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The expression of thyroid hormone transporters in the human fetal cerebral cortex during early development and in N-Tera-2 neurodifferentiation

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Non-technical summary Thyroid hormones are important in brain development and they enter cells through thyroid hormone transporters at the cell membrane. Thyroid hormone transporters are thought to play an important role since gene defects in one of these transporters, MCT8, have been associated with severe mental retardation. This paper describes the expression of a range of thyroid hormone transporters in the human fetal brain during early pregnancy, and suggests that these transporters could regulate the supply of thyroid hormones into brain cells from very early in development. Surprisingly, the reduction of thyroid hormones and MCT8 expression do not affect the differentiation of an unspecialised cell to a specialised human nerve cell in the brain. Thyroid hormones and MCT8 are thus likely to affect other processes during human brain development. To find out how thyroid hormones influence human fetal brain development requires further research.

Abstract Associations of neurological impairment with mutations in the thyroid hormone (TH) transporter, MCT8, and with maternal hypothyroxinaemia, suggest that THs are crucial for human fetal brain development. It has been postulated that TH transporters regulate the cellular supply of THs within the fetal brain during development. This study describes the expression of TH transporters in the human fetal cerebral cortex (7–20 weeks gestation) and during retinoic acid induced neurodifferentiation of the human N-Tera-2 (NT2) cell line, in triiodothyronine (T3) replete and T3-depleted media. Compared with adult cortex, mRNAs encoding OATP1A2, OATP1C1, OATP3A1 variant 2, OATP4A1, LAT2 and CD98 were reduced in fetal cortex at different gestational ages, whilst mRNAs encoding MCT8, MCT10, OATP3A1 variant 1 and LAT1 were similar. From the early first trimester, immunohistochemistry localised MCT8 and MCT10 to the microvasculature and to undifferentiated CNS cells. With neurodifferentiation, NT2 cells demonstrated declining T3 uptake, accompanied by reduced expressions of MCT8, LAT1, CD98 and OATP4A1. T3 depletion significantly reduced MCT10 and LAT2 mRNA expression at specific time points during neurodifferentiation but there were no effects upon T3 uptake, neurodifferentiation marker expression or neurite lengths and branching. MCT8 repression also did not affect NT2 neurodifferentiation. In conclusion, many TH transporters are expressed in the human fetal cerebral cortex from the first trimester, which could regulate cellular TH supply during early development. However, human NT2 neurodifferentiation is not dependent upon T3 or MCT8 and there were no compensatory changes to promote T3 uptake in a T3-depleted environment.

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Abbreviations AMV, avian myeloblastosis virus; BBB, blood–brain barrier; CNS, central nervous system; D2, iodothyronine deiodinase type 2; D3, iodothyronine deiodinase type 3; LAT, system-L amino acid transporter; MCT, human monocarboxylate transporter; mct, animal monocarboxylate transporter; NT2, N-Tera 2 cells; OATP, organic anion transporting polypeptide; TH, thyroid hormone; TR, thyroid hormone receptor; T3, triiodothyronine; T4, thyroxine; RA, retinoic acid.

Introduction

The sensitivity of the human fetal central nervous system (CNS) to small reductions in thyroid hormone (TH) exposure has been suggested by studies associating maternal subclinical hypothyroidism and hypothyroxinaemia with long-term neurodevelopmental deficits in offspring (Haddow *et al.* 1999; Pop *et al.* 2003). Current evidence suggests a direct effect of maternal THs upon fetal CNS development, particularly in the first half of pregnancy before the onset of endogenous fetal TH production (de Escobar *et al.* 2004). In rodents, THs are known to affect the proliferation and differentiation of CNS cells (Jones *et al.* 2003; Garcia-Silva *et al.* 2002), neuronal migration (Lavado-Autric *et al.* 2003), synaptogenesis (Gilbert & Paczkowski, 2003) and dendritic branching (Heuer & Mason, 2003). These effects are mostly mediated by nuclear TH receptors (TRs), which bind the active TH ligand, triiodothyronine (T3), to influence TH-responsive gene transcription.

Whilst circulating THs are the major determinants of TH supply, effective TH action requires the uptake of THs into cells and pre-receptor regulation of local T3 bioavailability by iodothyronine deiodinases. It is postulated that astrocytes express deiodinase type 2 (D2), which activates circulating thyroxine (T4) to T3 locally. Subsequently, T3 is transported into neurons, these cells being the ultimate target for TH action and shown to express TRs (Kilby *et al.* 2000) and deiodinase type 3 (D3), which inactivates any excess T3 (Tu *et al.* 1999).

Specific transport mechanisms facilitate the cellular influx and efflux of THs (Hennemann *et al.* 2001). TH transporters are required for TH passage across the blood–brain barrier (BBB) and choroid plexus and into neuronal and glial cells. Interest has focused on monocarboxylate transporter 8 (MCT8), whose gene mutations in humans have been associated with severe global neurological impairment including psychomotor, speech and cognitive deficits with radiological evidence of cortical and subcortical atrophy, alongside elevated serum free T3 but normal or low free T4 concentrations (Dumitrescu *et al.* 2004; Friesema *et al.* 2004; Schwartz *et al.* 2005). Post-mortem examination of an affected boy has revealed loss of neurons in the cerebral cortex, cerebellum and basal ganglia (Dumitrescu *et al.* 2004).

TH transport has also been demonstrated by monocarboxylate transporter 10 (MCT10), several organic anion transporting polypeptides (OATP) and System-L amino acid transporters (LAT). They have slightly different affinities for THs and transport other compounds (Table 1). Their expressions have been described in human adult brains and rodent brains but there have been few studies of the early human fetal brain.

It has been hypothesised that the regulation of cellular TH entry by TH transporters is critical to timely TH action during human fetal brain development, particularly neurodevelopment. We have, therefore, investigated changes in the expression of TH transporters within the human fetal cerebral cortex during the first half of gestation and during the neurodifferentiation of N-Tera-2 (NT2) cells, a human pluripotent embryonal cell line with characteristics of CNS precursors, which can be terminally differentiated into functional neurons (Andrews *et al.* 1984). We assessed if compensatory changes in TH transporter activity occurred with T3 depletion and, specifically, if MCT8 expression is important in NT2 neurodifferentiation.

Methods

Ethical approval

All tissue samples were obtained with the approval of the East Birmingham and South Birmingham Local Research and Ethics Committees, with written parental consent, and within the terms of the UK's Human Tissue Act (2004) and the *Declaration of Helsinki*.

Human tissues

Sixty-five human fetuses of gestational ages between 7 and 20 post-menstrual weeks (weeks) were examined after surgical termination of pregnancy, and cerebral cortices were biopsied as previously described (Chan *et al.* 2002; Kilby *et al.* 2000). In brief, the fetal cortex was identified at

Table 1. Characteristics of the thyroid hormone transporters present in the brain

Protein	Gene	K_m T4 (μM)	K_m T3 (μM)	Alternative substrates	Localisation in human brain	Localisation in rats and mice	References
MCT8	*SLC16A2*	4.7 (rat)	4.0 (rat)	Unknown	Adult and fetus: neurons, blood brain barrier, choroid plexus.	Adult and fetus/pup: neurons, blood brain barrier, choroid plexus,	(Friesema *et al.* 2003; Heuer *et al.* 2005; Trajkovic *et al.* 2007; Roberts *et al.* 2008; Wirth *et al.* 2009;
			0.86 (human)		Adult: folliculo-stellate cells of anterior pituitary, ependyma of 3rd ventricle, hypothalamus.	tanycytes of 3rd ventricle, cerebellar Purkinje cells, cortex, striatum, hypothalamus, amygdala, hippocampus.	Kinne *et al.* 2010; Fliers *et al.* 2006)
MCT10 (TAT1)	*SLC16A10*	*Higher than for T3	*Lower or the same as MCT8	Aromatic amino acids	Unknown	Adult: neurons Adult rabbits: hypothalamus	(Friesema *et al.* 2008; Ramadan *et al.* 2006; Mebis *et al.* 2009)
OATP1A2	*SLCO1A2*	8.0	6.5	Various organic anions, neutral compounds, cations	Adult: blood brain barrier	Adult: blood brain barrier	(Gao *et al.* 2000; Reichel *et al.* 1999)
OATP1C1	*SLCO1C1*	0.09	?	Bromosulfoph-thalein, oestrogens	Adult and fetus: blood brain barrier	Adult and fetus/pup: blood brain barrier, choroid plexus, neurons, Adult rabbits: hypothalamus	(Pizzagalli *et al.* 2002; Sugiyama *et al.* 2003; Roberts *et al.* 2008; Tohyama *et al.* 2004; Wirth *et al.* 2009; Mebis *et al.* 2009)
OATP3A1 variant 1	*SLCO3A1*	?	?	Prostoglandin E1 and E2, vasopressin, arachidonic acid	Adult: Astrocytes in white matter, choroid plexus	Adult: neurons, astroglia	(Huber *et al.* 2007)
OATP3A1 variant 2	*SLCO3A1*	?	?	As for variant 1	Adult: Neurons in grey and white matter, choroid plexus	Adult: neurons, astroglia	(Huber *et al.* 2007)
OATP4A1	*SLCO4A1*	?	0.9	Steroid conjugates, PGE2, penicillin	Adult: cerebral cortex	Adult: choroid plexus, neurons, cerebral cortex	(Tamai *et al.* 2000; Fujiwara *et al.* 2001; Choudhuri *et al.* 2003; Wirth *et al.* 2009)

