Monocarboxylate Transporter 8 in Neuronal Cell Growth

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Thyroid hormones are essential for the normal growth and development of the fetus, and even small alterations in maternal thyroid hormone status during early pregnancy may be associated with neurodevelopmental abnormalities in childhood. Mutations in the novel and specific thyroid hormone transporter monocarboxylate transporter 8 (MCT8) have been associated with severe neurodevelopmental impairment. However, the mechanism by which MCT8 influences neural development remains poorly defined. We have therefore investigated the effect of wild-type (WT) MCT8, and the previously reported L471P mutant, on the growth and function of human neuronal precursor NT2 cells as well as MCT8-null JEG-3 cells. HA-tagged WT MCT8 correctly localized to the plasma membrane in NT2 cells and increased T3 uptake in both cell types. In contrast, L471P MCT8 was largely retained in the endoplasmic reticulum and displayed no T3 transport activity. Transient overexpression of WT and mutant MCT8 proteins failed to induce endoplasmic reticular stress or apoptosis. However, MCT8 overexpression significantly repressed cell proliferation in each cell type in both the presence and absence of the active thyroid hormone T3 and in a dose-dependent manner. In contrast, L471P MCT8 showed no such influence. Finally, small interfering RNA depletion of endogenous MCT8 resulted in increased cell survival and decreased T3 uptake. Given that T3 stimulated proliferation in embryonic neuronal NT2 cells, whereas MCT8 repressed cell growth, these data suggest an entirely novel role for MCT8 in addition to T3 transport, mediated through the modulation of cell proliferation in the developing brain. (Endocrinology 150: 1961–1969, 2009)
Considerable physiological interest in MCT8 derives from the fact that mutations in the gene are associated with a condition of severe X-linked psychomotor retardation, accompanied by elevated serum T₃ concentrations (15–20). Patients with Allan-Herndon-Dudley syndrome, in which mutations in MCT8 have also been described (21–23), demonstrate a highly similar phenotype. MCT8 mutations have been reported in more than 20 families to date (24). Affected males demonstrate moderate to severe psychomotor delay, marked hypotonia (sometimes associated with progressive spasticity and movement disorders or epilepsy), and limited or absent speech. Given that MCT8 is expressed in murine neurons (12) and that serum T₃ concentrations are elevated in patients with Allan-Herndon-Dudley syndrome associated with MCT8 mutations, it has been proposed that MCT8 has a critical role in neuronal development. Specifically it is believed that this syndrome reflects a defect in the neuronal entry of T₃ and therefore in the action and metabolism of T₃ in these cells, resulting in impaired neurological development and a decrease in T₃ clearance. However, the exact mechanism of MCT8 action on neuronal cells has not been addressed, and the influence of mutation on neuronal cell growth is not known.

We have therefore examined several critical markers of MCT8 function in human embryonic neuronal N-Tera2 (NT2) cells, a pluripotent embryonal cell line that displays characteristics of central nervous system precursors (25) and which we have shown to be T₃ responsive (26). We also assessed identical markers in JEG3 choriocarcinoma cells, which are both unresponsive to T₃ (27–29) and MCT8-null (20, 30). As well as wild-type (WT) MCT8 function, we examined the reported MCT8 mutant L471P, described in a local patient with severe psychomotor impairment (16, 20). Our data suggest a novel neurodevelopmental role for MCT8 in addition to T₃ transport.

Materials and Methods

Cell lines and plasmids

Pluripotent human CNS precursor NT2 cells (25) (European Collection of Cell Cultures, Salisbury, UK) were maintained in DMEM-Ham’s F12 (1:1) growth medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin. JEG-3 choriocarcinoma cells were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. Before T₃ uptake, proliferation and caspase activation experiments, plated cells were washed with serum-free medium, and fresh media were added, containing 10% charcoal-stripped FCS (TH-deplete) and supplemented with 0, 10, or 50 nM T₃ as described. All experiments were carried out on a minimum of three separate occasions.

