



Research Paper

Overcoming Monocarboxylate Transporter 8 (MCT8)-Deficiency to Promote Human Oligodendrocyte Differentiation and Myelination



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ABSTRACT

Cell membrane thyroid hormone (TH) transport can be facilitated by the monocarboxylate transporter 8 (MCT8), encoded by the solute carrier family 16 member 2 (*SLC16A2*) gene. Human mutations of the gene, *SLC16A2*, result in the X-linked-inherited psychomotor retardation and hypomyelination disorder, Allan-Herndon-Dudley syndrome (AHDS). We posited that abrogating MCT8-dependent TH transport limits oligodendrogenesis and myelination. We show that human oligodendrocytes (OL), derived from the *NKX2.1*-GFP human embryonic stem cell (hESC) reporter line, express MCT8. Moreover, treatment of these cultures with DITPA (an MCT8-independent TH analog), up-regulates OL differentiation transcription factors and myelin gene expression. DITPA promotes hESC-derived OL myelination of retinal ganglion axons in co-culture. Pharmacological and genetic blockade of MCT8 induces significant OL apoptosis, impairing myelination. DITPA treatment limits OL apoptosis mediated by *SLC16A2* down-regulation primarily signaling through AKT phosphorylation, driving myelination. Our results highlight the potential role of MCT8 in TH transport for human OL development and may implicate DITPA as a promising treatment for developmentally-regulated myelination in AHDS.

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1. Introduction

Thyroid hormones (THs) play a vital role during mammalian embryonic brain development. Identification of a number of TH transporters, across cell plasma membranes, has expanded our understanding of how bioactive THs can elicit cell-specific developmental events (for review see Kapoor et al., 2015, Lee and Petratos, 2016). Membrane-bound transporters are members of the solute carrier (SLC) superfamily of proteins, now known to consist of 395 distinctive proteins categorized into 52 families (for review see Lin et al., 2015). The monocarboxylate transporters (MCTs), encoded by the *SLC16* gene family, consist of 14 members, of which MCT8 (encoded by *SLC16A2*) and MCT10 (encoded by *SLC16A10*) are two homologous 12 transmembrane helical proteins, responsible for TH transport (for review see Visser et al., 2008). Other important TH membrane transporters include the organic anionic transporter protein (OAT1P1C) and the L-type amino acid transporters

(LAT1 and LAT2) encoded by the *SLC01C1*, *SLC7A5* and *SLC7A6* genes respectively (Tamai et al., 2000; Pizzagalli et al., 2002; Hagenbuch and Meier, 2003; Friesema et al., 2001). These transporters seem to be of critical importance at the blood brain barrier (BBB) for T₄ transport from the peripheral circulation into the CNS parenchyma via astrocytes (Boado et al., 1999; van der Deure et al., 2008), although the levels of OAT1P1C in humans is considerably lower than that present in the rodent (Roberts et al., 2008), which may indicate why neurological phenotypes are present only in *Mct8/Oat1p1c* double knock out mice (Mayerl et al., 2014). Recently the identification of *Mct8* expression in a rodent oligodendroglial cell line (158N) has been reported but its significance has not been elucidated (Braun et al., 2011).

Of clinical importance, MCT8 has been identified as a high affinity TH cell membrane transporter, since the only substrates identified include tri-iodothyronine (T₃) and its pro-hormone thyroxine (T₄) (Friesema et al., 2003; Kinne et al., 2010). In humans, mutations at the *SLC16A2* gene locus (encoding MCT8) cause the severe congenital X-linked psychomotor retardation, known as Allan-Herndon-Dudley syndrome (AHDS) (Friesema et al., 2004; Dumitrescu et al., 2004; Schwartz et al., 2005). Along with the increased serum levels of free-

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T₃, developmentally delayed myelination shown by magnetic resonance imaging (MRI), is a common feature of this disorder (Armour et al., 2015; Vaur-Barriere et al., 2009; Gika et al., 2010). Although myelination was reported in T2-weighted MRI from follow-up longitudinal studies of AHDS patients, their brain development is incomplete as neurological phenotypes persist (Armour et al., 2015; Gika et al., 2010; Vaur-Barriere et al., 2009). However, myelin deficits have been reported in a recent post-mortem analysis of an 11-year-old AHDS boy which revealed prominent hypomyelination by myelin basic protein (MBP) immunostaining (Lopez-Espindola et al., 2014).

Despite TH-dependency during oligodendrocyte (OL) differentiation, cell entry of these hydrophobic hormones remains undefined within this cell lineage. Since *Slc16a2* mutant mice display no overt neurological abnormality, having only a mild behavioral phenotype (Dumitrescu et al., 2006; Wirth et al., 2009), we utilized oligodendroglial precursor cells (OPCs) derived from human embryonic stem cells (hESCs) to identify the expression profiles and physiological role of MCT8 during OL development. Although several protocols exist that derive OPCs from hESCs (for review see Alsanie et al., 2013), the efficiency to develop homogeneous cultures varies, preventing robust molecular analyses of derived OPCs and mature OLs. Therefore, we developed a modified technique to obtain high yields of oligodendroglial cells to clarify the role of MCT8 during OL development.

TH analogs that do not require MCT8 have been suggested as a potential therapy to treat AHDS. For example, di-iodothyropropionic acid (DITPA) can normalize peripheral hyperthyroidism and reduce hypermetabolism in AHDS patients (Verge et al., 2012). However, the exact mechanism by which DITPA acts is largely unknown. Considering our findings of reduced OL viability upon inhibition of MCT8, in this study we posit that the provision of DITPA upon knockdown of *SLC16A2* in hESC-derived OPCs may potentiate their proliferation and differentiation. Microarray analysis revealed up-regulation of OL-specific transcription factors upon DITPA administration to early OPCs. We tested the effect of DITPA upon OL development and found that it induced cell cycle exit, OPC differentiation and myelination in vitro. Importantly, DITPA administration rescued these cells from apoptosis mediated by *SLC16A2* down-regulation and promoted their myelination of axons, likely due to downstream phosphorylation of AKT and ERK1. Collectively, these data suggest that MCT8 is a physiological TH transporter in OLs and that early intervention using DITPA holds therapeutic promise in enhancing myelination in AHDS.

2. Materials and Methods

2.1. hESC Culture

We used two distinct lines of hESC for this study, HES3 and *NKX2.1*-GFP reporter line derived from HES3 (Goulburn et al., 2011). hESC studies were approved by Monash University Human Research Ethics Committee.

2.2. Derivation of OPCs

OPCs were generated from *NKX2.1*-GFP reporter line using our protocol modified from (Chaerkady et al., 2011; Kerr et al., 2010). A detailed protocol is described in Supplemental Experimental Procedures and defined within the International (PCT) Patent Publication No. WO2016/101017A1 (Petras et al., 2016).

2.3. Immunocytochemistry

Preparations of cultures for immunolabeling are described in Supplemental Experimental Procedures. See Table 1 for definition of markers used to assess oligodendrocyte development.

2.4. Flow Cytometry

Preparations of cells for flow cytometry are described in Supplemental Experimental Procedures. See Table 1 for definition of markers used to assess oligodendrocyte development.

2.5. Fluorescence-Activated Cell Sorting (FACS)

The rationale and detailed protocol for *NKX2.1*-based sorting is described in Supplemental Experimental Procedures. At the end of stage III of hESC differentiation, *NKX2.1*-GFP + cells were sorted using a BD Influx (BD Biosciences). The sorted cells; GFP – and GFP + cells were collected in stage IV medium with 10 μM Y27632 (Enzo) then further differentiated.

2.6. Microarray Analysis

H9 cell line-derived human (h)OPCs (Merck/Millipore) were differentiated for 4 weeks and the following treatments were administered to the cells for 48 h: Medium with 0.01% absolute ethanol (diluent control); 1 ng/mL DITPA (DITPA 1 ng/mL); 10 ng/mL DITPA (DITPA 10 ng/mL); or 100 ng/mL DITPA (DITPA 100 ng/mL). DITPA was diluted in absolute ethanol. 1 μg of collected mRNA from each population was hybridized to Human HT-12 v3.0 Gene Expression BeadChip (Illumina) according to the manufacturer's instruction (For detailed procedure see Supplemental Experimental Procedures).

2.7. Cell Cycle Analysis with BrdU

BrdU cell cycle analysis was performed according to the manufacturer's protocol (BD Biosciences). For a detailed protocol, see Supplemental Experimental Procedures.

2.8. Generation of Lentivirus Carrying *SLC16A2*-shRNA

psi-LVRU6MP vectors carrying either scrambled shRNA or 4 different shRNA sequences for *SLC16A2* were generated by Genecopoeia, USA. These vectors have two different promoters, U6 promoter for shRNA and EF1α for the mCherry reporter, and a puromycin resistance for stable selection (8357 bp). We tested 4 different shRNA sequences for our *NKX2.1*-GFP hESC lines and the most efficient *SLC16A2*-shRNA sequence was selected for the generation of lentivirus. The most efficient target sequence for our cells is as follows: GCTTCGCGCCGTAGCTTA. This specific sequence or scrambled shRNA sequence were packaged into a lentivirus (Genecopoeia).

