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Hippocampus and Hypothalamus RNA-sequencing of WFS1-deficient Mice

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Abstract—Wolfram syndrome is caused by mutations in the *WFS1* gene. WFS1 protein dysfunction results in a range of neuroendocrine syndromes and is mostly characterized by juvenile-onset diabetes mellitus and optic atrophy. WFS1 has been shown to participate in membrane trafficking, protein processing and Ca^{2+} homeostasis in the endoplasmic reticulum. Aim of the present study was to find the transcriptomic changes influenced by WFS1 in the hypothalamus and hippocampus using RNA-sequencing. The WFS1-deficient mice were used as a model system to analyze the changes in transcriptional networks. The number of differentially expressed genes between hypothalami of WFS1-deficient (Wfs1KO) and wild-type (WT) mice was 43 and between hippocampi 311 with False Discovery Rate (FDR) <0.05. *Avpr1a* and *Avpr1b* were significantly upregulated in the hypothalamus and hippocampus of Wfs1KO mice respectively. *Trpm8* was the most upregulated gene in the hippocampus of Wfs1KO mice. The functional analysis revealed significant enrichment of networks and pathways associated with protein synthesis, cell-to-cell signaling and interaction, molecular transport, metabolic disease and nervous system development and function. In conclusion, the transcriptomic profiles of WFS1-deficient hypothalamus and hippocampus do indicate the activation of degenerative molecular pathways causing the clinical occurrences typical to Wolfram syndrome.

Key words: Wfs1, hypothalamus, hippocampus, RNA-sequencing.

INTRODUCTION

Wolfram syndrome (WS, OMIM 222300) is a rare autosomal recessive neurodegenerative disorder with main features of juvenile-onset diabetes mellitus, progressive optic atrophy, diabetes insipidus and deafness. The mutations in the wolframin gene (*WFS1*) cause WS (Barrett and Bundey, 1997; Inoue et al., 1998; Strom et al., 1998). The clinical presentation varies a lot from patient to patient, including several neurological abnormalities such as seizures, mental retardation and nystagmus (Barrett et al., 1995). The patients usually die because of central respiratory failure due to brain stem atrophy, indicating the significance of neurodegeneration in WS (Barrett et al., 1995; Scolding et al., 1996). In addition, psychiatric illnesses like depression, psychosis, impulsivity and aggression have often been found in WS patients (Swift et al., 1990; Takei et al., 2006). *WFS1* mutation carriers have a higher possibility of psychiatric hospitalization, primarily due to depression (Swift and Swift, 2000). WS is underdiagnosed because the clinical features are very heterogeneous and the small number of patients makes research on WS difficult. Therefore, not all pathophysiological mechanisms and clinical features of WS have been fully characterized.

WFS1 is a transmembrane glycoprotein localizing in the endoplasmic reticulum (ER) (Takeda et al., 2001; Hofmann et al., 2003; Philbrook et al., 2005). WFS1 has been shown to participate in membrane trafficking, processing proteins and/or regulation of the Ca^{2+} homeostasis in the ER (Takeda et al., 2001; Hatanaka et al., 2011). WFS1 could be a Ca^{2+}-channel or a regulator in the ER (Osman et al., 2003). WFS1 is expressed at highest levels in brain, heart and pancreatic β-cells (Inoue et al., 1998; Strom et al., 1998; Hofmann et al., 2003; Ishihara et al., 2004). In the brain the highest expressions have been found in the hippocampus and cerebral cortex, also in the nucleus accumbens, striatum, amygdala, thalamus and in several hypothalamic nuclei, such as the suprachiasmatic nucleus and paraventricular nucleus (Kato et al.,

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Abbreviations: AVP, arginine vasopressin; *Avpr1a*, arginine vasopressin receptor subtype V1a gene in mouse; *Avpr1b*, vasopressin receptor subtype V1b gene in mouse; ER, endoplasmic reticulum; FDR, False Discovery Rate; LogFC, Log2 Fold Change; PVH, paraventricular nucleus of the hypothalamus; RNA-seq, RNA sequencing; RT-PCR, quantitative real-time PCR; TRP, transient receptor potential channel family; *Trpm8*, transient receptor potential cation channel, subfamily M, member 8 gene in mouse; *Trpv3*, transient receptor potential cation channel, subfamily V, member 3 gene in mouse; *WFS1*, wolframin-1 gene in human; *Wfs1*, wolframin-1 gene in mouse; WFS1, wolframin-1 protein; Wfs1KO, WFS1-deficient mice; WS, Wolfram syndrome; WT, wild-type mice.

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2008). WFS1 expression in limbic structures and hypothalamus indicates its deficiency should have considerable impact on endocrine regulation, cognitive and emotional behavior.

WFS1 dysfunction leads to ER calcium depletion (Takei et al., 2006), increased cytosolic Ca^{2+} level and expression of pro-apoptotic molecules, leading to cell death (Hara et al., 2014). Its interaction with the ion pumps Na + K^+ ATPase and vacuolar-type H^+ ATPase support the role in protein folding, biosynthesis and secretion (Zatyka et al., 2008; Gharanei et al., 2013).

