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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.

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 *SLCO1C1*, *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 T_4 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 (T_3) and its pro-hormone thyroxine (T_4) (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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T_3, developmentally delayed myelination shown by magnetic resonance imaging (MRI), is a common feature of this disorder (Armour et al., 2015; Vaurs-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; Vaurs-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 (Petratos 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: GCTTCGCGCCGTAGTC TTA. 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

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