2.9. Stable Knockdown of *SLC16A2*

NKX2.1-GFP sorted cells at the end of stage VI or V were transduced with psi-LVRU6MP vectors carrying either scrambled shRNA or shRNA sequences for *SLC16A2* (Genecopoeia, USA) with multiplicity of infection (MOI) of 10 in appropriate stage-specific medium containing polybrene (5 μg/mL, Sigma-Aldrich). Efficiency of transduction was validated by analyzing mCherry + cells by flow cytometry and efficiency of knockdown was validated by analyzing the *SLC16A2* transcript level by qRT-PCR 5 days post-transduction. An apoptosis assay was performed on cells either, treated with or without DITPA for 3 days after 72 h post-transduction, and then fixed. Cells were stained with monoclonal rat anti-mCherry (M11217, Life Technologies, 1:1000), polyclonal rabbit anti-cleaved caspase-3 (9661, Cell Signaling Technology, 1:400) and DAPI (Life Technologies) then analyzed by confocal microscopy (Nikon A1 Inverted using a ×20 water objective lens). Apoptotic OLs were defined as those mCherry-positive cells with cleaved caspase-3 + nuclei that were also condensed and fragmented as assessed by DAPI. The data were plotted as the number of cleaved caspase-3/mCherry + cells divided by total number of mCherry + cells. For the

Table 1
Neural lineage markers utilized throughout this study.

Name of the markers	Abbreviation	Expressed in	According to hESC differentiation (see Fig. 1)
Nestin	Nestin	Neural precursor cells	Stage IV onwards
Glial fibrillary acidic protein	GFAP	Neural precursor cells, Astrocytes	
Epcam	Epcam	Neuroepithelial cells	
β -III tubulin	β -III tubulin	Neurons	Stage IV and V
Microtubule-associated protein 2	MAP2	Neurons	
Sex-determining region Y-box 10	Sox10	Oligodendrocyte precursor cells	
Oligodendrocyte transcription factor 2	Olig2	Oligodendrocyte precursor cells	
Platelet-derived growth factor receptor alpha	PDGFR α	Glial precursor cells, Oligodendrocyte precursor cells	
Chondroitin sulfate proteoglycan (CSPG4), Neural/glia antigen 2	NG2	Glial precursor cells, Oligodendrocyte precursor cells	Stage V and VI Stage VI
O4	O4	Pre-myelinating oligodendrocytes	
Myelin basic protein	MBP	Mature oligodendrocytes	

Western blotting performed to assess AKT and ERK1/2 signaling, see Supplemental Experimental Procedures.

2.10. Co-culture and Myelination Assays with Rat RGC and hESC-Derived OPCs

Retinae were dissected from P6 Sprague-Dawley rat pups (AMPREP Animal Ethics Committee #E-1602/2015/M). RGCs were purified according to the published immunopanning protocol (Watkins et al., 2008; Deliyanti and Wilkinson-Berka, 2015). Details of the RGC preparation are described in Supplemental Experimental Procedure. The RGC growth medium was changed every third day and cultures were maintained for 9 days. Pre-OLs from *NKX2.1*-GFP+ cells at stage VI day 10, were FACS sorted according to O4 antibody labeling. O4+ sorted cells were then co-cultured with RGCs at a density of 20,000/well under 3 different conditions: T₃ with 0.01% ethanol, 10 ng/mL DITPA, and 40 ng/mL T₃ with DITPA in myelination medium (see Supplemental Experimental Procedure). The medium was changed every 3 days. At day 7, all co-cultures were fixed with 2% paraformaldehyde (PFA) for 10 min at room temperature. Cells were stained with a monoclonal rat anti-mCherry (Life Technologies, M11217, 1:1000); monoclonal mouse anti-NF-200 (Sigma-Aldrich, N0142, 1:200); and polyclonal rabbit anti-MBP (Millipore, AB980, 1:200). Subsequently cells were stained with appropriate Alexa Fluor-labeled secondary antibodies (Life technologies).

2.11. Quantification of Myelination in Culture

In RGC-OPC co-cultures, myelin segments were counted manually. 10 fields ($\times 20$ objective) were randomly selected and captured from each coverslip with a confocal microscope (Nikon A1 inverted). For representative images, 10 z-stack images (0.25 μ m intervals) were captured, analyzed and processed for 3D volume rendering by Imaris version 7.6.4. OLs were scored according to their morphology and defined as either “resting” (processes not touching axons); “contacting” (processes touching axons but not wrapping); and “ensheathing” (processes aligned with and wrapping axons). A myelination index (i.e. percentage of myelinated axons) was calculated by: the number of MBP+ membrane segments that are ensheathing the NF200+

axons divided by the total number of NF200+ axons and multiplied by 100 (see Supplemental Experimental Procedure).

2.12. Statistical Analysis

Data are presented as mean \pm SEM. Two-way ANOVA with Tukey's multiple comparison test determined statistical significance, unless otherwise stated. A *P* value of <0.05 was considered as statistically significant. GraphPad Prism version 6.0c software was used for statistical analysis of the data.

3. Results

3.1. Patent

All data presented in this manuscript are defined within the International Patent Cooperation Treaty (PCT), International publication No. WO2016/101017A1 in the name of NeuOrphan Pty Ltd. Entitled: Improvements in oligodendroglial cell culturing methods and in methods for treating neurodegenerative disorders by using thyroid hormones or analogues (Petraatos et al., 2016). NeuOrphan Pty Ltd. has commercial interests in the current technology for research and development purposes.

3.2. *NKX2.1*-GFP-based Sorting Enhances OPC Yield

We utilized two hESC lines in this study; Hes3 and Hes3-derived *NKX2.1*-GFP reporter lines (Goulburn et al., 2011). The previous protocol for generating hESC-derived OPCs (Chaerkady et al., 2011) was modified with the addition of sonic hedgehog (shh) (Pringle et al., 1996) during the neural precursor stage of differentiation (Fig. 1) [see also the International (PCT) Patent Application No. PCT/AU2015/000770]. We successfully derived PDGFR α + /NG2+ OPCs, O4+ pre-OLs and MBP+ pre-myelinating OLs (Fig. 1). By flow cytometry and immunocytochemistry, we identified the induction of PDGFR α + /NG2+ OPCs and O4+ pre-OLs at the end of stage IV and VI, respectively but at low yields. We then sorted *NKX2.1*+ cells at the peak timepoint of GFP expression during hESC differentiation. By live imaging and flow cytometry, we observed *NKX2.1*-GFP induction at day 8 of stage II (Fig. S1). During stage III, we identified that the maximal induction of *NKX2.1*-GFP occurred

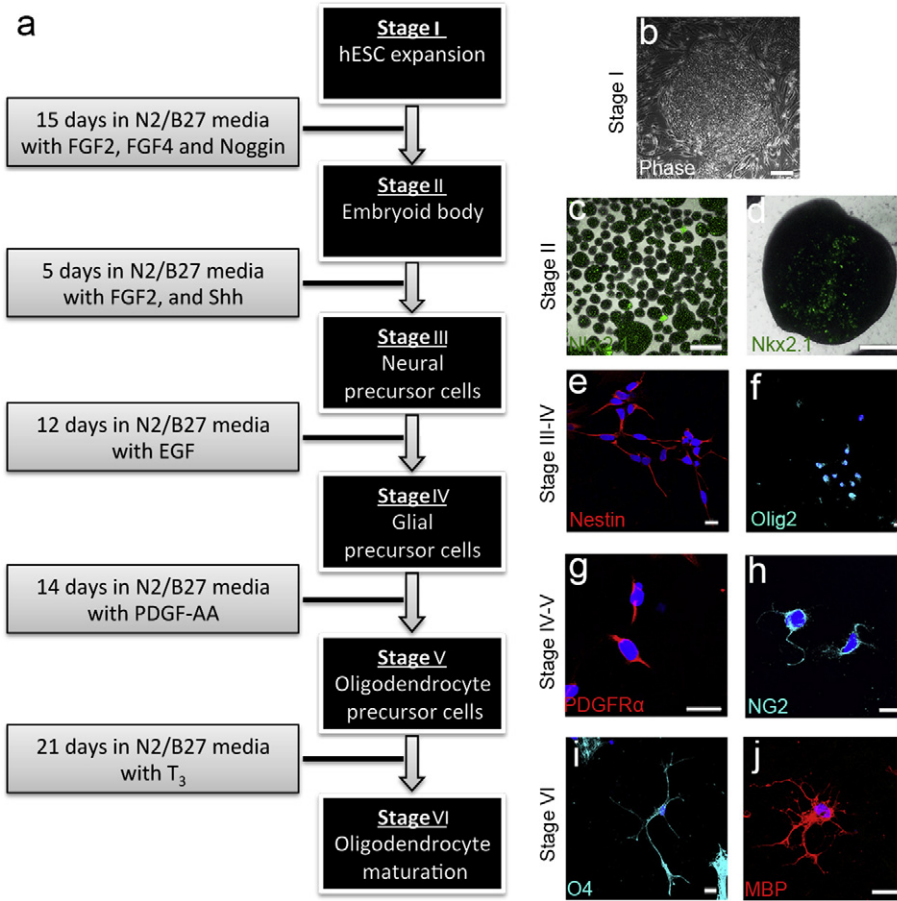


Fig. 1. *NKX2.1*-GFP hESC reporter line can be directed toward an OPC fate. (a) Directed differentiation of hESC into OPCs. (b) hESCs (stage I) were differentiated to neural embryoid bodies (EBs) (stage II) expressing (c, d) *NKX2.1*. (e) Nestin + neural precursor cells (stage III) were generated. (f) Olig2 + Glial precursor cells (stage IV) appeared under the influence of EGF. These were further differentiated into OPCs (stage V) expressing (g) PDGFRα and (h) NG2 through the addition of PDGF-AA. (i, j) T₃ promoted terminal differentiation of OPCs into (i) O4 + pre-OLs (stage VI), and (j) MBP + pre-myelinating OLs. (e–j) Counterstaining was performed by DAPI. Scale bar = 100 μm for (b–d) and 20 μm for (e–j).