Table 1. Continued

Protein	Gene	K_m T4 (μM)	K_m T3 (μM)	Alternative substrates	Localisation in human brain	Localisation in rats and mice	References
LAT1	*SLC7A5*	7.9	0.8	Large neutral amino acids	Adult: cerebral cortex	Adult and fetus/pup: neurons, astrocytes, blood brain barrier, choroid plexus	(Friesema *et al.* 2001; Kido *et al.* 2001; Broer *et al.* 2000; Heckel *et al.* 2003; Blondeau *et al.* 1993; Wirth *et al.* 2009)
LAT2	*SLC7A8*	?	?	As for LAT1	Adult: cerebral cortex, neurons Fetus: microglia	As for LAT1	

Only some of the K_m values have been determined mainly from studies using *Xenopus laevis* oocytes: MCT8, LAT-CD98, OATP1A2, OATP4A1.*The independent expression of the human MCT8 or MCT10 in COS1 cells both showed a greater preference for T3 than T4 uptake. Whilst MCT10 showed a greater ability to transport T3 compared to MCT8, MCT8 was found to be a more potent transporter of T4.

operation and a biopsy taken as histological evaluation of paraffin embedded sections, and further samples snap frozen immediately in liquid nitrogen. Fetuses were grouped thus: early 1st trimester 7–9 weeks ($n = 11$), late 1st trimester 10–12 weeks ($n = 25$), early 2nd trimester 13–16 weeks ($n = 20$) and mid 2nd trimester 17–20 weeks ($n = 9$). In addition, samples of normal adult cerebral cortex ($n = 10$) taken at post-mortem and donated to the UK National Brain Bank were obtained (The Institute for Neurological Diseases, Queen's Square, London, UK). All samples were snap-frozen in liquid nitrogen and stored at -80°C until required for RNA and protein evaluation.

RNA extraction, reverse transcription and real time quantitative TaqMan PCR

Total RNA was extracted from 100 mg frozen tissue after homogenisation, using TRIagent (Sigma, Poole, UK), as described previously (Chan *et al.* 2002). RNA (1 μg) was reverse transcribed using avian myeloblastosis virus (AMV) reverse transcriptase (Promega Corp., Madison, WI, USA) in a total reaction volume of 20 μl, with random hexamer primers according to the manufacturer's instructions then diluted 1:2 with RNAse free water.

RT-PCR was carried out in 25 μl reactions on 96-well plates in a reaction buffer containing 1× TaqMan Universal PCR Master Mix, 150 nmol TaqMan probe and 900 nmol primers using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Warrington, UK), as described previously (Chan *et al.* 2002). Primers and FAM-labelled probe sets for all genes were as previously validated and described (Chan *et al.* 2006; Loubiere *et al.* 2010) except for OATP3A1 variants 1 and 2, which were newly designed with Primer Express v3.0 (Applied Biosystems) (Table 2) and validated alongside control RT reactions without AMV. To provide an internal reference for RT efficacy, 18S ribosomal RNA and cyclophilin VIC-labelled-probes and primers (Applied Biosystems) were selected for normalisation based upon preliminary tests using the TaqMan Human Endogenous Control Array plate (Applied Biosystems) and model-based calculations as previously described (Andersen *et al.* 2004). All reactions were performed single-plexed in duplicate. Relative quantifications of each gene were determined, as previously described (Loubiere *et al.* 2010), and normalised to the geometric means of the two control genes. The mean mRNA expression for adult samples was assigned the arbitrary value of 1. The relative mRNA expression for each sample compared to the mean for adults was calculated and used to perform statistical analysis.

Immunohistochemistry

Formalin-fixed paraffin-embedded sections (5 μm) of forebrain (telencephalon) from three surgically terminated pregnancies between 8 and 11 weeks gestation were obtained from the London Neurogenerative Disease Brain Bank (Institute of Psychiatry, King's College London). Sections were immunostained for MCT8 or MCT10 using an avidin–biotin peroxidase technique (Vectastain Elite, Vector Laboratories, Peterborough, UK) as we have previously described (Kilby *et al.* 2000). Briefly, after de-waxing with Histoclear and serial rehydration with ethanol, sections were incubated in 10 mM sodium citrate buffer (pH 6.0) at 95°C for 5 min for antigen retrieval. After washing in 50 mM Tris–0.15 M saline (pH 7.5; TBS), the sections were blocked with 10% normal goat

Table 2. TaqMan® Real-time RT-PCR primers and probes sequences

Protein	Accession number	Forward primer	Reverse primer	Probe	Amplicon length (bp)	Exon-exon boundary
MCT8	NM_006517	CAACGCACTTACC-GCATCTG	GTAGCCCCAATACA-ACCAAGAG	TCCACATACTTCATCAGG-TGTACATAGGGAACAAA	150	3 – 4
MCT10	NM_018593	GATTCATGTCTATA-CCCATGACTGTTG	CACATCATAGGAG-CCCAGTTTGT	CCCACCCATTGCAGGG-TTACTTCGT	77	5 – 6
OATP1A2	NM_134431	GTGAACACAGATG-ATCTGATCATAACTC	AACTCCTGCACAA-ATCAGAAAGC	ATGCACCGACCCAAC-GAGTGTCAGT	87	5 – 6
OATP1C1	NM_017435	TTTTGCCTTGGGTATCT-ACACATTAG	CATGAAGTATCAAT-CAAAACTCCAAAAT	AATAAGAGTTCTTGCA-GGAATCCCAGCTCCA	90	13 – 14; detects all four isoforms
OATP3A1 variant 1	NM_013272	CCTTCGTTTGTTGGGC-TTCAT	GTCAGAGTAGAGGC-AAAGAACTCACT	CTGGCATCGACTCCA-CCTGCCTG	282	9 – 10 and splice site in exon 10
OATP3A1 variant 2	NM_001145044	GAAAAACTATAAACG-CTACATCAAAAACC	GGTTTTCTCAGTCTC-AATGTCTTGGTA	CTCTGTGCTGG-TGCTCAGCCCG	85	splice site in exon 10 – exon 11
OATP4A1	NM_016354	GCAGCCACGGA-GACGAA	AAAGATTCTGAGGG-ATACAGCTACAGT	CTCGGTACACCTT-CTGGCCGTCCA	70	7 – 8
LAT1	NM_003486	AAATGATCAACCCC-TACAGAAACCT	ACGTACACCAGCG-TCACGAT	CCTGGCCATCATCAT-CTCCCTGCC	73	4 – 5
LAT2	NM_012244	AAATCTGGAGGTGA-CTACTCCTATGTC	GATCACCAGCACA-GCAATCC	AGCCTCAGGAACCCA-GCCAGTCCT	87	2 – 3
CD98	NM_001012661	TGAGATTGGCCT-GGATGCA	GGACTCATCCCACA-GCATGAC	CCCTTCCTGGACA-GCCTATGGAGGC	73	11 -12
Pou5F1 (variant 1)	NM_002701	AGAAGGAGAAGC-TGGAGCAAAA	TGGCAAATTGCTCG-AGTTCTT	CCGGAGGAGTCCC-AGGACATCAAA	78	1–2
Nestin	NM_006617	GCGGTGGCTCC-AAGACTTC	ACTGGGAGCAAAG-ATCCAAGAC	CTTTCAGGACCCCAAG-CTGGAGCTG	100	3–4
Neurofilament-light	NM_006158	GACAAGCAGAACG-CCGACAT	CTTTGTGGTCCTCAA-TTCATTTTCTA	AGCGCTATGCAGGA-CACGATCAACAAA	75	1–2

serum (Sigma-Aldrich, Poole, UK) in diluting buffer (TBS, 0.3% Tween 20, 2% BSA) for 20 min before incubation overnight at 4°C with the primary antibody MCT8 (1:800) or MCT10 (1:800). The manufacture and specificity of the MCT8 (4790) (Loubiere *et al.* 2010) and MCT10 (2198) antibodies (Vasilopoulou *et al.* 2010) (Fig. 1*C* and *D*) have been previously described. Specificity of immunostaining was further confirmed using adjacent sections as negative controls treated with the primary antibody pre-absorbed by an excess of blocking peptide. With TBS-Tween washes between each step, the sections were incubated with biotinylated goat anti-rabbit secondary antibody (1:200; Vector Laboratories) for 30 min, followed by 5% hydrogen peroxide in TBS (5 min), then avidin–biotin–peroxidase complex application (30 min) and developed by incubation in 3,3′-diaminobenzidine (Vector Laboratories) for 5–15 min. Sections were rinsed in distilled water and mounted in Vectashield mounting media (Vector Laboratories).

Neurodifferentiation of NT2 cells

NT2 cells (European Collection of Cell Cultures, Salisbury, UK) were maintained in Dulbecco's modified Eagle's medium (DMEM):Ham's F12 (1:1) growth medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine and 100 U ml^{-1} of penicillin–streptomycin (Invitrogen Ltd, UK) as previously described (Andrews *et al.* 1984). Cells were passaged and split

1:4 twice weekly, and cultured at 37°C and 5% CO_2. Differentiation of NT2 precursor cells to terminally differentiated neurons with retinoic acid (RA) treatment was conducted based on methods previously described (Pleasure *et al.* 1992; Chan *et al.* 2003). Charcoal-stripped FBS (First Link Ltd, Birmingham, UK), which is virtually devoid of THs, was used to create a T3-depleted environment. Since NT2 cells were unable to tolerate 5 weeks in culture with media supplemented with charcoal-stripped FBS alone, differentiation experiments were performed in media containing 2% of normal FBS with 8% of charcoal-stripped FBS (combination media) with the addition of 10 nM T3 (T3-replete) and without T3 (T3-depleted) along with 10 μM RA (Sigma-Aldrich). We had previously shown that the dose of 10 nM T3 was optimal in the induction of T3-responsive gene expression in NT2 cells (Chan *et al.* 2003).