The expression vector for human MCT8 was obtained from Professor Theo Visser (Erasmus Medical Centre, The Netherlands) and was modified to contain a C-terminal hemagglutinin (HA) tag. This new plasmid (WT MCT8-HA) was subject to site directed mutagenesis to generate a HA-tagged expression vector for the L471P MCT8 mutant. Additionally, cDNA encoding CD8 was kindly provided by Dr. S. Munro (MRC Laboratory of Molecular Biology, Cambridge, UK) and human MCT4 generously donated by Professor Andrew Halestrap (University of Bristol, Bristol, UK). cDNA encoding human μ-crystallin was obtained from Imagenes (Berlin, Germany). All cDNA was subcloned into the pcDNA3.1 expression vector (Invitrogen, Paisley, UK).

Transfections were carried out using FuGENE6 transfection reagent (Roche, Indianapolis, IN) at a 1:3 ratio FuGENE to DNA. Transfection efficiency was assessed using β-galactosidase staining concurrent with all other transfections and observed to be approximately 20–25% in NT2 and 50% in JEG-3 cells.

Immunofluorescence assays

NT2 cells were plated onto 22-mm² glass coverslips and transfected with WT or L471P MCT8-HA, with and without cotransfection of CD8. Forty-eight hours after transfection, cells were fixed in phosphate buffer containing 0.02% sodium azide, 2% glucose, and 2% paraformaldehyde; permeabilized in 100% methanol; and blocked in 10% normal goat serum. Cells were then incubated in rabbit anti-HA antibody (1 μg/μl) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with either mouse monoclonal anti CD8 antibody (Ancell, Bayport, MN) or mouse monoclonal anti-protein disulfide isomerase (PDI) antibody (Stressgen Bioreagents, Ann Arbor, MI). They were washed in PBS and incubated in Alexa Fluor 488 goat-antimouse and Alexa Fluor 594 goat antirabbit secondary antibodies (Molecular Probes, Eugene, OR). Confocal immunofluorescence microscopy was performed on two-dimensional images obtained using an LSM510 laser-scanning confocal microscope (Zeiss, Thornwood, NY).

Western blot analysis

NT2 and JEG-3 cells were grown in 6-well plates and transfected with 2.5 μg WT or L471P MCT8 or with vector only (VO) or hMCT4 plasmids as controls. As a positive control for endoplasmic reticulum (ER) stress, JEG-3 cells were treated with 1.5 μM thapsigargin. Forty-eight hours after transfection or treatment, proteins were prepared in lysis buffer containing enzyme inhibitors (1 mM phenylmethylsulfonylfluoride, 0.3 μM aprotinin, and 0.4 mM leupeptin) and denatured (30 min, 37 °C) in loading buffer. Western blot analyses were performed as described previously (31–33), using rabbit antimunoglobulin heavy chain-binding protein (BiP; Cell Signaling Technology, Danvers, MA), mouse anti-HA (Covance, Vienna, VA) or mouse anti-CCAAT/enhancer-binding protein homologous protein (CHOP; Affinity BioReagents, Golden, CO) primary antibodies. β-Actin expression was determined in all Western blot analyses.

Caspase-3 and -7 activity assays

The effect of MCT8 overexpression on caspase activity was determined using the luminescence based Caspase-Glo 3/7 assay (Promega Corp., Madison, WI), according to the manufacturer’s guidelines. NT2 or JEG-3 cells were seeded at densities of 5000 NT2 cells/well or 6000 NT2 cells/well into white 96-well plates (Corning Corp., Midland, MI), grown in T₃-deplete or replete media and transfected with 0.15 μg WT or L471P MCT8 plasmids or with VO or hMCT4 plasmids as controls. Forty-eight hours after transfection, Caspase-Glo 3/7 assay reagent was added to the cells, and plates were shaken for 30 sec at 300 rpm. Fluorescence was detected 90 min after addition of the homogenous caspase-3/7 reagent at an excitation wavelength of 485 nm and emission wavelength of 520 nm using an Orion luminoimeter (Flowgen, Nottingham, UK). All samples were assessed in triplicate.