ER perturbations have been acknowledged to induce cell death in neurodegenerative disorders (Ilieva et al., 2007; Hoozemans et al., 2012) and diabetes mellitus (Cardozo et al., 2005; Gwiazda et al., 2009). In several pathological conditions affecting the nervous system a defective calcium homeostasis has been noted (Tessitore et al., 2004; Pelled et al., 2005). Mutations in *Wfs1* cause selective destruction of pancreatic β-cells and neuronal cells (Inoue et al., 1998). The underlying mechanism is thought to be dysfunctional unresolved ER stress (Ishihara et al., 2004; Fonseca et al., 2005, 2010; Riggs et al., 2005). WFS1 is upregulated in case of ER stress and its deficiency leads to more distinct apoptosis promoting progression of WS symptoms (Ishihara et al., 2004; Fonseca et al., 2005, 2010).

Aim of the present study was to find transcriptomic changes influenced by WFS1 in the brain structures with highest expression, hypothalamus and hippocampus, using RNA-sequencing and WFS1-deficient mice to understand the mechanisms underlying the WS symptoms.

EXPERIMENTAL PROCEDURES

Animals

The animal experiments described in this study were performed with permission from Estonian National Board of Animal Experiments (No. 71, April 8th, 2011) and in accordance with the European Communities Directive (86/609/EEC).

Generation of *Wfs1* mutant (Wfs1KO, $Wfs1^{tm1Koks}$) mice has been described elsewhere (Koks et al., 2009). In the current study two genotypes of littermate mice were used for RNA-sequencing (RNA-seq): wild-type (WT) and homozygotes for *Wfs1* mutation (Wfs1KO). All studies were performed on F2 generation male mice (129S6/SvEvTu x 129S6/SvEvTu) and mice were 6–7 months old at the time of the experiment. Mice were housed in groups of 6–8 at 20 ± 2 °C under 12-h/12-h light/dark cycle with free access to food and water.

RNA extraction

Mice were sacrificed by cervical dislocation, hippocampi and hypothalami were dissected out from the brain and snap-frozen in liquid nitrogen. For hippocampus four animals and for hypothalamus five animals were used from both genotypes. Total RNA was extracted from hippocampus and hypothalamus using mirVana miRNA Isolation Kit (Life Technologies/Thermo Fisher Scientific Inc, Waltham, MA, USA) according to the manufacturer's protocol. DNase I treatment was performed on column according to manufacturers' protocol (Qiagen Inc, Valencia, CA, USA). The RNA quality was assessed using Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies Inc, Santa Clara, CA, USA).

RNA-sequencing

For hippocampi (n = 4 for both genotypes) whole transcriptome sequencing a total of 10 μg of RNA was treated with RiboMinus Eukaryote Kit for RNA-seq (Invitrogen/Thermo Fisher Scientific Inc, Waltham, MA, USA) to eliminate ribosomal RNA from the rest of the transcriptome. The SOLiD Total RNA-Seq Kit and 500 ng of ribodepleted RNA was used according to the manufacturer's protocol (Life Technologies/Thermo Fisher Scientific) for whole transcriptome RNA-seq library preparation. The libraries were barcoded and pooled together for the following template preparation. The sequencing was performed with SOLiD 4 platform (Life Technologies/Thermo Fisher Scientific) using paired-end DNA sequencing chemistry (50 bp forward and 35 bp reverse).

For hypothalami (n = 5 for both genotypes) whole transcriptome sequencing cDNA was synthesized using Ovation RNA-Seq System V2 (NuGEN Technologies Inc, San Carlos, CA, USA). Fifty ng of total RNA was used. SOLiD DNA Fragment library kit (cDNA input 2 μg) was used to generate libraries and quality was controlled with the Agilent Bioanalyzer 2100 and High Sensitivity DNA Kit (Agilent Technologies) before sequencing. The libraries were barcoded and pooled together for the template preparation. The template was prepared with automated SOLiD EZ Bead System and SOLiD EZ Bead E80 System Consumables (Life Technologies/Thermo Fisher Scientific). The SOLiD 5500xl System and paired-end (75 bp forward and 35 bp reverse) chemistry for DNA sequencing were applied (Life Technologies/Thermo Fisher Scientific).

Quantitative real-time PCR validation

The confirmatory quantitative real-time PCR (RT-PCR) was performed using TaqMan gene expression assays and chemistry (Life Technologies/Thermo Fisher Scientific). Five hippocampal and hypothalamic samples from each genotype were analyzed thrice. Total RNA from each sample was subjected to cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Life Technologies/Thermo Fisher Scientific) following the manufacturer's protocol. The RNA input for hippocampus was 2 μg and for hypothalamus 1 μg. The expression of *Wfs1* (Wolfram syndrome 1 (wolframin), Mm01220326_m1) and *Hspa5* (heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa) (*Bip*, *Grp78*), Mm00517691_m1) were analyzed from both tissues. Additionally for hippocampus data *Trpm8* (transient receptor potential cation channel, subfamily M, member 8, Mm01299593_m1), *Cyb5r2* (cytochrome b5 reductase 2, Mm00623496_m1), *Ccl28* (chemokine