Quantitative RT-PCR

At time 0, 1, 3, 7, 14 and 21 days after RA treatment ($n = 6$ experiments, each condition in duplicate), total RNA was extracted and reverse transcribed for real time quantitative RT-PCR performed as described above. These time points were chosen based on when significant differences in neurodifferentiation marker expression in NT2 cells has been previously described (Przyborski *et al.* 2003). Primers and FAM-labelled probes for the neurodifferentiation markers Pou5F1, nestin and neurofilament-light were designed and validated (Table 2). All reactions were performed single-plexed alongside VIC-labelled-probe and primers for 18S ribosomal RNA (Applied Biosystems) as an internal reference. Relative mRNA expression was calculated as above within each experiment with the mean expression at time 0 assigned the arbitrary value of 1.

Western blotting and flow cytometry

Protein was extracted on Days 0, 3, 7 and 14 ($n = 4$ experiments) using RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 1 mM EDTA, 0.25% sodium deoxycholate and 50 mM Tris, pH7.4) with protease inhibitors then quantified (Bradford assay). Western immunoblotting was performed as we have previously described (Boelaert *et al.* 2003; Loubiere *et al.* 2010). Protein (30 μg) was denatured (30 min at 37°C or 95°C) in Laemmli buffer containing DTT and 1% SDS, then separated by electrophoresis in 8% or 10% SDS-PAGE gel and blotted onto nitrocellulose membranes. Blots were incubated in 5% non-fat milk in 20 mM Tris/137 mM NaCl, Tween 0.025% (TBS-Tween; pH 7.6) then probed with primary antibody overnight. The manufacture and specificity of the OATP antibodies have also been previously described (Huber *et al.* 2007; Gao *et al.* 2005). Rabbit polyclonal primary antibodies were used at 1:500 for MCT8, 1:250 for MCT10, 1:1000 for OATP3A1v1, OATP3A1v2 and OATP4A1 and goat polyclonal anti-human CD98 (Santa Cruz, CA, USA) at 1:250. Following washes with TBS-Tween immunoblots were then probed with secondary antibody conjugated to horseradish peroxidase and bands visualised using the ECL Plus chemiluminescence detection kit (GE Healthcare Life Sciences, Little Chalfont, UK). β-Actin expression was used to assess protein loading. Relative densitometry was performed using the ImageJ soft ware (NIH, USA). Given the specific interest in MCT8, we also used flow cytometry to quantify MCT8 protein at time 0, 1, 3, 7, 14 and 21 days after RA treatment ($n = 3$ experiments). Cells were detached with 5 mM EDTA, fixed in 4% paraformaldehyde (10 min at 37°C), permeabilized with ice cold 90% methanol (30 min), blocked with 5% normal goat serum then stained with anti-MCT8 (1:100) followed by goat-anti-rabbit-FITC (1:500; Abcam plc, Cambridge, UK). Staining and washes were performed in 1% FBS in PBS at room temperature. Cell fluorescence was acquired on a BectonDickinson FACSCalibur multicolour flow cytometer with the CellQuest software (BD Biosciences, Frederick, MD, USA) and analysed with the FlowJo software (Tree Star Inc., Ashland, OR, USA).

T3 uptake experiments

T3 uptake by NT2 cells was measured in duplicate at time points coinciding with neurodifferentiation marker assessments which were following 0, 1, 3, 7, 14 and 21 days' culture with RA in T3-replete or T3-depleted medium in 12-well plates. Following a 1 h wash-out in combination medium, cells were incubated for 0, 2, 10 or 30 min with serum free medium supplemented with 1 nM T3 containing approximately 1.5×10^5 cpm of [^{125}I]T3 (Perkin Elmer, Waltham, MA, USA) then rapidly washed three times with ice-cold serum free medium containing 0.1% BSA and lysed with 2% SDS. The radioactivity in the cell lysates (intracellular radioactivity) were expressed as a percentage of the radioactivity in the incubation media and corrected for total protein content quantified by the Bradford assay (Friesema *et al.* 2008; James *et al.* 2009).

Morphological assessments of differentiated neurons

To isolate fully differentiated neurons, cells were RA treated three times a week for 5 weeks then replated in fresh combination medium without RA for 48 h. This conditioned medium was subsequently stored for use later in the experiment. Cells were then fed with combination medium containing 10 μM 5-fluoro-2′-deoxyuridine (FudR), 10 μM Uridine (Urd) and 1 μM cytosine β-D-arabinofuranoside (araC) (Sigma-Aldrich, UK) four

times over 9–10 days followed by a single feed of conditioned medium without mitotic inhibitors for 24 h, as previously described (Pleasure *et al.* 1992; Chan *et al.* 2003). Differentiated neurons were detached by mechanical tapping and plated onto poly-D-lysine and laminin (Sigma-Aldrich) coated chamber slides (0.9 cm^2) then passaged in 50:50 conditioned medium (i.e. equal amounts of combination and conditioned media) every 3–4 days for 1 week, to yield a purer population of neurons. Neurons were fixed in 4% formaldehyde, blocked with 5% goat serum plus 0.1% Triton X-100 and immunostained with β3-tubulin (1:200; Sigma-Aldrich) followed by Alexa Fluor 488 goat anti-rabbit (1:1000; Invitrogen) and mounted with Vectashield with DAPI (Vector Laboratories). Neurons were examined under an epifluorescence microscope (Zeiss) and neurite outgrowth analysed using an image analysis software (Axiovision 4.0, Carl Zeiss, USA). Individual neurons with clearly visible neurites in their entirety were analysed for the length of the longest primary neurite, the total length of all primary neurites, total number of primary neurites per neuronal body and number of secondary neurites measuring greater than 20 μM in length (Ahmed *et al.* 2005). Fifteen non-overlapping high-powered fields per well ($n = 7$ wells for each condition) obtained over two separate differentiation experiments were assessed with measurements obtained for a total of 83 neurons in a T3-depleted environment and 43 neurons with T3-repletion. Average results for each well were used for statistical analysis.

Repressing MCT8 expression using siRNA

MCT8 knock-down experiments were carried out using predesigned MCT8-specific siRNAs (ID no. 117397, Ambion Inc., Huntingdon, UK) as previously described (James *et al.* 2009). A 'scrambled' siRNA (siRNA no. 1, ID no. 4611, Ambion) was used as negative control. NT2 cells were transfected using siPORT NeoFX transfection reagent (Ambion) as per the manufacturer's instructions. In brief, cells were trypsinized and suspended in culture medium and mixed with siRNA (10 nM) and NeoFX transfection reagent (4 μl ml^{-1} of medium) before plating and incubation for 48 h prior to RA treatment in T3-replete or T3-depleted medium. Total RNA and protein were harvested on Days 0, 1, 3, 7, 14 and 21 after RA treatment and assessed by TaqMan RT-PCR and Western immunoblotting, respectively (as described above) to confirm the silencing of endogenous MCT8 ($n = 4$ experiments). Messenger RNA expression of the neurodifferentiation markers Pou5F1, nestin and neurofilament-light were quantified by TaqMan RT-PCR as described above. We had previously confirmed that MCT8 repression by MCT8-specific siRNAs resulted in a significant reduction in T3 uptake by 19% at 72 h post-transfection compared with 'scrambled' controls (James *et al.* 2009).

Statistical analyses

Data were analysed using SigmaStat software v. 3.1 (SPSS Science Software UK Ltd). For the ontogeny data, the Kruskal–Wallis ANOVA was used for between group comparisons with Dunn's *post hoc* multiple comparisons testing to isolate groups that differed from others. For the NT2 cell work, data were analysed by the general linear model repeated measures ANOVA taking into account all variables with Tukey's *post hoc* multiple comparisons analysis. Data from the MCT8 knock-down experiments required logarithmic transformation to pass normality tests as determined using the Kolmogorov–Smirnov test prior to analysis of variance. Significance was accepted as $P < 0.05$.

Results

Expression of TH transporters in the human fetal cerebral cortex

Monocarboxylate transporters (MCTs). The levels of mRNA encoding both MCT8 and MCT10 did not demonstrate any statistically significant differences in the fetal cerebral cortex across gestation (7–20 weeks) and when compared with adult (Fig. 1*A* and *B*).

The presence of both MCT8 and MCT10 protein from early gestation was confirmed by immunohistochemistry (Fig. 1*E* and *F*). MCT8 was localised within the microvasculature and in undifferentiated CNS precursor cells within the fetal cerebral cortex from 8 weeks with similar localisation at 11 weeks. MCT10 staining was evident in undifferentiated CNS cells and occasionally in early differentiated neurons, recognisable by a short axonal process emanating from the perikaryon, at 8 weeks but there was a reduced proportion of undifferentiated cells staining positive at 10 weeks. In contrast to MCT8, faint but clear MCT10 staining of the microvasculature was only observed at 10 weeks gestation but not at 8 weeks. For both MCT8 and MCT10 only a subpopulation of undifferentiated cells clearly stained positive, which could represent particular stages of neuronal or glia development. No immunostaining was observed with pre-absorption of the primary antibody with the respective blocking peptides.