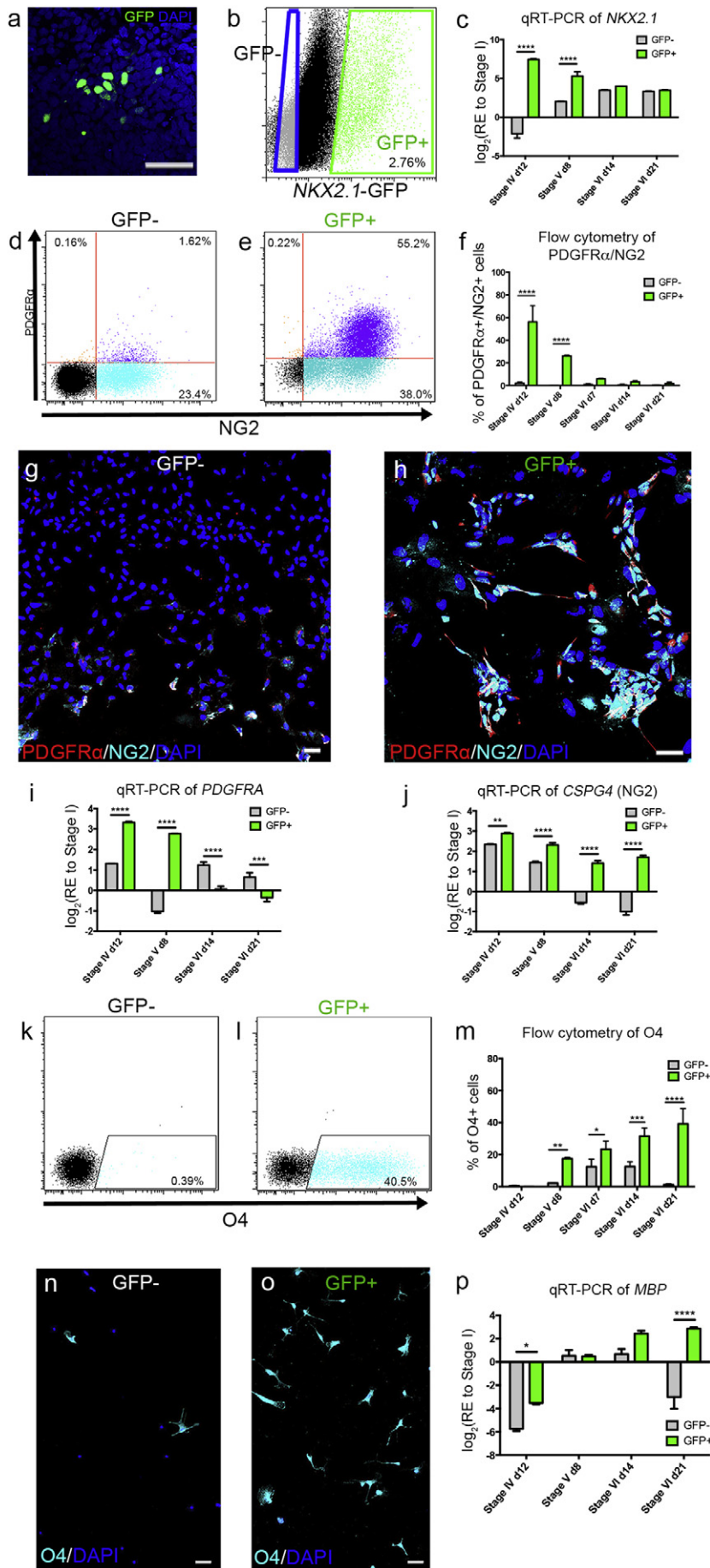
by day 5, served as a time-point for cell sorting (Fig. 2A,B). Therefore, both GFP + and GFP – cells were sorted by FACS at day 5 of stage III, then differentiated under the same culture conditions upon which their oligodendrogenic potential was addressed. Following sorting of the GFP + and GFP – populations of differentiating hESCs according to maximal induction of *NKX2.1*-GFP, qRT-PCR analysis of the *NKX2.1* transcript identified significant up-regulation of this gene in GFP + populations at stages IV–V (Fig. 2C). First, the yield of PDGFRα + /NG2 + OPC derivation was analyzed. At the end of stage IV, the yield of OPC derivation was significantly higher in the GFP + (~55.2%) compared with GFP – (~1.62%) isolated cells (Fig. 2D,E). This trend was also evident at stage V day 6, although the percentage of PDGFRα + /NG2 + OPCs significantly decreased as the cells differentiated (Fig. 2F, Fig. S2A–B). Immunocytochemistry for PDGFRα and NG2 at stage IV day 12, supported the flow cytometry data (Fig. 2G,H). Furthermore, in line with these data we also showed significant up-regulation in *PDGFRα* from stage IV day 12, to stage V day 8, whereas the *CSPG4* (encoding NG2) gene was significantly up-regulated early from stage IV onwards in GFP + compared with GFP – sorted cells (Fig. 2I,J).

Next, we analyzed the yield of O4 + pre-OL derivation from stage IV to stage VI. We found that the percentage of O4 + pre-OLs was significantly higher in GFP + compared with GFP – sorted cells from stages V–VI (Fig. 2K–O). Immunocytochemistry analysis also demonstrated a greater derivation of O4 + pre-OLs at the end of stage VI (Fig. 2N,O). In support of these data, qRT-PCR analysis for the *MBP* gene showed a ~4-fold up-regulation in GFP + sorted cells when compared with GFP – isolated cells at stage VI day 21 (Fig. 2P). Further to these surface markers, we analyzed an essential transcription factor for oligodendrogenesis, Sox10

(Fig. S2C–F). By performing immunocytochemistry, we determined that significantly more Sox10 + cells were generated from the isolated GFP + cells by stage IV (day 12; ~49%), and VI (day 21; ~82%), than GFP-negative cells (<20% at either stage; Fig. S2E). qRT-PCR analysis supported these immunocytochemistry data, showing significantly up-regulated *SOX10* gene expression levels in GFP + when compared with the GFP – sorted cells from stage IV day 12, to stage V day 8 (Fig. S2F).

3.3. MCT8 is Expressed on Oligodendroglial Lineage Cells

Having successfully generated human OPCs, immature OLs and OLs, we next aimed to determine which of these cell types expressed MCT8 was indeed co-expressed with various oligodendroglial lineage markers (Fig. 3). Firstly, we showed MCT8 expression on O4 + pre-OLs by flow cytometry during stage VI, where significant numbers of O4 + cells were labeled with MCT8 among the GFP + sorted cultures (Fig. 3A,B). Moreover, western blot analysis of cell lysates from stage VI, revealed a specific monomeric form of MCT8 (approximately 60 kDa) (Friesema et al., 2006) in both the GFP – and GFP + isolated cells (for commercial antibody validation assay see Fig. S4). We also detected this in the conditioned medium, indicating MCT8 may be released extracellularly (Fig. 3C), possibly enriched in oligodendroglial cell-derived exosomes (Fruhbeis et al., 2013). This possibility is currently under investigation. qRT-PCR analysis for *SLC16A2* revealed increasing levels of this gene from the OPC stage (stage IV day 12) through to the pre-OL stage (stage VI day 21) in GFP + isolated cells (Fig. 3D). These results indicate that MCT8 is expressed in maturing oligodendroglial lineage cells.