Caspase-3 expression using small interfering RNA (siRNA)

MCT8 siRNA experiments were carried out using predesigned MCT8-specific siRNAs (sense strand, 5’-GCCUCGGCUACUUCGCUAUU-3’; Ambion Inc., Huntsville, TX). A scrambled siRNA (sense strand 5’-GGGGCCAGUUCUCAGUUCU-3’; Ambion) was used as a negative control. NT2 cells were transfected using siPORT NeoFX transfection reagent (Ambion). In brief, cells were trypsinized and suspended in culture medium and mixed with siRNA (to final dose of 10 nM) and NeoFX transfection reagent (4 μM/ml of medium) before plating and incubation for 72 h. In the absence of a specific MCT8 antibody, this technique was verified using JEG-3 cells transfected with HA-tagged WT MCT8. Forty-eight hours after transfection, cells were trypsinized and
treated with siRNA. Protein was harvested after 72 h to confirm silencing of transfected HA-MCT8.

**T$_{3}$ uptake assays**

Cells were seeded in 12-well plates at densities of 75,000 cells/well for NT2 or 60,000 cells/well for JEG-3 cells. Cells were grown in hormone-free media and subject to MCT8 silencing or transfected with a total of 500 ng of plasmid, of which 50% was WT, L471P MCT8, or human MCT4 or its empty vector as controls, and the other 50% was human $\mu$-crystallin (CRYM) or VO control. T$_{3}$ uptake assays were performed 48 h after transfection or 72 h after siRNA treatment. Cells were rinsed with DMEM:F12 containing 0.1% BSA and then incubated for 10 min at 37°C with DMEM:F12, supplemented with 0.1% BSA and either 1 or 1000 nM total T$_{3}$ (containing 10$^5$ cpm $^{[125]T}$, high specific activity; GE Healthcare, Chalfont St. Giles, Bucks, UK). Incubation was stopped by removing the medium and washing the cells quickly three times in DMEM:F120.1% BSA. Cells were lysed in 2% sodium dodecyl sulfate and lysates were measured in a $\gamma$-counter. T$_{3}$ uptake is expressed as a percentage of the $\gamma$-count apparent in sodium dodecyl sulfate lysates compared with the total $\gamma$-count added to the cells. Protein concentrations of lysates, measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK), were used to correct uptake reads for protein concentration relative to that of VO transfection or scrambled siRNA-treated controls.

**Cell survival and proliferation analyses**

3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assays were carried out on JEG-3 or NT2 cells seeded at densities of 5000 or 6000 cells/well, respectively, in 96-well plates, grown in T$_{3}$, replete media. Forty-five hours after transfection or 72 h after siRNA treatment, 100 $\mu$g of MTT was added to each well and incubated for about 3 h. Cell medium was removed and cells lysed in dimethylsulfoxide (DMSO) to solubilize the purple formazan crystals produced by the reduction of MTT in active mitochondria. The absorbance of the DMSO solution was then measured at 545 nm to estimate cell number. Experiments comprised four to eight replicates each and were performed on at least three separate occasions.

Mitogenesis was also estimated from the measurement of nuclear $^{3}$H-thymidine incorporation. Cells, seeded at densities of 25,000 NT2 or 20,000 JEG-3 cells/well of a 24-well plate, were cultured in T$_{3}$, replete media and transfected with 0.5 $\mu$g/well WT or L471P MCT8, MCT4, or their VO. Cells were incubated with 0.2 $\mu$Ci $^{3}$H-thymidine in serum-free media for the last 5 h of culture incubation. Cells were then washed twice in ice cold PBS, followed by 1 ml of cold 5% trichloroacetic acid, and left on ice for 20 min. Then 0.1 M sodium hydroxide was added to the cells before transfer to 4 ml of scintillant. Radioactive counts were determined by scintillation counting. Mitogenesis was assessed at 48 h. Experiments comprised three replicates each and were performed on at least three separate occasions.

**Statistical analyses**

Data were analyzed using Sigma Stat software (SPSS Science Software UK Ltd., Birmingham, UK). Data that followed a normal distribution were analyzed using a Student $t$ test or one-way ANOVA. Nonparametric data were analyzed using the Mann-Whitney test for comparison between two groups and the Kruskal-Wallis ANOVA for between-group comparison of more than two groups. Dunn’s method was used as multiple comparison procedure to isolate groups that differed from others. Significance was taken as $P < 0.05$.