Organic anion transporting polypeptides (OATPs). Messenger RNAs encoding OATP1A2, OATP1C1 and OATP3A1v2 were significantly lower in the fetal cerebral cortex compared with adults (ANOVA, $P < 0.001$ for each, Fig. 2*A*–*C*) with mean expression at 7–9 weeks

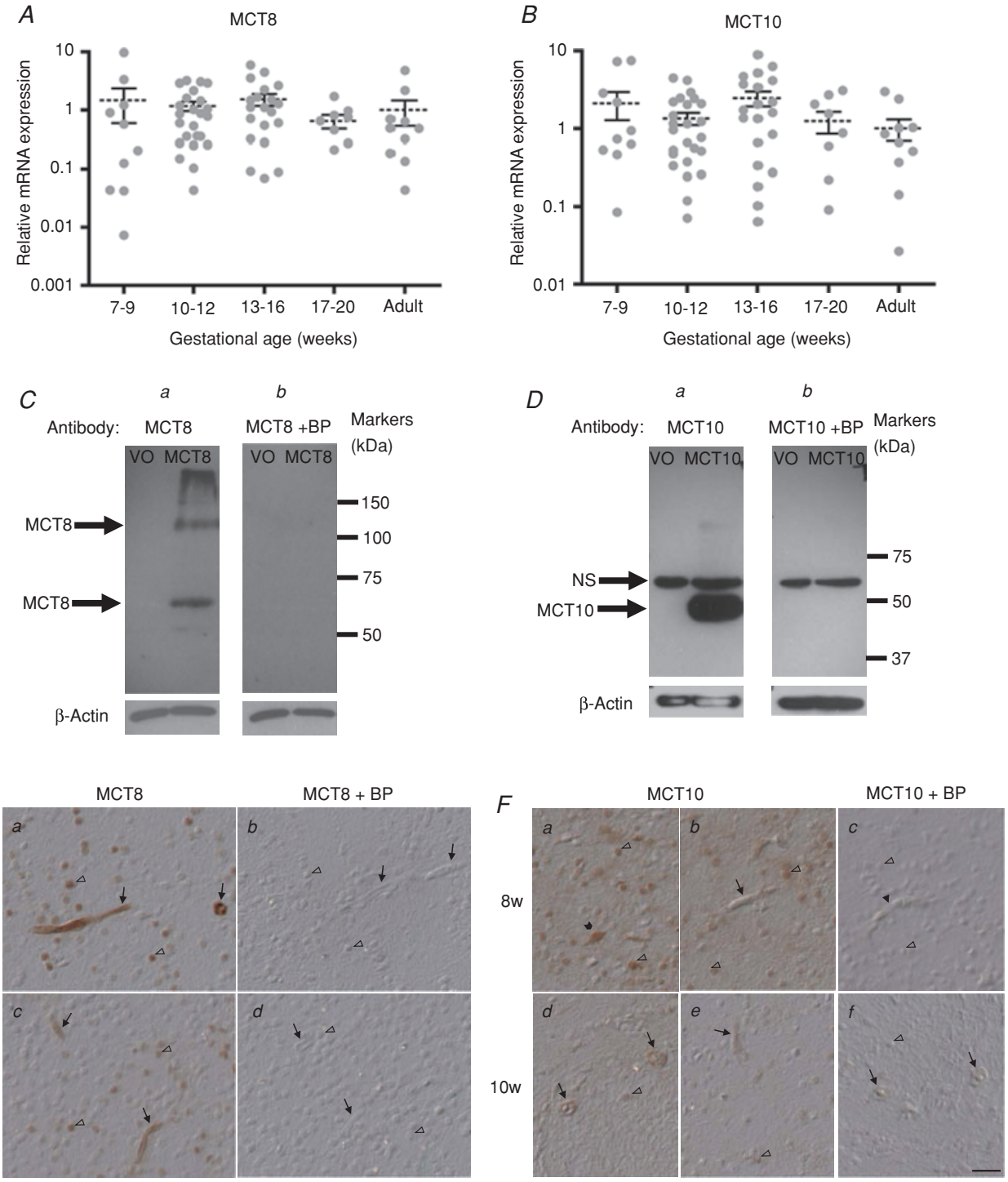


Figure 1. The expression of MCT8 and MCT10 in the human fetal cerebral cortex
A and *B*, scatter plots showing the relative expression of mRNA (means ± SEM) encoding the monocarboxylate transporters MCT8 (*A*) and MCT10 (*B*) in human fetal cerebral cortex from 7 to 20 weeks gestation compared with the adult cerebral cortex, given an arbitrary value of 1. *C* and *D*, Western blots demonstrating the specificity of the MCT8 antibody 4790 (*C*), and MCT10 antibody 2198 (*D*); specificity of the MCT8 antibody (1.6 μg ml^{-1}) was tested in MCT8 null JEG3 cells transfected with empty vector (VO) or plasmid encoding human MCT8 (James *et al.* 2009). There are two bands at approximately 60 kDa and 120 kDa seen in MCT8 transfected cells only (*Ca*), consistent with the predicted molecular weights of the MCT8 monomer (63 kDa) and homodimer (Visser *et al.* 2009). Both bands disappeared when the MCT8 antibody was preabsorbed with excess of blocking peptide (BP; *Cb*). Specificity of the MCT10 antibody (0.86 μg ml^{-1}) was determined by transfecting the HTR8/SVNeo cells

being 92%, 96% and 95% lower than adults, respectively ($P < 0.05$). Whilst the mean levels of OATP1A2 and OATP1C1 mRNAs remained significantly lower until 16 weeks of gestation and began to increase in mid-gestation at 17–20 weeks, the mean level of OATP3A1v2 mRNA expression remained suppressed at 97% even at 17–20 weeks compared with adult ($P < 0.05$). In contrast, OATP3A1v1 mRNA expression in the fetal cerebral cortex from 7 to 20 weeks was similar to adult (Fig. 2*D*). Mean OATP4A1 mRNA expression (ANOVA $P < 0.05$) was similar to adult levels in the early 1st trimester but dropped to a nadir at 10–12 weeks, reaching a 70% reduction compared with adult ($P < 0.05$), then subsequently increased to adult levels by mid-gestation (Fig. 2*E*).

System L-type amino acid transporters (LATs and CD98). Messenger RNA encoding LAT1 did not show any statistically significant differences in expression in the fetal cerebral cortex between 7 and 20 weeks and when compared with adult (Fig. 3*A*). In contrast, both LAT2 (ANOVA $P < 0.01$) and the LATs' obligatory associated protein, CD98 (ANOVA $P < 0.05$), demonstrated a significant decrease in mRNA expression in the fetal cerebral cortex at 17–20 weeks with a 92% and 82% reduction in mean expression, respectively, compared with adult ($P < 0.05$; Fig. 3*B* and *C*). LAT2 mRNA expression at 17–20 weeks was also significantly lower compared with that at 10–12 weeks and 13–16 weeks ($P < 0.05$).

Expression of TH transporters and response to T3 during NT2 neurodifferentiation

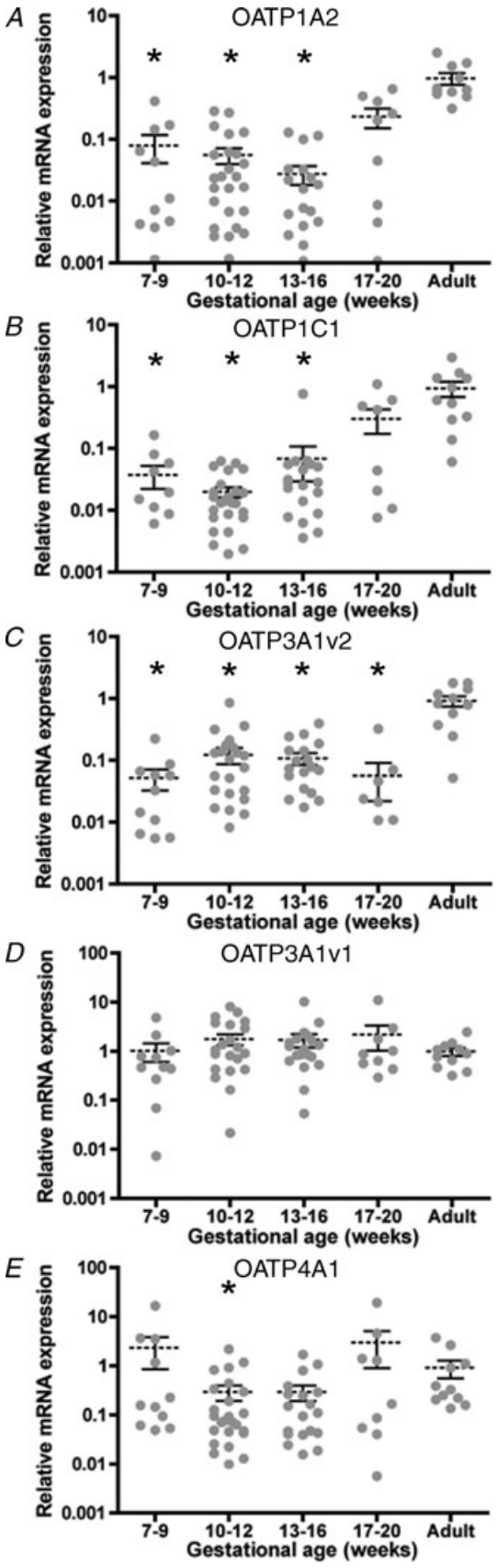
With the localisation of MCT8 and MCT10 in CNS precursor cells and neurons being the postulated ultimate target cell of T3 action in the brain during development, we went on to investigate human neurodifferentiation *in vitro* using human CNS precursor NT2 cells, which express the TR isoforms, α1, α2 and β1, and are T3-responsive. They demonstrate increased mRNA expression of the thyroid hormone responsive gene, TRβ1 (Chan *et al.* 2003), and increased cell proliferation in response to T3 treatment (James *et al.* 2009).