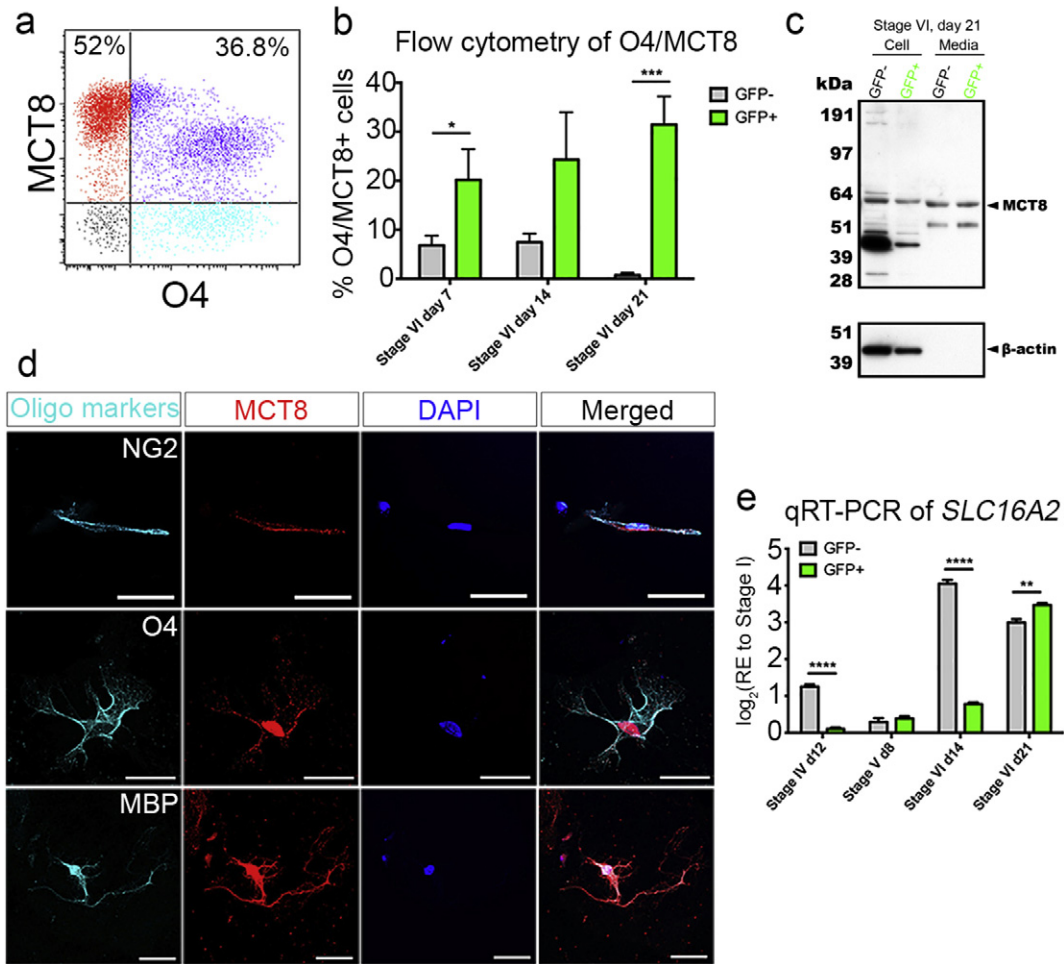


Fig. 3. MCT8 is expressed on immature and pre-myelinating OLs. (a) Flow cytometric analysis showing expression of MCT8 in O4 + immature OLs derived from the GFP + sorted population at stage VI, day 21. (b) The proportion of O4 +/MCT8 + cells during differentiation (c) Prior to cell lysate protein analysis, commercial antibody validation as performed (see Fig. S4). Western blot of cell lysates and conditioned medium from the GFP – and GFP + sorted population at stage VI, day 21 with MCT8 and β-actin antibodies. (d) qRT-PCR analysis of *SLC16A2* during differentiation. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; (*n* = 3; mean ± SEM). Scale bar = 50 μm.

3.4. DITPA Potentiates OL Development in Mixed Neural Cultures

To identify whether DITPA can promote oligodendrogenesis in the same manner as T₃, we first utilized the human OPC differentiation kit (Merck-Millipore). Using this kit, we were able to derive ~30% of NG2 + OPCs at the end of differentiation (Week 4) (Fig. 4A). Further analysis revealed that there was a mixed neural population of Nestin + neural precursors, GFAP + astrocytes and β-III-tubulin + neurons at the end of differentiation (Week 4, data not shown), indicating these cells are at the early stage of OPC specification from multipotent neural precursors. From this time-point, we treated cells with 1 ng/mL and 10 ng/mL of DITPA for 48 h and performed microarray analysis. Cells grown in differentiation medium with 0.01% ethanol (dissolvent of DITPA) served as a negative control.

Gene expression profiles of cells treated with either DITPA at 1 ng/mL or DITPA at 10 ng/mL when compared to control cells treated with Ethanol; showed that DITPA regulated 3385 genes (Fig. 4C), illustrated in the heatmap (Fig. 4D). Gene ontology (GO) analysis showed

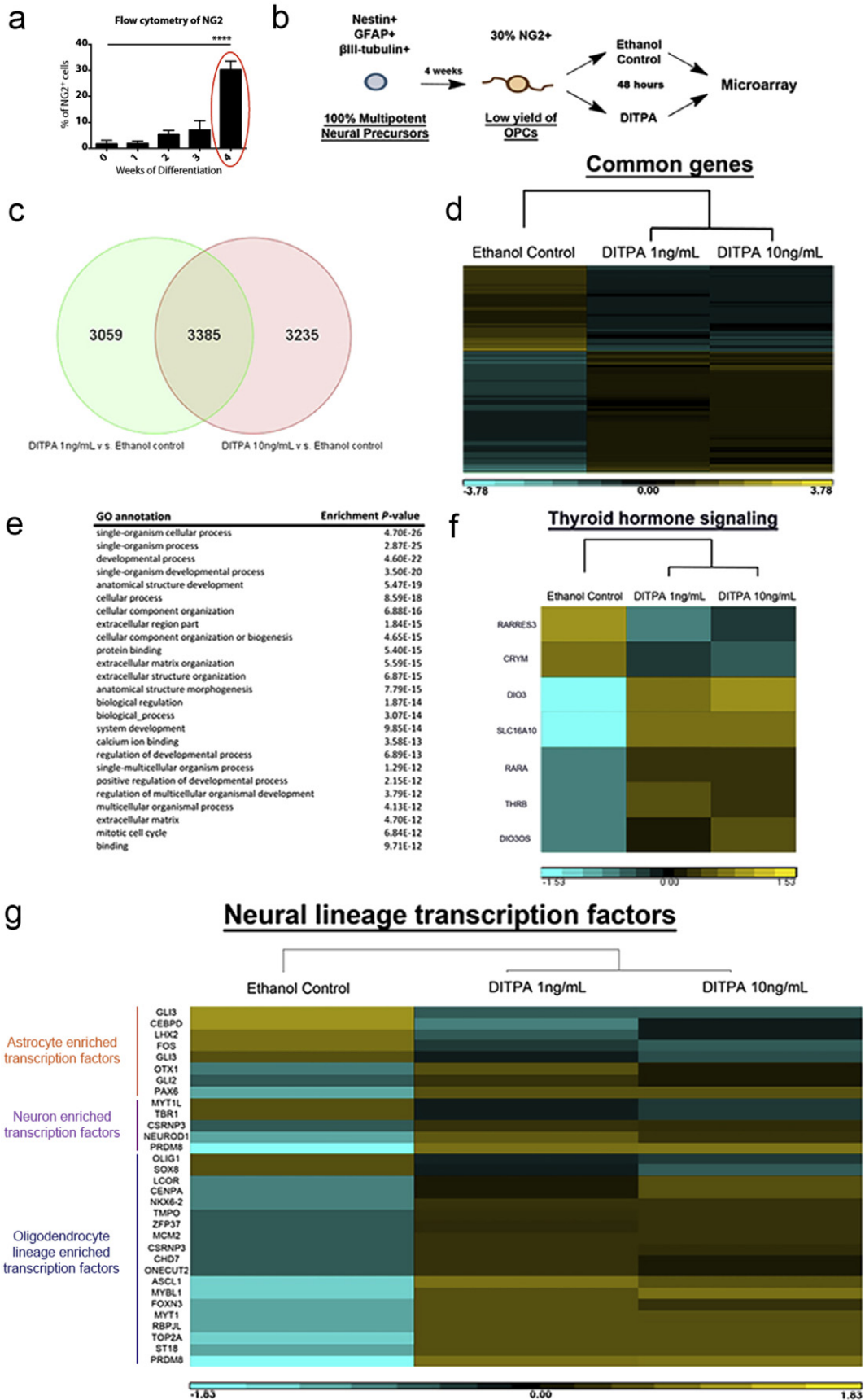
that genes related to developmental processes were regulated upon DITPA treatment (Fig. 4E). From this GO analysis, we selected the GO term ‘TH signaling’ and found a down-regulation of retinoic acid response element 3 (*RARRES3*) and intracellular TH-binding protein, μ-crystallin (*CRYM*). Moreover, there was an up-regulation of deiodinase 3 (*DIO3*), nuclear retinoic acid receptor alpha (*RARA*), nuclear TH receptor β (*THRB*) and *DIO3* opposite strand (*DIO3OS*) (Fig. 4F). These results indicate that DITPA may not be transported by μ-crystallin in the cell since its cellular levels are required for appropriate TH signaling (Suzuki et al., 2007, Takeshige et al., 2014). Considering that *DIO3*, *RARA* and *THRB* were all upregulated in our GO analysis, it would suggest that TH signaling is maintained in these cell lines. However, one plausible hypothesis is that DITPA may bind to the retinoic acid receptor α and TH receptor β to transcribe OL-related genes (Baas et al., 2002, Baas et al., 2000, Laeng et al., 1994).

Importantly, we found up-regulation of an array of transcription factors (lists from Najm et al., 2013) enriched in oligodendroglial cells such as achaete-scute homolog 1 (*ASCL1*) and myelin transcription factor 1

Fig. 2. *NKX2.1*-GFP + sorted cells were differentiated toward an oligodendroglial lineage. (a) hESC-derived *NKX2.1*-expressing cells were identified by immunolabeling for GFP followed by DAPI counterstaining. (b) GFP + cells were FACS-sorted from GFP-cells at stage III, day 5. (c) qRT-PCR analysis of *NKX2.1* post-sorting throughout differentiation. (d–e) Flow cytometric analysis of PDGFRα +/NG2 + OPCs in (d) the GFP – and (e) GFP + populations at stage IV, day 12. (f) The proportion of PDGFRα +/NG2 + OPCs during differentiation. (g–h) Immunostaining for PDGFRα and NG2 on (g) GFP – and (h) GFP + sorted cells at stage V, counterstained with DAPI. (i–j) qRT-PCR analyses of (i) *PDGFRA* and (j) *CSPG4* throughout differentiation. (k–m) Flow cytometric analysis of O4 in (K) GFP – and (l) GFP + sorted populations at stage VI, day 21. (M) The proportion of O4 + immature OLs throughout differentiation. (n–o) Immunostaining for O4 and DAPI on (n) GFP – and (o) GFP + sorted populations. (p) qRT-PCR analysis of *MBP* throughout differentiation (RE; relative expression). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; (*n* = 3–4; mean ± SEM). Scale bar = 50 μm.