**Results**

**Subcellular localization of WT and mutated MCT8**

The nature of MCT8 as a transporter protein implies that for the protein to be functional, it needs to be located in the plasma membrane. Hence, we began our studies by assessing the localization of WT MCT8, and its L471P mutant, in our cellular model of NT2 cells, a pluripotent embryonal cell line that displays characteristics of CNS precursors. In transiently transfected NT2 cells, HA-tagged WT MCT8 showed intense cell surface expression and colocalized with the plasma membrane marker CD8, confirming previous studies (Fig. 1A). A small proportion of protein colocalized with the ER marker PDI (Fig. 1B), indicating the continued synthesis of this protein. We next investigated the subcellular expression of the HA-tagged MCT8 mutant L471P. Whereas a small quantity of the L471P mutant protein did reach the membrane, showing limited colocalization with CD8 (Fig. 1C), the overall patterns of expression of L471P MCT8 and CD8 were different, with a larger quantity of L471P MCT8-CD8 retained intracellularly. Indeed, when HA staining for L471P MCT8-HA was compared with that for PDI, a greater degree of colocalization was evident (Fig. 1D), suggesting that a higher proportion of this protein was retained in the ER. Further examination of WT and L471P localization is provided in supplemental Fig. 1 (published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org).
MCT8 and ER stress

Expression of mutant or folding-incompetent proteins, or exhaustion of the protein folding capacity of cells by the overexpression of WT proteins, may cause ER stress and trigger the unfolded protein response (34). Thus, we addressed the hypothesis that cells overexpressing WT MCT8, or the L471P mutant, which is predominantly retained in the ER (see Fig. 1), may be prone to ER stress. We transfected NT2 or MCT8-null JEG-3 cells and performed Western blotting for two established markers of ER stress. BiP (GRP78) is involved in the recognition of unfolded proteins and is up-regulated in protective ER stress pathways (35, 36), whereas CHOP is up-regulated in apoptotic pathways of the unfolded protein response (37–39). As a positive control, JEG-3 cells were treated with 1.5 μM thapsigargin, which triggers ER stress through the inhibition of calcium ATPases in the ER (40, 41). Furthermore, we transfected cells with MCT4, a structural homolog of MCT8, which transports monocarboxylates but not THs (42).

We were unable to detect any significant changes in the expression of either CHOP or BiP in response to transfection with either WT or mutant MCT8 plasmids or with MCT4 compared with VO controls (Fig. 2A). The two cell lines investigated had differing endogenous levels of cellular ER stress, with CHOP and BiP both showing higher expression in JEG-3 cells than NT2s. Despite this, neither of the overexpressed MCT8 genotypes induced obvious changes in either marker in either cell line. Thus, the influence of WT or L471P mutant MCT8 on cell growth or neurodevelopment is unlikely to derive from the induction of ER stress.

The influence of WT and mutated MCT8 on caspase activation

Although MCT8 expression did not significantly influence ER stress, we considered the possibility that MCT8 mutation may alter cell apoptosis as a potential mechanism underlying neurodevelopmental delay in Allan-Herndon-Dudley syndrome. We therefore transfected NT2 and JEG-3 cells with WT or L471P MCT8 or MCT4. Functional caspase-3/7 assays demonstrated that transfection induced a mild increase in caspase activation compared with untreated controls (Fig. 2B). However, in assays carried out 24 h (data not shown) or 48 h after transfection, WT and L471P mutant MCT8 failed to significantly alter caspase-3/7 activity in both NT2 [Fig. 2B(i)] and JEG-3 [Fig. 2B(ii)]. Thus, neither WT nor L471P mutant MCT8 exert major influences on the caspase-3/7 apoptosis pathway of neural precursor NT2 cells or MCT8-null JEG-3 cells.