T3 does not affect NT2 neurodifferentiation. The changes in mRNA expression of three neurodifferentiation markers, Pou5F1, nestin and neurofilament, across 21 days of RA treatment (Fig. 4*A*–*C*) were consistent with that previously described in NT2 cells (Przyborski *et al.* 2003) and were similar for NT2 cells differentiated in T3-depleted compared with those in T3-replete medium. Pou5F1 mRNA expression was significantly down-regulated by Day 1 ($P < 0.01$) compared with Day 0 with further reductions on Day 3 and Day 7, then remained suppressed at Days 14 and 21 ($P < 0.001$; Fig. 4*A*). Nestin mRNA expression peaked on Day 3 ($P < 0.05$) followed by a significant reduction by Day 7 ($P < 0.001$) after RA treatment which was sustained until Day 21 (Fig. 4*B*). Neurofilament mRNA showed a gradual rise in expression showing significantly increased expression on Day 14 and 21 by 7-fold ($P < 0.05$) and 11-fold ($P < 0.001$), respectively, compared with Day 0 (Fig. 4*C*). The morphology of neurons assessed by measuring the lengths of primary neurites and counting the number of primary neurites and branch points (ratio of the number of secondary neurites to the number of primary neurites per neuron) showed no significant differences between neurons terminally differentiated in T3-depleted medium compared with T3 repletion (Fig. 4*D* and *E*).

TH transporter expression with NT2 neurodifferentiation. We then investigated if the lack of effect of T3 depletion upon NT2 neurodifferentiation was due to compensatory up-regulation in TH transporter expression and function. Only two TH transporters, MCT10 and LAT2, demonstrated significant changes in expression when NT2 cells were differentiated in T3-depleted medium compared with those in T3-replete medium (ANOVA for effect of T3 were $P < 0.05$ for both; Fig. 5*A* and *B*). However, contrary to our hypothesis, T3 depletion resulted in time-specific suppression in mRNA expression: MCT10 on Days 1 ($P < 0.05$) and 14 ($P < 0.001$) and LAT2 on Day 14 ($P < 0.01$). None of the other thyroid hormone transporters demonstrated alterations in expression with T3 depletion.

With RA-induced neurodifferentiation, the TH transporters displayed four different patterns of changes in mRNA expression. Firstly, MCT10 and LAT2 mRNA

with empty vector (VO) or plasmid encoding human MCT10 (a kind gift from Dr Theo Visser, Erasmus University Rotterdam (Friesema *et al.* 2008). A single band at 50 kDa was obtained with transfection of MCT10 (*Da*), which disappeared when the antibody was preabsorbed with excess of blocking peptide (*Db*). Immunoblotting for β-actin on the same blots was used to assess protein loading. NS: non-specific band. *E* and *F*, representative sections of the fetal forebrain (telencephalon) from 8 to 11 weeks gestation (w) showing MCT8 (*Ea* and *Ec*) and MCT10 (*Fa*, *b*, *d* and *e*) immunoreactivity, which is lost with pre-absorption of the primary antibody with the corresponding blocking peptides (BP; *Eb*, *Ed*, *Fc* and *Ff*). Microvessels (arrows), undifferentiated CNS cells (open triangles) and a differentiating neuron (filled arrowhead) are indicated. Bright field micrographs taken with DIC optics. Scale bar applies to all panels and corresponds to 20 μm.



expression demonstrated a nadir on Days 3 and 7 (ANOVA for effect of time were $P < 0.001$ for both; Fig. 5*A* and *B*). MCT10 protein expression assessed by Western blotting, however, did not show any statistically significant changes with RA treatment nor with T3 treatment (data not shown). Secondly, there was a zenith in OATP3A1v2 mRNA expression on Days 7 and 14 of RA treatment (ANOVA $P < 0.01$; Fig. 5*C*). In the third group, MCT8 mRNA declined significantly (ANOVA $P < 0.01$; Fig. 5*D*) from Day 3 to Day 14 ($P < 0.01$) whilst OATP4A1 (ANOVA $P < 0.01$; Fig. 5*E*) and LAT1 (ANOVA $P < 0.001$; Fig. 5*F*) mRNA declined significantly from Day 1 to Day 7 then all remained suppressed until Day 21. MCT8 protein assessed by flow cytometry was already significantly down-regulated by Day 3 compared to Day 0 and remained suppressed (ANOVA $P < 0.001$; Fig. 4*G*). However, OATP4A1 protein assessed by Western blotting showed no statistically significant changes (data not shown). In the final group, CD98 and OATP3A1v1 mRNA expression remained unchanged throughout the course of RA treatment (data not shown) but CD98 protein assessed by Western blotting was significantly down-regulated by Day 7 compared to Day 3 (ANOVA $P < 0.01$; Fig. 4*H* and *I*). Since CD98 is the obligatory associated protein of the System-L transporters, CD98 down-regulation could reduce the activities of both LAT1 and LAT2. NT2 cells did not express OATP1C1 mRNA.

T3 uptake by NT2 cells during neurodifferentiation. To assess the combined effects of the TH transporters upon net T3 uptake over the course of RA induced neurodifferentiation, comparisons of T3 uptake were made from data obtained after 10 min of incubation in [^{125}I]T3. There was a significant decline of 52–55% in T3 uptake between Day 0 compared with Days 7 and 14 of RA treatment ($P < 0.05$ for both; Fig. 5*J*). However, no differences in T3 uptake were observed between cells cultured in T3-depleted medium compared with T3-replete medium at 2, 10 and 30 min after incubation in [^{125}I]T3 (only data at 10 min are shown in Fig. 5*J*).

MCT8 repression does not affect NT2 neurodifferentiation. Given the association of MCT8 mutations with severe neurological impairment, that

Figure 2. The expression of the OATPs in the human fetal cerebral cortex
Scatter plots showing the relative expression of mRNA (means ± SEM) encoding organic anion transporting polypeptides OATP1A2 (*A*), OATP1C1 (*B*), OATP3A1 variants 2 (*C*) and 1 (*D*) and OATP4A1 (*E*), in human fetal cerebral cortex from 7 to 20 weeks gestation compared with the adult cerebral cortex, given an arbitrary value of 1. Statistically significant differences were $^*P < 0.05$, compared with adults.

MCT8 represses NT2 proliferation independently of T3 (James *et al.* 2009), that early overexpression of Mct8 in murine embryonic stem cells can accelerate neurodifferentiation (Sugiura *et al.* 2007) as well as this study's findings of MCT8's localisation in undifferentiated CNS cells in the human fetal brain and a down-regulation of MCT8 with neurodifferentiation, we hypothesised that MCT8 plays a critical role during early NT2 neurodifferentiation. Thus, we proceeded with MCT8 knock-down prior to RA treatment. At the start of RA treatment (Day 0), significant repression in the mRNA and protein expression of endogenous MCT8 in NT2 cells was already achieved, with a 72% reduction in MCT8 mRNA expression in MCT8-specific siRNA transfected cells compared with cells transfected with scrambled siRNA ($P < 0.00001$; Fig. 6*A*). This significant reduction in MCT8 mRNA expression was sustained at least until Day 3 and accompanied by suppression of both MCT8 monomer and homodimer protein expression (Fig. 6*B*). By Day 7, there was still some suppression in MCT8 monomer protein expression but the homodimer expression was now similar to controls. Repression of MCT8 expression did not, however, result in any significant changes in the mRNA expression of the neurodifferentiation markers Pou5F1, nestin and neurofilament both in T3-replete and T3-depleted media (Fig. 6*C–E*).

Discussion

This study describes for the first time the ontogeny of nine different TH transporters simultaneously in a series of human fetal cerebral cortex samples from the first half of pregnancy. The presence of MCT8, OATP1C1, OATP1A2, OATP4A1, LAT1 and LAT2 is consistent with previous reports based on studies of the human fetal brain at later gestations (Table 1). Descriptions of the expression of OATP3A1 (variants 1 and 2) and MCT10, and the localisation MCT8 and MCT10 within the human fetal cerebral cortex in the 1st trimester of pregnancy are novel.