(*MYT1*) with DITPA treatment whereas, minimal astrocyte and neuron enriched transcription factors were regulated upon DITPA treatment (Fig. 4G). Since T_3 has been previously shown to promote cell cycle exit

and OL differentiation (Barres et al., 1994), we further analyzed cell cycle associated gene pathways such as *BMP*, *TGF β* , *WNT*, and Notch signaling (within the GO term). From this analysis, we found genes



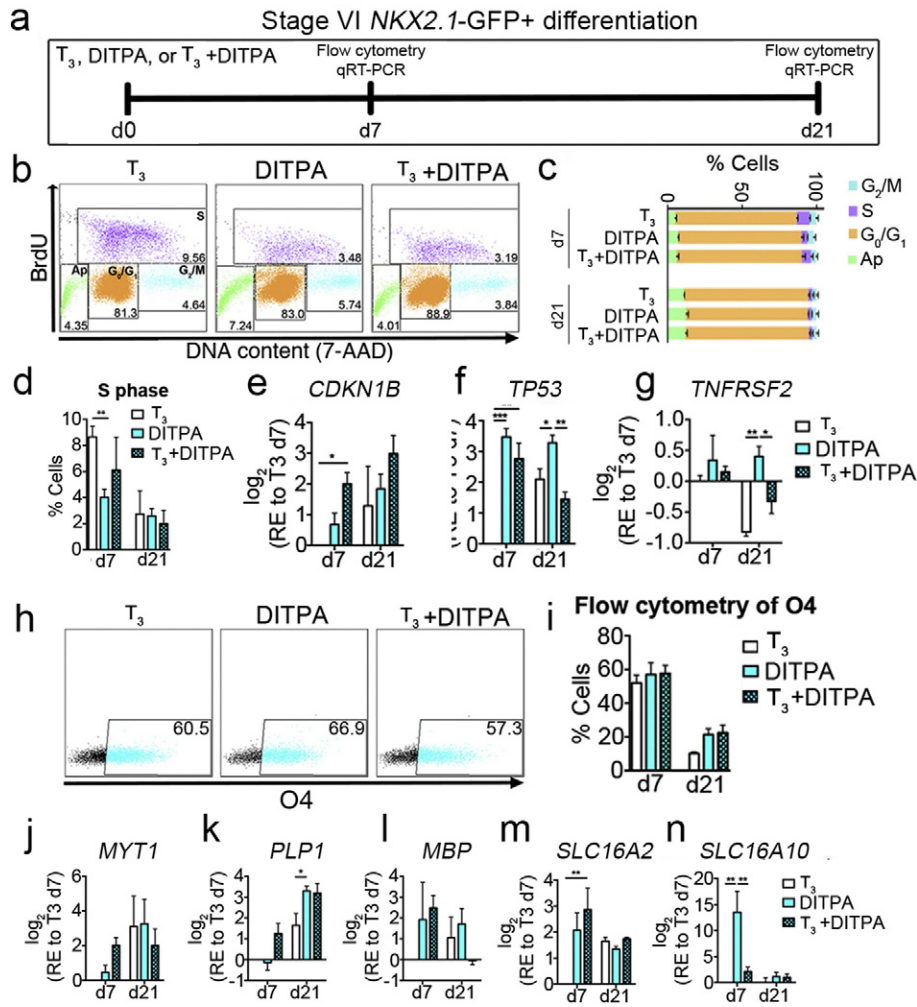


Fig. 5. DITPA potentiates OPC cycle exit promoting differentiation in enriched OL cultures derived from *NKX2.1-GFP+* sorted hESCs. (a) DITPA treatment (10 ng/mL) regime throughout Stage VI. Cells were treated with either T_3 alone (T_3 , 50 ng/mL), DITPA alone (DITPA, 10 ng/mL) or T_3 with DITPA (T_3 , 50 ng/mL + DITPA, 10 ng/mL) then these were analyzed on day 7 and 21 post-treatments by flow cytometry and qRT-PCR. (b) Flow cytometric dot plots upon BrdU incorporation at day 7 of treatments (Ap; apoptotic cells). (c) The percentage of cells at the different cell cycle stages at day 7 and 21 post-treatments. (d) The percentage of cells in S phase (BrdU-positive) for the different treatment groups are shown. (e–g) qRT-PCR analyses of cell cycle-associated genes; (e) *CDKN1B*, (f) *TP53* and (g) *TNFRSF2*, 7 and 21 days post-treatments. (h) Flow cytometric analysis of O4+ cells analyzed by flow cytometry at day 7 and 21 post-treatments. (i) The percentage of O4+ cells analyzed by flow cytometry at day 7 and 21 post-treatments. (j–l) qRT-PCR analyses of myelin genes; (j) *MYT1*, (k) *PLP1*, (l) *MBP*, 7 and 21 days post-treatments. (m and n) qRT-PCR analyses of TH transporter genes; (m) *SLC16A2* (MCT8), and (n) *SLC16A10* (MCT10) 7 and 21 days post-treatments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ($n = 3$; mean \pm SEM).

associated with these pathways to be regulated by DITPA (Fig. S5A–B). Furthermore, we found: (i) a significant up-regulation of the repressor (*RPRM*) (~13 fold), a p53-dependent G2 arrest mediator (Laeng et al., 1994); (ii) up-regulation of metallothionein (*MT1A*) (~8 fold), a binding partner of p53 to modulate p53-dependent apoptosis (Ostrakhovitch et al., 2006); and (iii) the up-regulation of *P53* (~2 fold) upon DITPA treatment (Fig. S5B). These results may indicate that DITPA promotes cell cycle exit and potentiates OL development at the early stage of differentiation.

3.5. DITPA Promotes Cell Cycle Exit to Potentiate OL Differentiation

To examine the effect of DITPA on cell cycle exit, we treated stage VI *NKX2.1-GFP+* sorted cells with T_3 alone (T_3 control), DITPA alone

(DITPA), or co-administration of T_3 and DITPA ($T_3 + DITPA$) for 21 days and analyzed BrdU incorporation by flow cytometry at day 7 and 21 (Fig. 5A). At day 7 and 21, the cells were pulsed with BrdU for 1 h and treated with DNase I for 45 min. At day 7, we found a significant reduction in the numbers of cells that were in S phase upon both the administration of DITPA in the cultures, compared with those treated with T_3 alone. However, at day 21 we found no significant differences among all three groups (Fig. 5B–D). These data indicate that DITPA potentiates cell cycle exit faster than T_3 . However, the possibility exists that T_3 administration may also be generating other proliferating neural lineage cells.

TH-responsive cell cycle arrest associated genes such as *CDKN1B* (encodes for p27) (Fig. 5E) and *TP53* (encodes for p53) (Fig. 5F)

Fig. 4. Transcriptome analysis for human mixed neural cell cultures differentiated toward OPC lineage with DITPA. (a) The proportion of NG2-positive cells during differentiation of Merck-Millipore human mixed neural cell cultures. One-way ANOVA with Tukey's post-hoc test; **** $P < 0.0001$ ($n = 3–4$; mean \pm SEM). At week 4, ~30% of NG2+ cells were derived and DITPA (10 ng/mL) was treated (circled in red). (b) DITPA treatment (10 ng/mL) regime upon human OPC specification from neural precursors. (c) Comparative gene expression profiles from different concentrations of DITPA (10 ng/mL) versus ethanol control (including T_3 in differentiation medium) treatments. (d) Genome-wide transcriptional profile heatmap obtained for the different concentrations of DITPA (10 ng/mL) and ethanol control treatments. (e) Highly enriched GO terms of commonly expressed genes following the administration of DITPA at 1 ng/mL and 10 ng/mL versus ethanol control. (f and g) Heatmap of (f) genes related to TH signaling and (g) Neuronal, astroglial and oligodendroglial enriched transcription factors (Gene lists are from Najm et al., 2013) following the administration DITPA (10 ng/mL) and ethanol control.

were both up-regulated by DITPA and T_3 + DITPA when compared to the T_3 treated cultures. As overexpression of p53 has been identified to potentiate apoptosis via death receptors (DRs) (Wosik et al., 2003), we analyzed one of the DRs, previously reported to promote oligodendrocytopathy, DR6 (Mi et al., 2011), and found almost negligible up-regulation of *TNFRSF2* (encodes DR6) upon DITPA treatment. Hence, the up-regulation of *TP53* by DITPA treatment is likely to promote cell cycle exit for the purpose of OL differentiation, not cell death (Fig. 5G). Along with these data, further gene expression analysis by qRT-PCR revealed that down-regulation of the essential genes for BMP signaling, *BMP7*, JNK signaling *JUN*, TGF β signaling, *TGFB3*, and WNT signaling *WNT5A* upon DITPA treatment occurs, corroborating the hypothesis that DITPA can promote differentiation. Furthermore, up-regulation of the WNT antagonists such as *FRZB*, *SFRP1*, and *SFRP2* were found upon DITPA treatment supporting this notion (Fig. S5C). These data indicate that DITPA potentiates oligodendroglial cell cycle exit, promoting differentiation at the late stage from OPCs toward O4+ pre-OLs.

In line with these data demonstrating modulation of genes regulating cell development, further qRT-PCR-based gene expression analysis revealed that master regulators of OL differentiation such as *OLIG1*, *OLIG2* and *SOX10* were up-regulated following DITPA treatment. Following DITPA treatment at day 7, the greatest increase in gene expression was demonstrated to occur in *OLIG1* and *OLIG2*, far greater than that seen for T_3 . Moreover, important genes for OL development such as *ASCL1*, *PDGFRA*, and *NKX6.2* were up-regulated following all treatment regimes. On the other hand, as cells mature, the immature OPC gene, *CSPG4* was down-regulated in all three treatment groups (Fig. S5C). Moreover, myelin genes such as *MYT1*, *PLP1*, and *MBP* were up-regulated following the treatment of differentiating cultures with DITPA (Fig. 5J–N). In particular, at day 21 of DITPA treatment, we found substantial up-regulation of *PLP1* than that documented for the cells treated with T_3 alone (Fig. 5J–L). These results suggest that DITPA promotes OL differentiation and this is far greater than what T_3 can facilitate.