T₃ uptake studies in vitro

To examine the influence of our MCT genotypes on T₃ uptake in vitro, NT2 and JEG-3 cells were transfected with MCT4 and MCT8 plasmids as before, but in the presence and absence of a human CRYM cDNA. CRYM is a cytosolic T₃ binding protein that acts to regulate T₃-mediated transactivation and increases intracellular T₃ binding capacity (43–45). Experiments were carried out in the presence of 1 nM total T₃. Basal T₃ uptake by NT2 cells was approximately 2.4% and increased to 3.0% in the presence of CRYM. In NT2 cells in the absence of CRYM, MCT4 failed to alter T₃ uptake, whereas MCT8 yielded a 1.6 ± 0.2-fold induction to approximately 4.0% uptake (n = 3, P < 0.001) compared with VO control (Fig. 3A). The L471P mutant failed to augment T₃ uptake. In the presence of cotransfected CRYM (Fig. 3A), WT MCT8 demonstrated enhanced T₃ uptake to 9.2% (3.8 ± 0.5-fold increase compared with empty vector transfected cells, n = 3, P < 0.001). In contrast, L471P MCT8 failed to induce significant T₃ uptake, despite cotransfection with CRYM, indicating a failure of initial T₃ influx rather than subsequent increased efflux. All experiments were repeated at a subsaturating dose of 1 μM T₃ and, whereas percent uptake was decreased, showed similar results to those performed at 1 nM T₃ (supplemental Fig. 2).

JEG-3 cell data were in agreement with findings in NT2 cells (Fig. 3B). Basal T₃ uptake by JEG-3 cells was approximately
2.6% and increased to 3.1% in the presence of CRYM. At a T₃ concentration of 1 nM, WT MCT8 elicited a 1.7 0.1-fold increase in T₃ uptake to 4.3%, whereas L471P mutant MCT8 did not significantly alter uptake. MCT4 showed similar results to the L471P mutant. Cotransfection of CRYM augmented T₃ uptake mediated by WT MTC8 to 6.0% (2.3 0.1-fold increase compared with VO transfected cells, n = 3, P < 0.001) but did not significantly alter the activities of the other MCT genotypes. Again, experiments were carried out in parallel in 1 μM total T₃ and demonstrated identical trends (supplemental Fig. 3).

**Dose-dependent influence of MCT8 on NT2 cell survival**

The influence of MCT8 on cell survival and proliferation has not been investigated previously. NT2 cells were transiently transfected with a range of doses (0–200 ng/well of a 96 well plate) of MCT8-expressing vector and mitochondrial viability assessed by MTT assay after 48 h, in the absence of T₃. In comparison with matching quantities of empty vector, doses of 100 ng DNA and above resulted in significant repression of cell number (Fig. 4A). In contrast, the L471P mutant failed to significantly repress cell number at any of the doses examined (Fig. 4B). To examine whether such differences in cell viability reflected altered protein expression of WT and L471P MCT8, we examined exogenous protein levels at the doses (100, 150, and 200 ng/well) associated with significant proliferative effects (Fig. 4). Although the L471P protein was detected at on average approximately 30% lower efficiency than WT MCT8 in NT2 cells, forced high expression of the mutant (150 and 200 ng/well) failed to significantly alter cell survival (n = 3). **, P < 0.01; ***, P < 0.001.

**The effect of WT and mutant MCT8 on NT2 cell survival**

To investigate the unexpected finding that MCT8 suppressed cell proliferation in a dose-dependent manner, we examined the effect of our MCT8 genotypes on cell viability by MTT assay in response to a range of T₃ concentrations. VO

![FIG. 3. T₃ uptake studies in NT2 (A) and JEG-3 (B) cells. Cells were transfected with empty vector or MCT4 or with WT and mutated MCT8, both in the presence (CRYM) and absence (VO) of cotransfected β-cryallin. CRYM should bind to T₃ in the cytoplasm, altering the balance of equilibrium to promote T₃ uptake. Cells were incubated with 1 nM T₃ containing 10⁶ cpm [¹²⁵I]T₃ for 10 min and cell lysates counted on a γ-counter. T₃ uptake is expressed as a percentage of the total T₃ added, corrected for protein concentration (n = 3). **, P < 0.01; ***, P < 0.001.](https://academic.oup.com/endo/article-abstract/150/4/1961/2455890