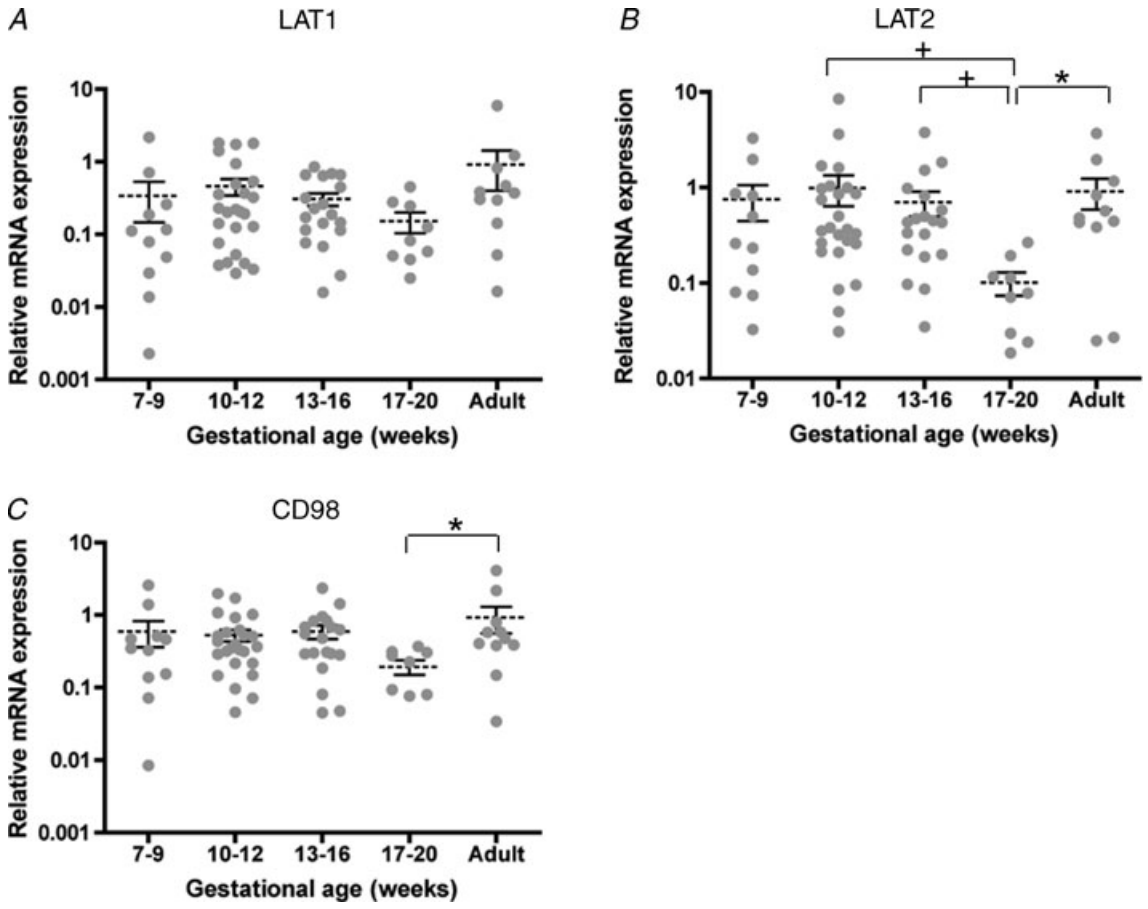


Figure 3. The expression of System L in the human fetal cerebral cortex
Scatter plots showing the relative expression of mRNA (means ± SEM) encoding System L amino acid transporters LAT1 (*A*) and LAT2 (*B*), and their obligatory associated protein, CD98 (*C*), in human fetal cerebral cortex from 7 to 20 weeks gestation compared with the adult cerebral cortex, given an arbitrary value of 1. Statistically significant differences were $^*P < 0.05$ compared with adults, $+P < 0.05$ between gestational age groups.

Even though NT2 cells express multiple TH transporters and demonstrate T3 uptake, T3 and specifically MCT8 are not critical for NT2 neurodifferentiation.

Minor aberrations in maternal thyroid function occurring before the onset of endogenous fetal TH production in the midtrimester of human pregnancy are associated with long-term neurodevelopmental sequelae in offspring (Haddow *et al.* 1999; Pop *et al.* 2003). The mechanism by which this occurs is speculatively through the inadequate supply of transplacentally derived maternal TH, which can directly impact upon the early developing fetal CNS. Maternally derived T4 can already be found at biologically relevant concentrations in the coelomic and amniotic fluids from 5 weeks (Calvo *et al.* 2002).

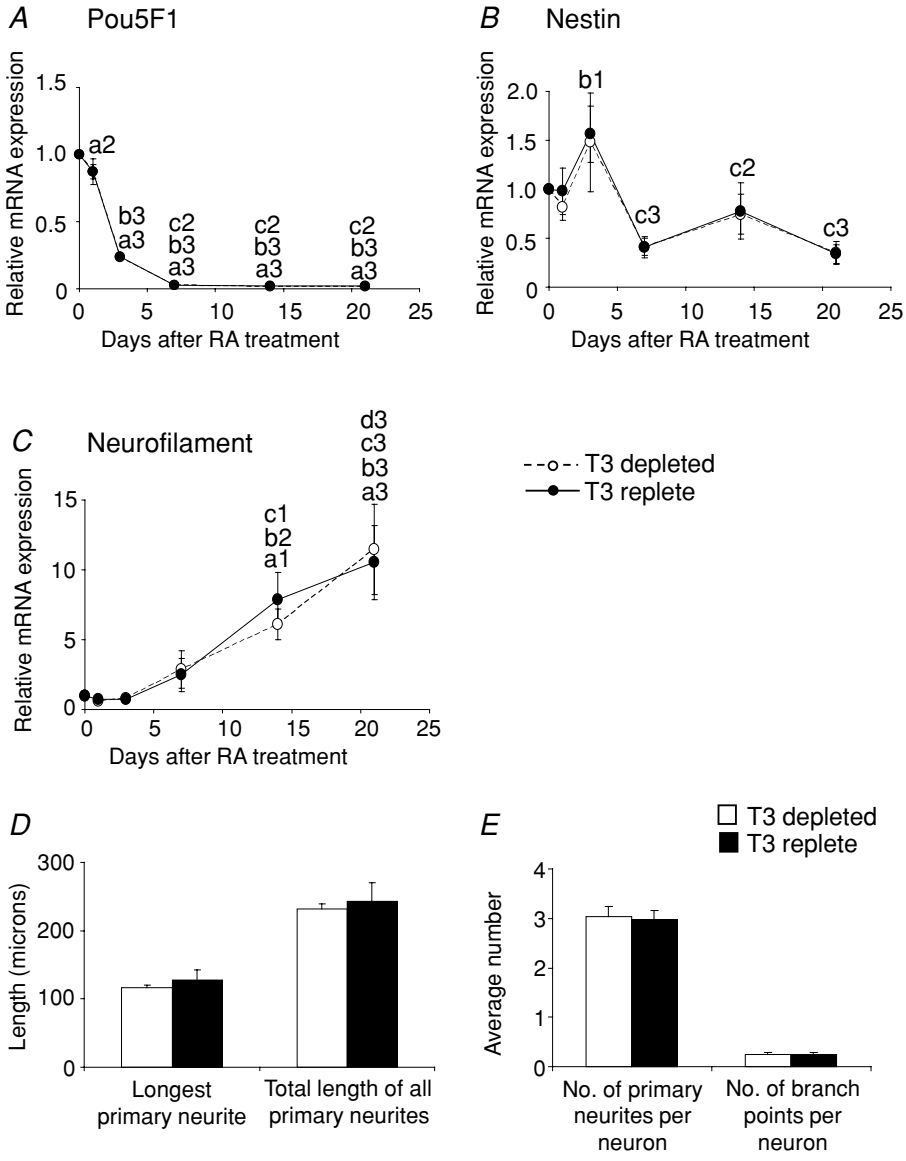


Figure 4. Neurodifferentiation marker expression and neuronal morphology in NT2 cells with retinoic acid treatment in T3-replete and T3-depleted media
The relative expression (means ± SEM) of mRNA encoding the neurodifferentiation markers Pou5F1 (*A*), nestin (*B*) and neurofilament (*C*), with RA-induced neurodifferentiation of NT2 cells in T3-replete and T3-depleted media. All data are presented relative to values on Day 0 of RA treatment, given an arbitrary value of 1, within each experiment ($n = 6$). Measurements of the lengths of primary neurites (*D*) and the number of primary neurites and branch points (ratio of the number of secondary neurites to the number of primary neurites per neuron; *E*) of neurons terminally differentiated in T3-replete and T3-depleted media are shown. All statistically significant differences over time (taking both T3 conditions into account) are indicated as follows: a, different from Day 0; b, different from Day 1; c, different from Day 3; d, different from Day 7. Numbers represent the following: 1, $P < 0.05$; 2, $P < 0.01$; 3, $P < 0.001$.

The expression of multiple TH transporters from 7 weeks suggests that CNS cells have the capability for TH uptake from the early 1st trimester. These findings taken together with our previous data showing that human fetal cerebral cortices express all of the major TR isoforms and display D2 and D3 activities from 7 weeks (Chan *et al.* 2002) support the notion that the early developing human CNS possesses all of the necessary apparatus for TH uptake, metabolism and utilisation. Indeed in a rat model, the impact of a significant rise in maternal T4 upon TH responsive gene expression has been demonstrated within the fetal brain before endogenous fetal TH production (Dowling *et al.* 2000). Our data support the hypothesis that the human fetal brain can respond to maternal TH from as early as 7 weeks, which could influence human CNS development.

Using Mct8 knockout mouse models, Mct8 has been shown to play an important role in TH entry across the BBB (Ceballos *et al.* 2009), the main route of TH entry into the brain (Dratman *et al.* 1991), and into CNS neurons (Trajkovic *et al.* 2007; Wirth *et al.* 2009). Thus, in human subjects with a MCT8 mutation, impaired cerebral TH uptake is very likely to contribute to the severe neurological phenotype described (Heuer, 2007). This suggests the importance of TH transporters in the regulation of both the entry of TH into brain parenchyma and the passage of TH across the plasma membranes of neuronal cells. The localisation of MCT8 and MCT10 in the microvasculature and in undifferentiated CNS cells in the1st trimester human fetal cerebral cortex supports this notion and emphasizes the possible early role played by these transporters in human cortical neurogenesis, which begins at 6 weeks and continues into midgestation with evidence of heterogeneity of progenitor cells in the entire telencephalic wall throughout this time (Howard *et al.* 2006).

