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Characterization of energy and neurotransmitter metabolism in cortical glutamatergic neurons derived from human induced pluripotent stem cells: A novel approach to study metabolism in human neurons

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ABSTRACT

Alterations in the cellular metabolic machinery of the brain are associated with neurodegenerative disorders such as Alzheimer's disease. Novel human cellular disease models are essential in order to study underlying disease mechanisms. In the present study, we characterized major metabolic pathways in neurons derived from human induced pluripotent stem cells (hiPSC). With this aim, cultures of hiPSC-derived neurons were incubated with [U-^{13}C]glucose, [U-^{13}C]glutamate or [U-^{13}C]glutamine. Isotopic labeling in metabolites was determined using gas chromatography coupled to mass spectrometry, and cellular amino acid content was quantified by high-performance liquid chromatography. Additionally, we evaluated mitochondrial function using real-time assessment of oxygen consumption via the Seahorse XF^{e}96 Analyzer. Moreover, in order to validate the hiPSC-derived neurons as a model system, a metabolic profiling was performed in parallel in primary neuronal cultures of mouse cerebral cortex and cerebellum. These serve as well-established models of GABAergic and glutamatergic neurons, respectively. The hiPSC-derived neurons were previously characterized as being forebrain-specific cortical glutamatergic neurons. However, a comparable preparation of predominantly mouse cortical glutamatergic neurons is not available. We found a higher glycolytic capacity in hiPSC-derived neurons compared to mouse neurons and a substantial oxidative metabolism through the mitochondrial tricarboxylic acid (TCA) cycle. This finding is supported by the extracellular acidification and oxygen consumption rates measured in the cultured human neurons. [U-^{13}C]Glutamate and [U-^{13}C]glutamine were found to be efficient energy substrates for the neuronal cultures originating from both mice and humans. Interestingly, isotopic labeling in metabolites from [U-^{13}C]glutamate was higher than that from [U-^{13}C]glutamine. Although the metabolic profile of hiPSC-derived neurons *in vitro* was particularly similar to the profile of mouse cortical neurons, important differences between the metabolic profile of human and

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid; α-KG, α-ketoglutarate; Ac-CoA, Acetyl-CoA; bFGF, Basic fibroblast growth factor; BSA, bovine serum albumin; BNDF, Brain-derived neurotrophic factor; D-AP5, D-(-)-2-Amino-5-phosphonopentanoic acid; db-cAMP, dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt; DMF, *N,N*-dimethylformamide; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione; DMEM, Dulbecco's modified Eagle's medium; ECAR, Extracellular acidification rate; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; FCS, Foetal calf serum; GABA, γ-aminobutyric acid; GLUT1, Glucose transporter 1 (slc2a1); GDNF, Glial-derived Neurotrophic Factor; hiPSC, human induced pluripotent stem cells; HPLC, High-Performance Liquid Chromatography; ME, malic enzyme; MTBSTFA, *N*-methyl-*N*-(tert-butyldimethylsilyl) trifluoroacetamide; NMDA, *N*-methyl-D-aspartate; OCR, Oxygen consumption rate; PAG, phosphate activated glutaminase; PBS, phosphate-buffered saline; TCA, tricarboxylic acid.

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mouse neurons were observed. The results of the present investigation establish hallmarks of cellular metabolism in human neurons derived from iPSC.

1. Introduction

In spite of remarkable efforts to model neurodegenerative diseases, more suitable model systems are currently needed to elucidate the mechanisms underlying neurodegeneration and to develop therapies to treat these brain disorders. Primary cultures of cells isolated from the rodent brain have been instrumental to study in depth aspects of cellular function in physiological and pathological conditions. For instance, cultures of neurons and astrocytes from mouse brain have been critical to unravel pathways associated with energy metabolism, particularly metabolism of glucose, the predominant energy substrate for neurons (Lange et al., 2012; Mergenthaler et al., 2013; Schousboe, 2012). Moreover, cultures of cells obtained from different brain regions can provide information regarding localized specific cellular functions (Waagepetersen et al., 2005a). However, animal models cannot accurately reproduce the complex phenotypes in the human brain and the effect of potential drugs tested in systems of animal origin is often not translational to humans. This emphasizes the current need for brain disease models based on human cells. Human foetal brain cells (Giuliani et al., 2003; Nelander et al., 2013), neurons derived from human embryonic and adult stem cells (Glaser and Brustle, 2005; Jakel et al., 2004), dissociated adult human brain cells (Gibbons and Dragunow, 2010) and cell lines from human carcinomas (Haile et al., 2014; Lee et al., 2005) have already been successfully used to study neurological diseases *in vitro*. Nonetheless, the use of these models has been limited by scarce availability of source tissue, as well as ethical and methodological issues (Gibbons and Dragunow, 2010; Hyun, 2010; Jakel et al., 2004). Recently, the ability to model neurodegenerative diseases has been considerably improved through the use of human induced pluripotent stem cell (hiPSC). These have allowed the generation of different cellular subtypes, disease-specific phenotypes, and the development of isogenic controls by correcting patient-specific mutations (Dage et al., 2014; Freude et al., 2014; Odawara et al., 2014; Peng and Copray, 2016; Takahashi and Yamanaka, 2006). In line with this, we have generated forebrain-specific glutamatergic neurons from human iPSCs which have been effectively used to model frontotemporal dementia *in vitro* (Zhang et al., 2017).