![FIG. 4. Dose response of WT (A) or L471P (B) MCT8 transfection into NT2 cells in the absence of T₃. Cell survival was assessed 48 h after transfection of 0–200 ng of plasmid DNA/well of a 96-well plate through MTT assays. Data are expressed as OD at a 545 nm relative to that of matched dose VO-transfected NT2 cells. Dotted lines, WT and L471P protein expression was determined at transfected plasmid doses eliciting significant cell survival inhibition by WT MCT8. Whereas L471P was expressed marginally less efficiently than WT MCT8 in NT2 cells, forced high expression of the mutant (150 and 200 ng/well) failed to significantly alter cell survival (n = 3). **, P < 0.01; ***, P < 0.001.](https://academic.oup.com/endo/article-abstract/150/4/1961/2455890)
treatments demonstrated a mild proliferative effect of T3 on NT2 cells, with a 5% (P = 0.058) and 7% (P = 0.02) increase in turnover at 10 and 50 nM, respectively, compared with 0 nM (Fig. 5A).

To examine the possibility that MCT8, as a complex membrane protein, was inducing a nonspecific effect on cell viability, we also transfected cells with MCT4. MCT4 produced no significant inhibition of cell viability (Fig. 5A), in contrast to WT MCT8, which yielded an approximate 30% repression with each T3 dose (P < 0.001 in each case). The L471P mutation did not affect proliferation compared with VO controls.

To confirm that changes in cell survival were not artifacts of the MTT methodology, we repeated all experimental treatments using tritiated thymidine incorporation assays (Fig. 5B). Tritiated thymidine revealed a more pronounced proliferative response to T3 in VO transfected cells. However, WT MCT8 still inhibited mitogenesis at each dose of T3 examined (37, 37, and 26% at 0, 10, and 50 nM T3, respectively; P = 0.043, 0.006, and 0.004), and the L471P MCT8 mutant failed to replicate this inhibition of proliferation.

The effect of WT and mutant MCT8 on JEG-3 cell survival

To address the possibility that MCT8 may act to modulate cell survival in a manner independent of T3, or that NT2 cells exhibit an aberrant proliferative response to MCT8 due to endogenous MCT8 expression, we repeated viability and mitogenesis assays in an alternative cell line. JEG-3 cells, which have previously been shown to be MCT8-null (30) and are also null for functional thyroid hormone receptors, showed no evidence of a proliferative response to T3 in MTT (Fig. 5C) and 3H-thymidine incorporation assays (Fig. 5D). However, transient overexpression of WT MCT8 significantly repressed cell survival and mitogenesis, whereas MCT4 or the L471P mutant did not. Overall, there was a marked consistency of effect across both assays in the two cell types, indicating the novel finding that MCT8 represses cell proliferation independently of T3.

The effect of MCT8 silencing on NT2 cell proliferation

To further address the effects of MCT8 on cell proliferation, we used a specific MCT8-targeted siRNA construct to knock down endogenous expression of MCT8 in NT2 cells. The siRNA technique was first verified by silencing expression of WT MCT8-HA in transfected cells (Fig. 6A). We observed that 10 nM MCT8-targeted siRNA was sufficient to knock down expression of transfected MCT8-HA by 80–90%.

We therefore applied this methodology to untransfected NT2 cells. Cells were incubated for 72 h in 96-well plates after treatment with 10 nM scrambled or MCT8-targeted siRNA constructs before being subject to MTT assays. RNA was harvested from cells in parallel treatments, and real-time RT-PCR demonstrated up to 80% knockdown in endogenous MCT8 mRNA expression in MCT8-targeted siRNA-treated cells compared with scrambled controls (data not shown). In addition, T3 uptake assays revealed that MCT8 knockdown in response to treatment with 10 nM MCT8-targeted siRNA caused a 19% reduction in T3 uptake into NT2 cells (n = 3, P = 0.021; Fig. 6B). This transient knockdown was associated with 8.6, 7.2, and 8.3% inductions in mitochondrial activity, measured through MTT assays, at 0, 10, and 50 nM T3, respectively (n = 3, P < 0.02 in each case; Fig. 6C). Thus, in contrast to the overexpression studies, transient knockdown of MCT8 caused small but significant decreases in T3 uptake and increases in mitochondrial activity compared with scrambled siRNA-treated controls, supporting our observation that MCT8 acts to modulate cell survival and proliferation. Taken together, these data imply a critical role for MCT8 in the regulation of cell proliferation. Furthermore, this function is lost in the well-characterized L471P MCT8 mutation.

