mutation inhibits trafficking to the plasma membrane. Much less Leu471Pro protein is detected by IB and ICC, suggesting that loss of function is correlated with reduced expression of the protein. The premature Arg245stop found in patient 5 results in a severely truncated MCT8 protein that cannot be detected with our polyclonal MCT8 antibody and does not have any functional activity. Mutant ex3 −1G→C is not detected by IB, and only very limited protein is observed in ICC. This suggests that the expression of this splice variant, although clearly detectable at the RNA level, is very limited at the protein level. The lack of T_3 transport by mutant delPhe230 can also be explained by the low expression of this protein. Mutant Arg271His shows significant residual transport capacity. IB shows high expression of the protein, and ICC indicates expression at the plasma membrane. Possibly, the partial loss of function is caused by reduced affinity for T_3.

Our findings associate the psychomotor retardation observed in MCT8 patients with loss of T_3 transport capacity. This illustrates that MCT8 is crucial for normal thyroid hormone-dependent development of the central nervous system in humans. Thyroid hormone plays a crucial role in processes such as cell migration, dendritic outgrowth, the formation of synapses, and myelination (16). Neurons are the major target cells for thyroid hormone, expressing T_3 receptors (17) and D3 and MCT8 (8). Loss-of-function mutations in MCT8 lead to reduced or absent supply of T_3 to neurons, resulting in impaired neurological development as well as a reduced clearance of T_3 by neuronal D3. The role of MCT8 in neuronal T_3 uptake was recently studied in MCT8 knockout mice by Dumitrescu *et al.* (18) and Trajkovic *et al.* (19). They show reduced T_3 concentrations, increased type II deiodinase activity, and reduced D3 activity in brain, reflecting local hypothyroidism, despite elevated serum T_3. Trajkovic *et al.* (19) also show reduced expression of the thyroid hormone-regulated genes TRH and RC3 in neurons, supporting hypothyroid state at the cellular level. It must be noted, however, that, although MCT8-deficient mice show reduced T_3 concentrations in the brain, they do not show an apparent neurological phenotype. This suggests differences in the role of MCT8 in the development of the central nervous system between the two species.

In conclusion, the experiments presented here support the hypothesis of reduced supply of T_3 to neurons in patients with mutations in *MCT8*. The severe psychomotor retardation observed in these patients clearly illustrates the important role of thyroid hormone in human neuronal development.

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