Changes in the expression of the TH transporters across gestation may reflect developmental regulation in response to rising TH availability with advancing gestation (Kester *et al.* 2004) and to altering cellular need for TH within specific cell types and brain regions, as well as reflect their role in the transport of other compounds apart from THs. The 17–20th week of gestation appears to be a watershed for TH transporter mRNA expression, which coincides with onset of endogenous fetal TH production (de Escobar *et al.* 2004) and the formation of anatomically continuous effective endothelial tight junctions in the BBB (Virgintino *et al.* 2004). Both OATP1A2 and OATP1C1, which are largely confined to endothelial cells forming the BBB in the human cerebral cortex (Gao *et al.* 2000; Bronger *et al.* 2005; Roberts *et al.* 2008), show very low mRNA expression in the fetal brain and only begin to rise from 17–20 weeks, the time when transcellular transport of proteins across the BBB is required. Possible increased TH availability at 17–20 weeks may also signal the need for added regulation at the plasma membrane of neurons and glia. This may be reflected by a significant reduction in mRNA encoding CD98 and LAT2, which have been localised to human fetal microglia (Wirth *et al.* 2009) and in rat, to neurons and astrocytes (Heckel *et al.* 2003). The contrasting mRNA expression patterns of the splice variants of OATP3A1 may reflect their differential role during development and is suggested by their differential distribution in the adult cerebral cortex: OATP3A1v1 in grey matter astrocytes and OATP3A1v2 in grey and white matter neurons (Huber *et al.* 2007).

The variation in mRNA abundance between subjects, even within the same gestational age group, is partly attributable to slightly different brain areas being sampled. However, the strength of our study is the large numbers of fetal cortices being analysed, which could partially compensate for these variations and allow ontogenic patterns to emerge. Even for adult samples, which were biopsied from a similar area of the cortex, large variations in mRNA expression of some transporters are still present and this may be due to genuine biological variation.

The expression of multiple transporters in the fetal brain may suggest redundancy in TH uptake mechanisms but the severe neurological phenotype in individuals with an isolated non-functional MCT8 mutation implies the contrary argument. It has been postulated that very low OATP1C1 expression in the BBB and the lack of LAT2 expression in developing neurons cannot compensate for the lack of MCT8 function in humans. This is unlike in rodents where there is higher Oatp14 (rodent equivalent of OATP1C1) expression at the BBB (Roberts *et al.* 2008) and co-expression of Lat2 in Mct8-expressing neurons (Wirth *et al.* 2009), which may explain why Mct8 knockout mice show limited neurological impairment (Wirth *et al.* 2009). If any compensation occurs, it is incomplete since primary neurons isolated from Mct8 knockout mice still demonstrate reduced T3 uptake compared to wild-type (Wirth *et al.* 2009). As yet, studies comparing the relative expressions of the other TH transporters in wild-type and Mct8 knockout mouse brains have not been reported. However, it has been demonstrated that local D2 activity can compensate for the lack of T3 availability in Mct8 knockout mouse brain resulting in limited effects on TH responsive gene expression (Morte *et al.* 2010).

To assess the potential roles of the different TH transporters in human neuronal development and the possible compensatory effects with T3-depletion, we have used NT2 cells as a human CNS precursor model to focus specifically on neurodifferentiation. T3 has a pro-proliferative effect in NT2 cells (James *et al.* 2009), which express TRs and display D3 activity and minimal D2 activity, but deiodinase activities do not change with T3 treatment (Chan *et al.* 2003). Our finding that T3 uptake declines with neurodifferentiation, suggests that T3 may be more important in the maintenance of

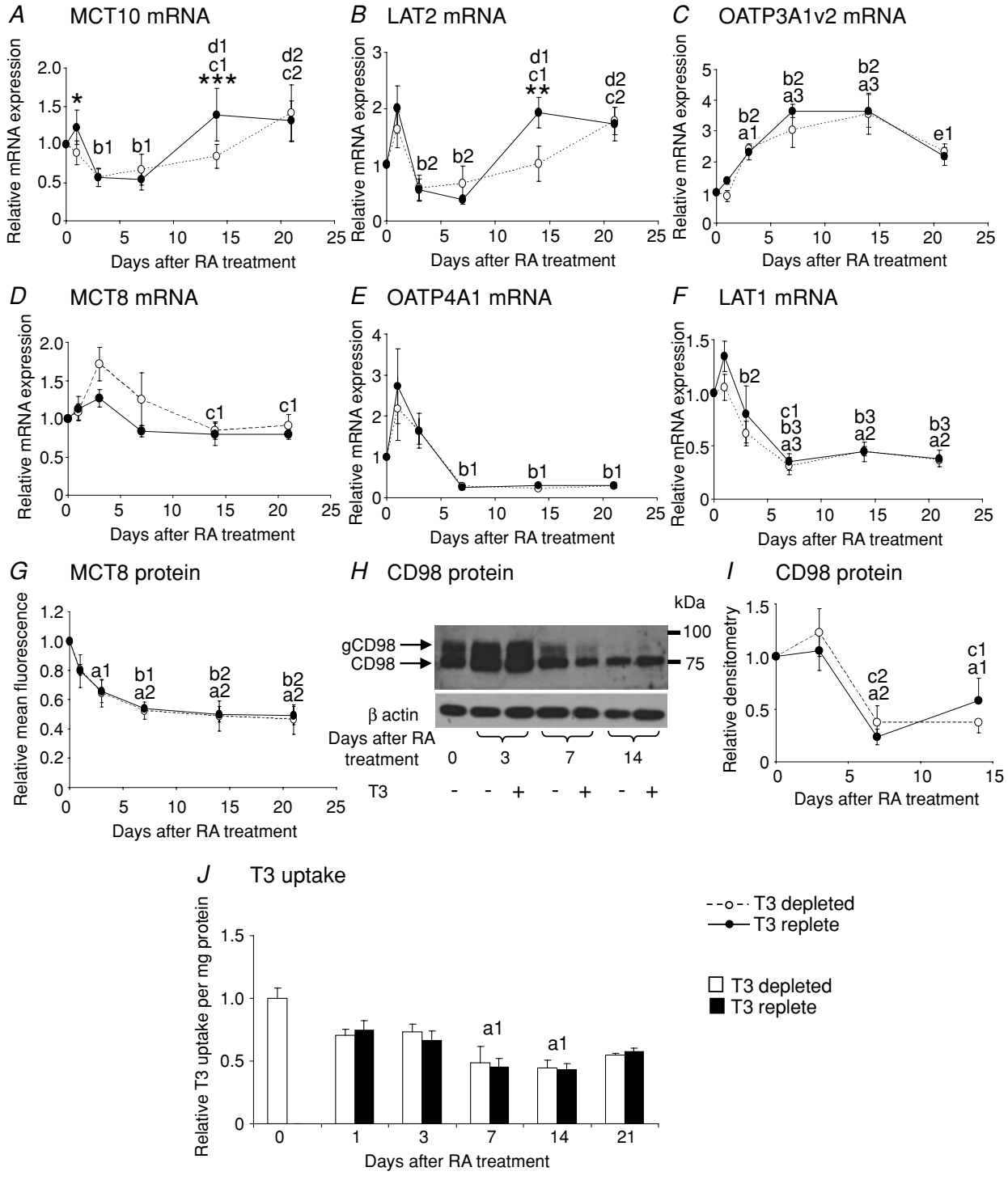


Figure 5. Thyroid hormone transporter expression and T3 uptake by NT2 cells with retinoic acid treatment in T3-replete and T3-depleted media

TH transporter expression with RA-induced neurodifferentiation of NT2 cells in T3-replete and T3-depleted media. All data (means ± SEM) are presented relative to values on Day 0 of RA treatment, given an arbitrary value of 1, within each experiment. Relative expressions of mRNA encoding MCT10 (*A*), LAT2 (*B*), OATP3A1v2 (*C*), MCT8 (*D*), OATP4A1 (*E*) and LAT1 (*F*) and relative expressions of MCT8 protein analysed by flow cytometry (*G*), a representative CD98 Western blot from one experiment (*H*) and combined CD98 relative densitometry data from 4 different experiments (*I*) are shown. Two bands for CD98 (*H*) were evident until Day 7, the larger band representing glycosylated CD98 (gCD98)(Ritchie & Taylor, 2001), and both bands were quantified for relative