Discussion

Our understanding of the transport of THs into mammalian cells is rapidly developing, with an increasing number of proteins identified as potential TH transporters (45–48). These include members of the amino acid
MCT8 repression was associated with decreased T3 uptake. C, NT2 cells were grown in media containing 0, 10, or 50 nM T3 and were treated with 10 nM scrambled or MCT8-targeting siRNA. Seventy-two hours after treatment, cells were subject to MTT 105 cpm [125I]T3 for 10 min and cell lysates counted on a β-counter. T3 uptake is expressed as a percentage of the total T3 added, corrected for protein concentration. MCT8 repression was associated with decreased T3 uptake. C, NT2 cells were grown in media containing 0, 10, or 50 nM T3 and were treated with 10 nM scrambled or MCT8-targeted siRNA. Seventy-two hours after treatment, cells were subject to MTT assay for cell survival. Data are expressed as an absorbance measured at 545 nm. Depletion of endogenous MCT8 resulted in mild increases in cell survival (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

FIG. 6. Knockdown of native MCT8 expression in NT2 cells represses T3 uptake but promotes cell survival. A, Verification of the efficacy of MCT8 siRNA. Cells were transfected with WT MCT8-HA in T25 flasks. Forty-eight hours after transfection, cells were trypsinized and reseeded either without further treatment or with treatment with 10 nM nonspecific (scrambled, scr) or MCT8-targeted siRNA. Proteins were extracted after a further 72 h of culture and subject to Western blot with an anti-HA antibody. β-Actin staining was used to assess protein loading. UT, Untransfected. B, Effects of MCT8 knockdown on T3 uptake. T3 uptake studies in NT2 cells were performed 72 h after treatment with 10 nM scrambled or MCT8-targeting siRNA. Cells were incubated with 1 nM T3 containing 106 cpm [125I]T3 for 10 min and cell lysates counted on a y-counter. T3 uptake is expressed as a percentage of the total T3 added, corrected for protein concentration. MCT8 repression was associated with decreased T3 uptake. C, NT2 cells were grown in media containing 0, 10, or 50 nM T3 and were treated with 10 nM scrambled or MCT8-targeted siRNA. Seventy-two hours after treatment, cells were subject to MTT assay for cell survival. Data are expressed as an absorbance measured at 545 nm. Depletion of endogenous MCT8 resulted in mild increases in cell survival (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

and organic anion transporter families as well as monocarboxylate transporters. However, the only TH transporter to date demonstrated to be spontaneously mutated and associated with a clinical phenotype in man is MCT8, providing strong evidence for an important physiological role for this protein in psychomotor development.

The crucial clinical phenotype of MCT8 mutation is catastrophic neurodevelopment and global neurological impairment (15, 23, 24). Males with MCT8 mutations have elevated circulating concentrations of T4, despite normal serum TSH levels, and display a more severe clinical phenotype than observed in cases of congenital hypothyroidism, in which there is low or absent T3 supply but no abnormality of T3 transport. It is therefore possible that MCT8 confers some other property on developing neurons in addition to that mediated solely by T3. To this end, we investigated the direct influence of WT and the previously investigated L471P mutant MCT8 on T3 uptake, cell growth, and ER stress in a model of human embryonal CNS precursor cells.

When we examined T3 uptake into NT2 cells, MCT8 transfection was associated with an approximately 60% increase in uptake, which was augmented in the presence of CRYM. This result is consistent with a facilitative diffusion of T3 catalyzed by MCT8, which predicts that accumulation would be increased by the binding of T3 to intracellular CRYM. The L471P mutation failed to significantly alter T3 uptake. Similar findings were apparent in JEG-3 cells. This finding for the L471P MCT8 mutation is confirmed by recent uptake studies carried out by Jansen et al. (20) in JEG-3 cells.