Having established the OL derivation potential of DITPA, we then asked whether these genes associated with TH signaling were also modified. Firstly, we found a significant up-regulation of *SLC16A2* and *SLC16A10* upon DITPA treatment at day 7 when compared with T_3 . Our results showed a ~14-fold up-regulation of *SLC16A10* following DITPA treatment (Fig. 5M and N). Further analysis revealed no significant differences in the gene expression for *THRB*, *DIO2* and *DIO3*, between T_3 and DITPA treatments, indicating that similar intracellular signaling is operative upon either T_3 or DITPA stimulation of cells. Co-administration of T_3 and DITPA showed significant up-regulation of *THRB* and *DIO3* (Fig. S5C), which may indicate that cells are in a hyperthyroid state.

3.6. DITPA Promotes Myelination in Co-culture

Since the evidence argued that DITPA promotes OL differentiation, we next asked whether it also potentiates CNS myelination. For this, we set up a co-culture system comprising rat retinal ganglion cells (RGCs) and *NKX2.1*-GFP+ sorted OPCs where, these cultures were treated with T_3 , DITPA, or T_3 + DITPA. Seven days following co-culture, we were able to detect subsets of MBP+ OLs that began initiating or wrapping axons with myelin membrane in all treatment groups, identified by MBP+ segments around NF-200+ axons (Fig. 6B). Quantification revealed that more contacting or ensheathing MBP+ OLs are observed in DITPA treated cultures than those observed following treatment with T_3 alone (Fig. 6C). In line with this, the percentage of myelinated axons (number of myelinated axons divided by the total number of axons and multiplied by 100) was significantly enhanced with DITPA compared with T_3 treatment alone (Fig. 6D) although, qualitatively the amount of myelin added per axon (MBP immunofluorescence intensity/axon) did not appear to be altered in the DITPA treated cultures (Fig. 6B). These data illustrate that DITPA drives myelination

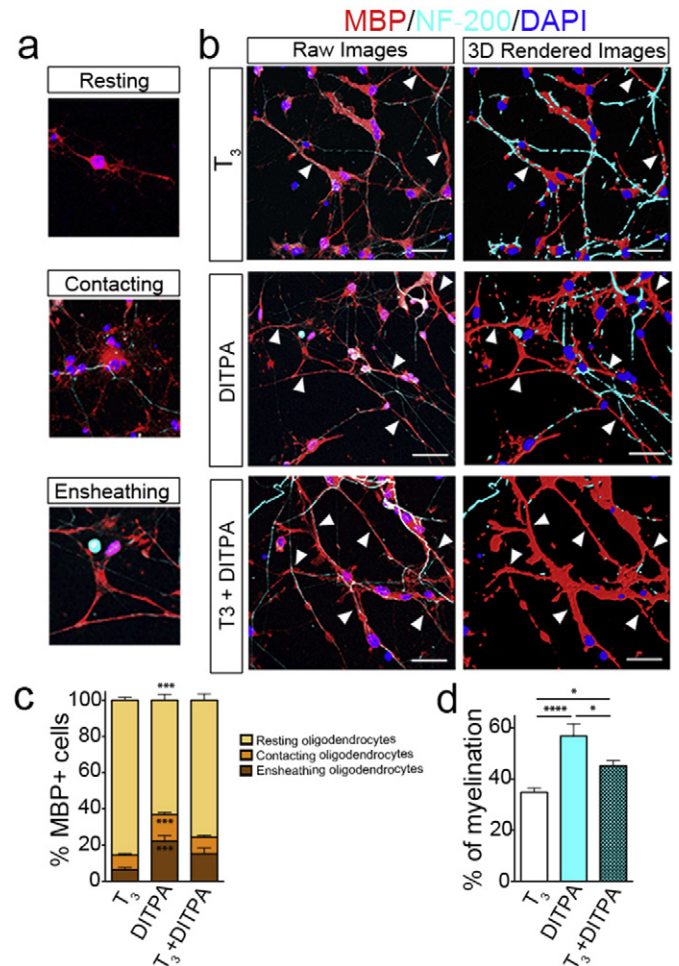


Fig. 6. DITPA promotes the myelination of rat RGCs from *NKX2.1*-GFP+ hESC derived OPCs. (a–d) At day 10, stage VI, OPCs from *NKX2.1*-GFP+ sorted cells were seeded on rat RGCs and maintained for 7 days with T_3 (50 ng/mL), DITPA (10 ng/mL), or T_3 (50 ng/mL) + DITPA (10 ng/mL) treatment, then immunostained with MBP, NF-200, and DAPI. MBP+ OLs were scored for their morphology as (a) “Resting”, “Contacting”, or “Ensheathing”. (b) Representative deconvoluted z-stack captured images from the myelinating co-cultures treated with T_3 (50 ng/mL), DITPA (10 ng/mL) or T_3 (50 ng/mL) + DITPA (10 ng/mL). For better representation, these z-stack images were rendered into an artificial 3D image and shown below as raw images (arrowhead indicates regions of myelination, scale bars = 50 μ m). (c) The percentage of MBP+ OLs that are resting, contacting, and ensheathing from the different treatment groups. (d) The percentage of myelinated axons within the different treatment groups. One-way ANOVA with Tukey's post-hoc analysis; * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$; ($n = 9$ –10, mean \pm SEM).

of axons from differentiating OLs expressing MCT8, in an expedited manner than can occur with T_3 alone, under normal differentiation conditions.

3.7. DITPA Limits OL Cell Death Caused by *SLC16A2* Down-regulation and Promotes Myelination Under These Conditions

DITPA is suggested to cross the plasma membrane in a MCT8-independent manner. To confirm that DITPA can therefore overcome MCT8 deficiency in cells of the oligodendroglial lineage, we reduced MCT8 function pharmacologically and expression using a gene-knockdown approach. Firstly, we applied different concentrations (1 ng/mL, 10 ng/mL and 100 ng/mL) of Bosutinib to pre-OLs from *NKX2.1*-GFP+ sorted cultures at stage VI, day 21 for 48 h. Bosutinib is a third generation tyrosine kinase inhibitor that was recently shown to non-competitively inhibit MCT8-dependent T_3 and T_4 uptake (Braun et al., 2012). By MTT cytotoxicity assay we showed increased

viability in the hESC-derived oligodendroglial cell populations that were treated with DITPA (see Supplemental information Fig. S6).

Moreover, we generated a lentivirus (LV) coding for *SLC16A2*- or scrambled shRNA (*SLC16A2*-shRNA – Fig. 7A). Pre-OLs from *NKX2.1*-GFP + sorted cultures at stage V, day 21 were transduced with either LV-*SLC16A2*-shRNA or LV-scrambled shRNA and harvested 5 days post-transduction, then these cells were used to examine gene expression by qRT-PCR and MCT8 protein expression by western blot (Figs. 7, S7). We found knock-down efficiency of ~10-fold down-regulation of *SLC16A2* by qRT-PCR and a substantial reduction in expression levels by western blot when compared with LV incorporating non-targeting shRNA (Fig. S7B).

Having demonstrated that these cells had significantly reduced MCT8 expression (Fig. S6), we proceeded to examine the effect of MCT8 knockdown on the level of apoptosis in these cultures, and determined that after 5 days of LV transduction, significantly more mCherry-labeled cells were positive for cleaved caspase 3 in the *SLC16A2*-shRNA treated cultures relative to scrambled controls. The effect of MCT8 knockdown was overcome by the administration of DITPA from 3 days post-LV-transduction (Fig. 7B,C), indicating DITPA prevents MCT8 deprivation-induced cell death.

We followed up this line of investigation by identifying prominent molecular signaling events that maybe activated to potentiate DITPA-mediated OL survival against *SLC16A2* knockdown. THs are known to regulate AKT and ERK1/2 signaling and these constitute downstream effectors of oligodendrocyte survival (Flores et al., 2000; Zaka et al., 2005). We analyzed the effect of DITPA on AKT and ERK1/2 signaling upon *SLC16A2* knockdown (Fig. 7D–J). We found a significant elevation in the levels of AKT phosphorylation (p-AKT) among *SLC16A2*-shRNA transduced OPCs following DITPA treatment when compared with transduced cells treated with T_3 alone or non-transduced OPCs. Furthermore, we observed a significant reduction in the levels of p-ERK1 upon *SLC16A2* knockdown in the presence of T_3 . However, this survival effector phosphoprotein was normalized to basal levels (non-transduced control) upon DITPA treatment of transduced cells (Fig. 7D–J). These results suggest that DITPA may prevent the death of OPCs following *SLC16A2* knockdown through the activation of AKT and ERK1 signaling.