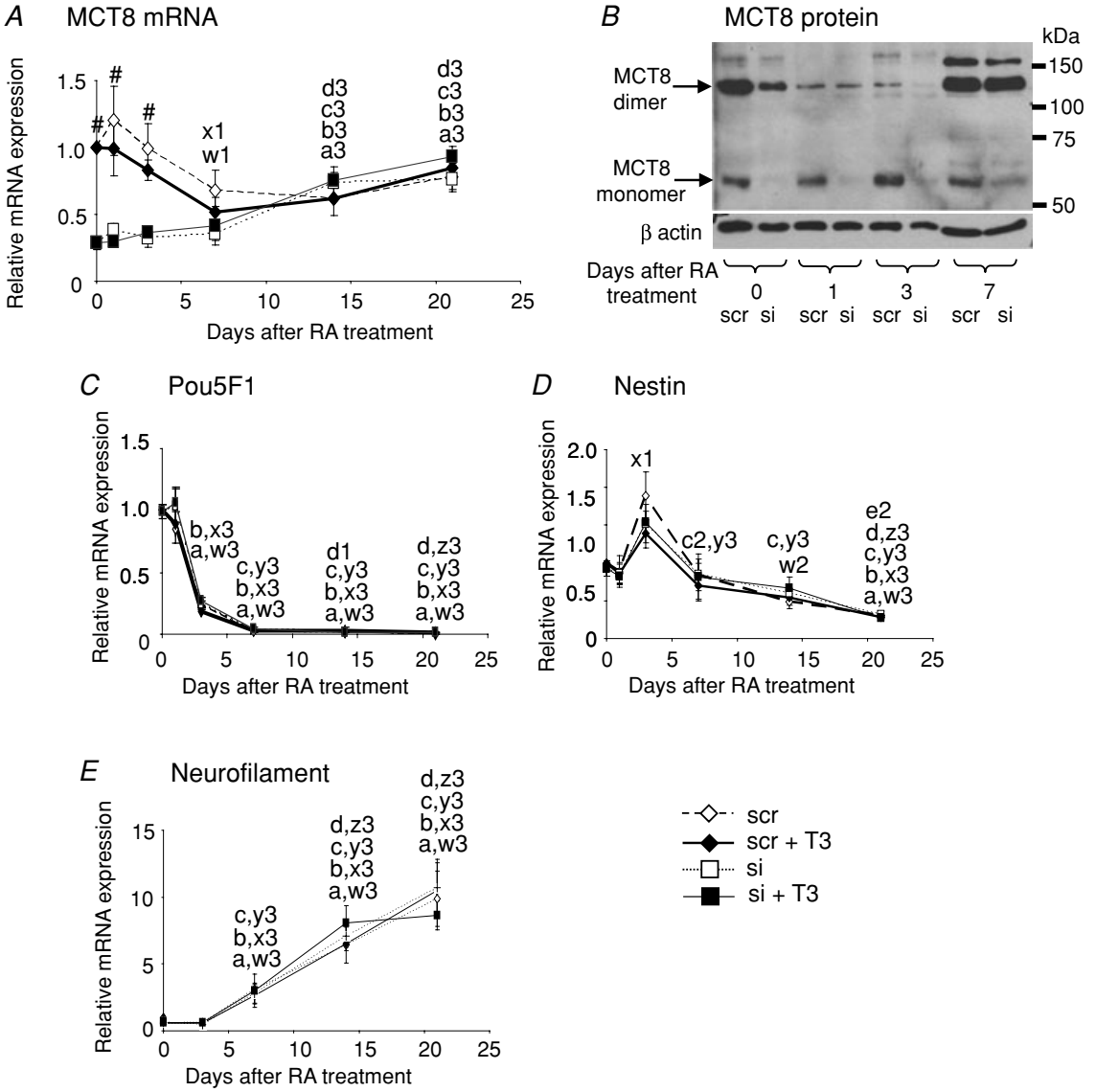


Figure 6. The expression of MCT8 and neurodifferentiation markers in NT2 cells during retinoic acid treatment in T3-replete and T3-depleted media following MCT8 knockdown

The relative expression (means ± SEM) of mRNA encoding MCT8 (*A*), Pou5F1 (*C*), nestin (*D*) and neurofilament (*E*), from 48 h (Day 0 of RA treatment) after NT2 transfection with MCT8-specific siRNAs (si) or 'scrambled' siRNAs (scr) followed by RA-induced neurodifferentiation in T3-replete and T3-depleted media. All data are presented relative to values for scr on Day 0 of RA treatment, given an arbitrary value of 1, within each experiment ($n = 4$). A representative Western blot from one experiment confirmed repression of MCT8 in NT2 cells transfected with si compared with scr over 7 days of RA treatment (*B*). Statistically significant differences between si and scr (taking both T3 conditions into account) were #$P < 0.00001$. All statistically significant differences over time (taking both T3 conditions into account) for scr are indicated as follows: w, different from Day 0; x, different from Day 1; y, different from Day 3; z, different from Day 7; and for si are indicated as follows: a, different from Day 0; b, different from Day 1; c, different from Day 3; d, different from Day 7. Numbers represent the following: 1, $P < 0.05$; 2, $P < 0.01$; 3, $P < 0.001$.

densitometry. Uptake of [^{125}I]T_3 after 10 min of incubation (*J*). All statistically significant differences over time (taking both T3 conditions into account) are indicated as follows: a, different from Day 0; b, different from Day 1; c, different from Day 3; d, different from Day 7; e, different from Day 14. Numbers represent the following: 1, $P < 0.05$; 2, $P < 0.01$; 3, $P < 0.001$. Asterisks indicate significant changes with T3 treatment at the specific time points $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

undifferentiated NT2 cells and during the earlier part of neurodifferentiation. Decreasing T3 uptake accompanied by the decline in MCT8, LAT1, CD98 and OATP4A1 expression with neurodifferentiation suggest that these may be the TH transporters regulating T3 uptake in NT2 cells. Further experiments using inhibitors of these specific TH transporters would be required to confirm this postulation.

When cultured in T3-depleted medium, NT2 cells did not demonstrate any compensatory rises in TH transporter expression nor in T3 uptake ability despite the absence of compensatory changes in deiodinase activities for intracellular T3 regulation. In fact, T3 depletion was associated with the suppression of MCT10 and LAT2 mRNA expression. Functional coordination of MCT10 and LAT2 activities may occur in the regulation of neutral amino acid efflux by early renal proximal tubules (Ramadan *et al.* 2007). The same could be occurring during neurodifferentiation under T3 regulation. The regulation of TH transporter expression by TH has been described in only a limited number of studies and appears to vary between transporters and tissue types. In rabbit euthyroid sick syndrome that is characterised by a low circulating T3 and low hypothalamic T4, Mct8 expression was unaltered but Mct10 and Oatp1C1 expression were increased in the hypothalamus (Mebis *et al.* 2009). In hypothyroid postnatal mice Mct8 mRNA expression is upregulated in the femur, and in a mouse osteoblastic cell line, T3 treatment downregulated Mct8 expression (Capelo *et al.* 2009). However, no alteration in Lat1 and Lat2 mRNA expression was observed *in vivo* or *in vitro* in this model.

In NT2 cells trends in TH transporter mRNA expression were not always reflected by trends in protein expression. Post-transcriptional modulation is likely to be involved and such discrepancies have been reported in plasma membrane transporters such as MCT8 in human placenta (Loubiere *et al.* 2010) and Oats in rat kidney (Zlender *et al.* 2009), although the mechanisms remain unknown.

The lack of difference in the rate of neurodifferentiation and neuronal morphology between the T3-depleted and T3-replete cells suggests that NT2 neurodifferentiation is not a T3-responsive process. Our findings challenge the prevailing notion that THs are critical to neurodifferentiation, conclusions which have been based upon research in rats and mice, predominantly on cerebellar neurons (Horn & Heuer, 2010). One study of non-CNS derived human fetal olfactory neuroepithelium did, however, demonstrate increased neurofilament-M expression assessed by immunocytochemistry in response to T3 treatment (Benvenuti *et al.* 2008) which is in contrast to NT2 cells. Apart from the fact that the Mct8 knockout mice show hardly any neurological deficits, unlike humans, the mouse and rat cerebral cortex differ from humans in that they demonstrate deiodinase type 1 (D1) activity (Visser *et al.* 1982), which can activate circulating T4 into T3, whilst human brains show no D1 activity (Chan *et al.* 2002). Thus, findings of TH action and metabolism in rodent CNS cannot readily be extrapolated to the human situation.

Since MCT8 also has T3-independent anti-proliferative effects (James *et al.* 2009), we specifically assessed the effects of MCT8 repression in NT2 neurodifferentiation. The absence of any changes in neurodifferentiation marker expression, despite significant MCT8 repression, suggests that MCT8 does not play a critical role in NT2 neurodifferentiation either. Mct8 has been shown to accelerate the neurodifferentiation of murine ES cells (Sugiura *et al.* 2007) and RA has been reported to stimulate Mct8 expression in murine F9 embryonal carcinoma cells (Kogai *et al.* 2010), but NT2 cells display neither of these phenomena, which may be indicative of yet more differences in neurodevelopment between mice and humans.

From these experiments of NT2 cell culture in isolation, we cannot exclude the possibility that in the presence of supportive glial cells, NT2 cells may respond differently to T3 or that possible T3 effects upon glial cells may indirectly impact upon NT2 neurodifferentiation. Also, charcoal-stripped FBS lacks some steroidal hormones, which may influence neurodifferentiation. Nonetheless, many *in vitro* and animal transplant studies support the view that NT2 cells are true representations of CNS neuronal progenitor cells (Trojanowski *et al.* 1997). Transplantation of NT2 cells into animal models of neurological disorders have shown benefit and phase II clinical trials of NT2 transplantation into human stroke patients are underway (Hara *et al.* 2008).

Since there is a lack of effect of T3 and MCT8 upon NT2 neurodifferentiation, it is conceivable that the neurological impairment seen in boys with MCT8 mutations is not a direct result of defective neurodifferentiation of cerebral neuroprogenitor cells. Apart from increased cerebral TH uptake at the BBB (Ceballos *et al.* 2009) and suppressed neuronal precursor proliferation (James *et al.* 2009), the impact of MCT8 upon other developmental processes within the human fetal brain such as neuronal cell transmission activity, synaptogenesis, neuronal migration and glia differentiation, remain to be investigated.

In conclusion, our demonstration of different expression patterns amongst the TH transporters in the human fetal cerebral cortex from very early gestation suggests that their concerted actions may result in the timely physiological regulation of cellular TH entry within the fetal CNS from early development. However, the precise role of TH and each TH transporter during human CNS development remains poorly understood, but they do not appear to directly impact upon human neurodifferentiation.

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Author contributions

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