Progressive decline of brain functions is a hallmark of neurodegenerative diseases. In particular, alterations in the cellular metabolic machinery have recently been associated with brain disorders such as Alzheimer's disease, Parkinson's disease, Huntington and epilepsy (Chassoux et al., 2016; Hertz et al., 2015; Patassini et al., 2016; Toulorge et al., 2016; Yin et al., 2016). Although patient-derived hiPSC neurons have been employed to model neurological disorders, a thorough metabolic characterization of hiPSC-derived neurons from both diseased and healthy individuals is lacking.

As mentioned above, Zhang et al., have recently developed a protocol to differentiate hiPSC from healthy and diseased individuals into cortical neurons with a prominent glutamatergic phenotype. Glutamatergic neurons release glutamate as their neurotransmitter, and most glutamate is believed to be taken up by surrounding astrocytes. Neurons do not have the enzymatic capability to refill the neurotransmitter pool from the synthesis of glutamate from glucose. Therefore, glutamine is produced and released by astrocytes to be taken up by glutamatergic neurons in order to act as precursor for neurotransmitter glutamate (Kreft et al., 2012). However, it is now acknowledged that glutamate is also taken up by neurons (Danbolt et al., 2016; Rimmele and Rosenberg, 2016). Thus, glutamine as well as glutamate is a source for refilling of neurotransmitter vesicles in glutamatergic neurons. In addition, glutamate and glutamine are vividly metabolized oxidatively in the mitochondria (Olstad et al., 2007a; Waagepetersen et al., 2005b). Glutamine is deamidated to glutamate by the activity of phosphate activated glutaminase (PAG), localized associated with the inner mitochondrial membrane (Bak et al., 2008; Roberg et al., 1995) while glutamate is entering the TCA cycle for oxidation via α-ketoglutarate.

In the present study we aimed to characterize several aspect of energy and neurotransmitter metabolism in neurons derived from hiPSC. With this purpose, we evaluated the capability of cultured cortical glutamatergic neurons derived from hiPSC, to metabolize various ^{13}C-labeled substrates, namely [U-^{13}C]glucose, [U-^{13}C] glutamate and [U-^{13}C]glutamine, and mapped metabolic pathways using mass spectrometric and HPLC analysis. Mitochondrial function of hiPSC-derived neurons was investigated using the Seahorse Extracellular XF^{e}96 Flux Analyzer. Furthermore, the metabolic profile of the human neurons in culture was compared with the profile of primary cultures of neurons isolated from cerebral cortex and cerebellum of mouse brain. The cortical and cerebellar primary cultures used here represent models of GABAergic and glutamatergic neurons, respectively (Drejer et al., 1985; Drejer and Schousboe, 1989; Hertz et al., 1989). It should be noted that a preparation of predominantly mouse cortical glutamatergic neurons is not available. We found that the metabolic profile of hiPSC-derived neurons in this study is comparable with mouse neuronal cultures. Our results show for the first time that the hiPSC-derived neurons are metabolically highly active cells that readily metabolize glucose via glycolysis and mitochondrial tricarboxylic acid cycle. We also found that glutamate is metabolized to a larger extent than glutamine in the hiPSC-derived neurons.

2. Material and methods

2.1. Materials

NMRI mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France) and housed in the animal facility at the Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen (Copenhagen, Denmark). Plastic ware for cell culturing was from NUNC A/S (Roskilde, Denmark). Dulbecco's modified Eagle's medium (DMEM) powder, poly-L-ornithine/laminin, db-cAMP, CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione), *N*-methyl-*N*-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), *N*,*N*-dimethylformamide (DMF), oligomycin, Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), rotenone, antimycin A and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Foetal calf serum (FCS) was from Gibco, Life Technologies (Carlsbad, CA, USA), D-AP5 (D-2-amino-5-phosphonopentanoic acid) from Tocris Bioscience (Bristol, ENG, UK) and SB431542 and LDN193189 from SelleckChem (Houston, TX, USA). [U-^{13}C]Glucose (all 99% [^{13}C]