Having demonstrated that DITPA can promote the survival of OPCs following acute downregulation of *SLC16A2*, we then set up the same co-culture experiment whereby rat RGCs were cultured with *SLC16A2* down-regulated OLs (post-LV-transduction) from *NKX2.1*-GFP + sorted cells at stage V day 14 (Fig. 7K–N). We treated the cultures with T_3 , DITPA or, T_3 + DITPA, on the day of co-culture and these were maintained for 7 days. mCherry +/MBP + myelinating OLs and mCherry +/MBP + myelin segments were found only in DITPA or T_3 + DITPA treated cultures (Fig. 7L–M). Electron microscopic imaging of the co-cultures demonstrated ultrastructural organization of myelin surrounding RGC axons in both control (non-targeting shRNA) and *SLC16A2*-shRNA transduced cultures with DITPA treatment but not in *SLC16A2*-transduced cultures with T_3 treatment (Fig. 7N). These data indicate that DITPA can promote the differentiation of OLs and moreover can potentiate the myelination of axons, even under the condition of acute MCT8 knockdown.

4. Discussion

Critical stages of the perinatal period govern brain development, where axons organized in fascicles are myelinated, thereby establishing time-dependent cognitive and motor functions. Hence, it is not surprising that the development of the fetal thyroid gland and circulating levels of THs are indeed elevated by the late gestational age, with maternal contributions delivered through the placenta (for review, see Moog et al., 2017; Bernal, 2007). Similarly, OL development coincides with circulating and CNS-specific fetal TH (Bernal, 2007). Despite the co-dependency of circulating and intracellular TH for brain development, oligodendrogenesis and myelination, the fact remains that both T_3 and

T_4 need to enter developing neural cells to exert their genomic and non-genomic effects. In this study, we show that OL development can be intrinsically controlled by the function of the TH membrane transporter, MCT8. We identified that specific MCT8-deficiency in human OPCs can promote their cell death and that addition of the TH analog, DITPA can bypass such a deficiency to salvage OPCs and still promote their maturity toward myelinating OLs.

4.1. DITPA Modulates Gene Expression toward OPC Differentiation

Our data argue that down-regulation of oligodendroglial differentiation repressor genes of the Wnt-Notch signaling pathways can be achieved upon the administration of DITPA to hESC-derived OPCs. This raises the tantalizing hypothesis that DITPA can lift the repression imposed on myelin gene expression during OL development (Chew et al., 2011). Supporting this contention, we identified the up-regulations of WNT antagonists *FRZB*, *SFRP1*, and *SFRP2* with DITPA treatment, again underpinning how DITPA can promote OL differentiation. However, recent evidence strongly suggests that in gastrointestinal tumors, the increased β -catenin-Tcf4 levels not only correlate with reduced TR α 1 transcriptional activity on its target genes but are also likely responsible for the shift of TR α 1 binding on Wnt targets (Sirakov et al., 2012) demonstrating a regulatory role for TH nuclear signaling in the cell cycle, all-be-it in a tumor cell line. Whether there is a causative effect of TH on Wnt or Notch pathways in neural cell differentiation including the derivation of mature OLs, has yet to be proven.

Along with the evidence supporting the action of TH directly on OL differentiation (Barres et al., 1994), T_3 may affect OPC proliferation dependent on the cells' specific stage of development (Baas et al., 1997). OPCs have been shown to exhibit a limited number of divisions before terminal differentiation, with TH and retinoic acid acting as external signals that influence this timing (Ahlgren et al., 1997). In particular, downstream signaling through TR α 1 following TH stimulation may be important in OPC differentiation, since *Tr α 1*^{-/-} mice isolated OPCs continue to divide even in the presence of TH (Billon et al., 2002). Our data show that the effect of T_3 on developing OLs derived from hESCs, is primarily seen from Stages V–VI, when the pre-OL marker O4 is expressed, coinciding with robust expression of the T_3 membrane transporter MCT8. We showed that these O4 + OPCs consisted of a higher proportion of cells in S-phase, suggesting that T_3 stimulation potentiates proliferation at this stage of development.

4.2. DITPA Promotes the Viability of Oligodendrocytes

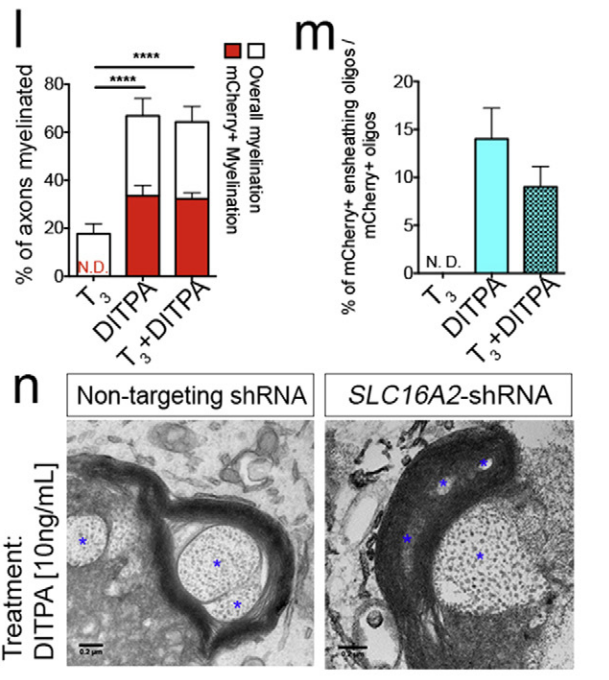
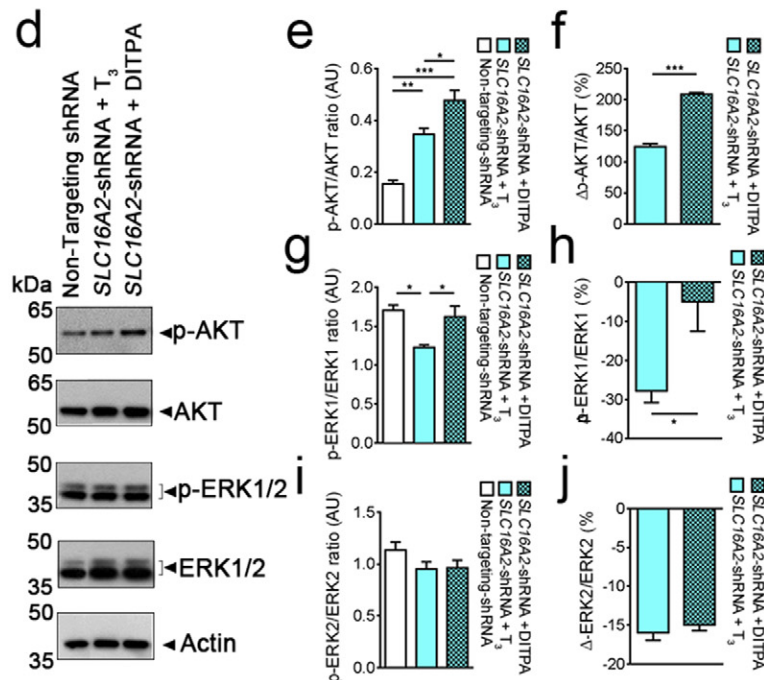
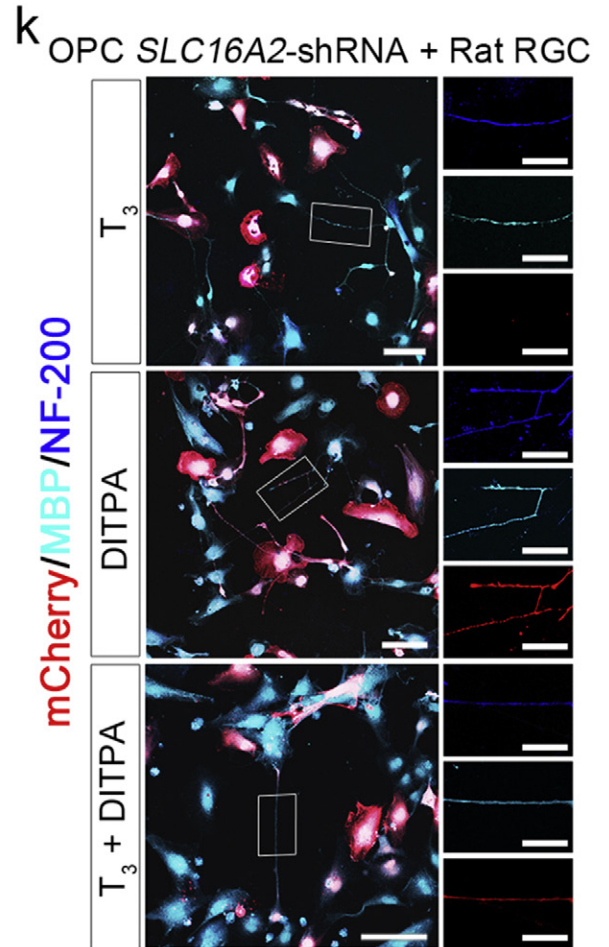
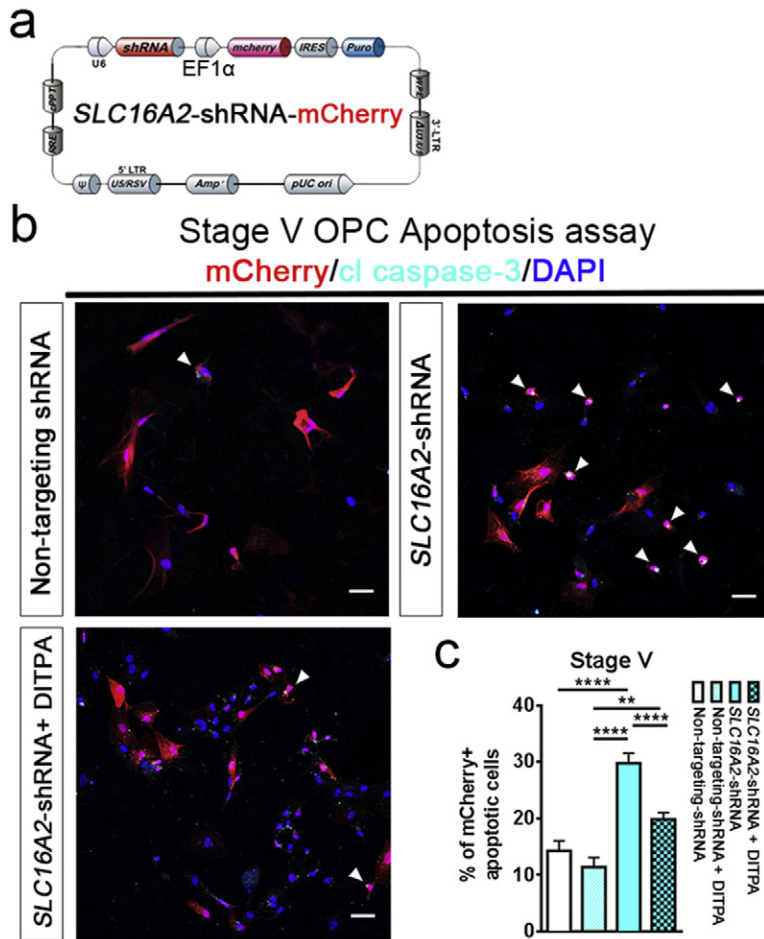
Our data demonstrated that the down-regulation of, or functional blockade of MCT8 promotes cell death of OPCs, identifying the cell membrane-transport of T_3 as integral to the expansion or depletion of OL populations. Intriguingly, we identified that the administration of DITPA to hESC-derived OPCs and other neural cell populations promoted the up-regulation of *THRB* and *DIO3* suggesting that bypassing MCT8 can still potentiate TH genomic signaling in the context of OL maturation and myelin gene expression, similar to that observed by other thyromimetics (Baxi et al., 2014). Moreover, the downstream activation of the AKT and ERK1 non-genomic pathways were identified as prominent downstream effectors of DITPA promoting OPC viability.