The most significant observation in our study is that MCT8 expression in neuronal precursor cells caused a dose-dependent decrease in cell survival or mitogenesis. This was unexpected, given that T3 treatment per se elicited a mild pro-proliferative effect on NT2 cells with increasing concentration. These findings were confirmed in two distinct cell lines by the two different methodologies of MTT assay and 3H-thymidine incorporation. Overall, WT MCT8 repressed cell survival or mitogenesis by approximately a third compared with controls, indicating a significant magnitude of effect. The converse experiment provided additional support for this finding, in that when we repressed endogenous MCT8 expression in NT2 cells using a specific siRNA, we observed small but consistent increases in cell proliferation. In addition, a recent report has indicated a role for MCT8 in promoting differentiation, in which overexpression of MCT8 in murine ES cells was found to enhance neural-lineage differentiation (49). Our own data, which examined proliferation rather than differentiation, are in agreement with this, suggesting together that MCT8 may be involved in the repression of proliferation through the induction of differentiation.

The effect of MCT8 on cell proliferation was not merely a nonspecific effect of overexpression, as confirmed by our studies of MCT4, which showed no influence on cell survival or mitogenesis. One caveat here is that technical constraints prevented us from examining MCT4 localization at the cell membrane. However, our data demonstrated that the parallel overexpression of a related MCT family member did not result in altered cell growth. Interestingly, transient overexpression of L471P MCT8 [a single nucleotide substitution resulting in a single amino acid change in the ninth transmembrane region of MCT8 (10)] failed to alter proliferation, demonstrating a loss of original WT function in terms of both T3 transport and cell survival or proliferative effects. Very recently Jansen et al. (50) reported a possible genotype-phenotype correlation in patients with Allan-Herndon-Dudley syndrome (50), in which less severe psychomotor retardation was observed in patients with residual T3 transport capacity (24). It is possible therefore that mutants showing residual T3 transport activity may also retain levels of alternative MCT8 function, which impact on cell proliferation, also contributing to less severe phenotypes. It would be interesting therefore in future studies to examine a wide spectrum of MCT8 mutations and assess their impact on cell survival and mitogenesis.

WT MCT8 showed strong plasma membrane staining, whereas L471P was predominantly located in the ER. One potential explanation of our data is that transient overexpression of MCT8 results in the accumulation of unfolded proteins in the ER and hence triggers ER stress and cell death. To address this, we examined expression of two different and well-characterized genes known to be up-regulated in different stages of ER stress.
However, neither of the MCT8 genotypes induced obvious expression changes in CHOP (up-regulated in apoptotic pathways of the unfolded protein response) or BiP (a molecular chaperone involved in protein folding and the recognition of unfolded proteins) (35, 37) in either JEG-3 or NT2 cells after 48 h. Similarly, caspase-3/7-mediated apoptosis was not significantly altered by MCT8. Thus, the influence of MCT8 on cell growth or development is unlikely to be via the induction of ER stress or apoptosis but rather some as-yet undefined mechanism.

It is intriguing to speculate on whether these data shed any light on the neurological discrepancy between murine knockouts and patients with MCT8 mutation. We did not perform studies in murine cells or with murine Mct8. However, it is conceivable that proliferative responses mediated by WT and mutated MCT8 may differ in human and mouse neural cells against a background of the altered expression of related transporters and deiodinases. Other members of the monocarboxylate transporter family rely on ancillary proteins to ensure their correct plasma membrane localization and function: MCT1 and MCT4 require Basigin, or CD147, whereas the ancillary protein for MCT2 is Embigin (51, 52). It is possible that MCT8 may bind other proteins at the plasma membrane and act to modulate their function. Hence, mutation or incorrect localization of MCT8 may affect the action of other alternative proteins, which may differ between man and mouse, and may in turn elicit different effects on neuronal development. Further experiments are required to test these speculative notions.

Taken together, these experiments confirm that MCT8 elicits increased T3 transport into cells and that mutations in the protein negate this effect. However, they also suggest a potent influence on cell proliferation. This must be via an alternative pathway than T3 and noradrenaline, compounds that play important roles in the action of other alternative proteins, which may differ between man and mouse, and may in turn elicit different effects on neuronal development. Further experiments are required to test these speculative notions.

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