4.3. DITPA Potentiates Oligodendrocyte Differentiation and Myelination in the Absence of MCT8

Despite the developmental dependency of OPCs on T_3 , no evidence exists for MCT8 during oligodendrogenesis and myelination. In mice, MCT8 facilitates the entry of TH into the brain parenchyma across the blood brain-barrier (BBB) (Ceballos et al., 2009) and, at a cellular level, the entry of TH into neurons in a region-specific manner (Trajkovic et al., 2007; Wirth et al., 2009). The importance of MCT8 for neurodevelopment was unequivocally demonstrated in patients

genetically identified as AHDS (Vaurs-Barriere et al., 2009; Biebermann et al., 2005; Visser, 2013). These infants are usually clinically identified at the 6-month postnatal time point by virtue of their poor head control, a consequence of their general hypotonia. The hypotonia persists during development throughout the trunk and the peripheral hypotonia

converts into dystonia and spasticity. These clinical manifestations are matched with a classically hypomyelinated brain profile which never reaches normal myelination throughout the individual's lifetime (Armour et al., 2015; Vaurs-Barriere et al., 2009). Currently, a clinical trial is underway using the TH analog, Triac (<https://clinicaltrials.gov/>)



ct2/show/NCT02060474) (Visser, 2014), and a previous off-label trial using DITPA for AHDS (Verge et al., 2012). There exists evidence that these MCT8-independent TH analogs can potentiate the resolution of peripheral hyperthyroid symptoms but their effect on human CNS cell development and importantly, clinical evidence of specific cognitive improvements, are still lacking (Verge et al., 2012; Kersseboom et al., 2014; Groeneweg et al., 2017; Ferrara et al., 2015; Ferrara et al., 2014). Our findings argue that the availability of T₃ to human OPCs specifically can be restricted by a lack of functional MCT8 and can potentiate OL dystrophy.

An important implication for the effect of limited MCT8 function on myelin formation was established in a developmental zebrafish model where the ablation of the *Slc16a2* gene rendered a OL maturation and myelin defect with associated locomotor and behavioral deficits (Zada et al., 2014). Indeed these resemble the neurological outcomes observed in AHDS patients under T₂-weighted MRI (Armour et al., 2015) or following neuropathological assessment (Charzewska et al., 2016). It therefore appears that the zebrafish model may mimic the human TH regulation more closely than that exhibited in the mouse. This is further ratified by the data derived from the *Slc16a2*^{-/-} mouse model that does not manifest the neurological phenotype that is exhibited in AHDS patients (Trajkovic et al., 2007). Moreover, it has since been identified that in the mouse CNS there is compensation for the loss of MCT8 through the upregulation of OATP1C1, an organic anion transporting polypeptide. The OATP1C1 transmembrane protein is capable of transporting T₄ across the BBB, with compensatory increase in the astrocytic deiodinase 2 occurring in the *Slc16a2*^{-/-} mouse model, converting T₄ to T₃ (Trajkovic et al., 2007). This was confirmed recently in a *Slc16a2* and *Oatp1c1* double knockout which exhibited the neurological deficits characteristic of AHDS, with reduced T₃ and T₄ uptake within the CNS and deiodinase activity with classical myelin delay (Mayerl et al., 2014). Our data support the effects seen in the zebrafish experimental paradigm since our hESC-derived OPCs had limited differentiation and myelinogenic potential following the acute LV-mediated shRNA knockdown of the *SLC16A2* gene. Of greatest importance was the finding that DITPA treatment of the *Slc16a2*^{-/-} zebrafish restored myelin deficiencies and locomotor behavioral outcomes (Zada et al., 2014). These data corroborate our findings that DITPA can potentiate OL differentiation and myelination in the absence of MCT8. The potential therapeutic window for use of DITPA in AHDS may relate to the heightened stages of oligodendrocyte maturation and myelination during the perinatal period (Groeneweg et al., 2017). In fact, it has been suggested that DITPA administration should be started in the mother in the last trimester if the MCT8 mutation is severe since transplacental passage of DITPA has been achieved in pregnant *Mct8*^{-/-} dams, modulating neurodevelopmental genes within the cerebral cortex of their pups. If early detection of *SLC16A2* mutations can be achieved within pregnant mothers in high-risk groups by genetic screening, then DITPA may be a viable option for parents carrying AHDS infants to term.

5. Conclusions

The current study has uncovered the biological outcomes of the DITPA when administered to differentiating OPCs. These cell-specific

effects were the direct result of facilitated transcriptional regulation of OL differentiation and eventual myelination of CNS axons that could not be matched by T₃ administration alone. The most profound pharmacological property of DITPA was that it was capable of salvaging OPCs deficient in MCT8 with a unique capacity to continue their differentiation toward myelination. These findings provide proof-of-principle data for the treatment of severe inherited neurodevelopmental disorders where TH metabolism is dysfunctional, such as the well-established CNS hypothyroid state occurring in AHDS afflicted children.

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Authors' Contributions

JYL and SP: Conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; MJK: Collection of data, manuscript writing; FR: Data analysis and interpretation; MFA, DD, JWB, AE and ES: Provision of study material.

Conflict of Interest

The authors declare that partial funding for the role of DITPA as a therapeutic molecule was through a commercial service agreement between Monash University and NeuOrphan P/L. No non-financial conflicts of interest exist for any of the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2017.10.016>.

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Fig. 7. DITPA rescues oligodendroglial cell death mediated by *SLC16A2* knock down, retaining its myelination capability. (a) Lentivirus (LV) was generated to carry the *SLC16A2*-shRNA with a mCherry reporter (b) Immunostaining for mCherry and cleaved caspase-3 (c) *NKX2.1*-GFP + sorted OPCs at stage V, day 12 (3 days post-transduction with non-targeting, *SLC16A2*-shRNA, or *SLC16A2*-shRNA + DITPA (10 ng/mL)) counterstained with DAPI. (b and c) The proportion of mCherry +/cl caspase-3 + apoptotic cells was increased upon *SLC16A2*-shRNA transduction. DITPA treatment (10 ng/mL) reduced the *SLC16A2*-shRNA-mediated apoptosis. Scale bar = 50 μm. (d–j) Western blot analysis of lysates from OPCs transduced either with non-targeting-shRNA or *SLC16A2*-shRNA assayed for p-AKT and p-ERK1/2. (e–f) Densitometric quantification of p-AKT/total AKT, (G–H) p-ERK1/total ERK1, and (i–j) p-ERK2/total ERK2. (k) 3 days post-transduction with *SLC16A2*-shRNA, OPCs from *NKX2.1*-GFP + sorted cells were seeded on rat RGCs and were maintained for 7 days with T₃ (50 ng/mL), DITPA (10 ng/mL), or T₃ (50 ng/mL) + DITPA (10 ng/mL) treatment, then immunostained with mCherry, MBP and NF-200 Scale bar = 100 μm. Magnified images of single axons are shown on the right hand side of each image Scale bar = 20 μm. (l) The percentage of overall myelination and mCherry + myelination and (m) the percentages of ensheathing mCherry+/MBP + OLs among the different treatment groups. (n) Representative electron microscopic images of rat RGCs and OPCs co-cultures transduced with non-targeting, or, *SLC16A2*-shRNA upon DITPA treatment (10 ng/mL), showing compact myelin. Asterisks indicate axon fibers. Scale bar = 0.2 μm. One-way ANOVA with Tukey's post-hoc analysis; **P < 0.01; ****P < 0.0001; N.D.: not detected; (n = 9–10, mean ± SEM).

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