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CENTRAL SEROTONERGIC NEURON DEFICIENCY IN A MOUSE MODEL OF ZELLWEGER SYNDROME

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Abstract—Zellweger syndrome (ZS) is a severe peroxisomal disorder caused by mutations in peroxisome biogenesis, or PEX, genes. A central hallmark of ZS is abnormal neuronal migration and neurodegeneration, which manifests as widespread neurological dysfunction. The molecular basis of ZS neuropathology is not well understood. Here we present findings using a mouse model of ZS neuropathology with conditional brain inactivation of the *PEX13* gene. We demonstrate that PEX13 brain mutants display changes that reflect an abnormal serotonergic system – decreased levels of tryptophan hydroxylase-2, the rate-limiting enzyme of serotonin (5-hydroxytryptamine, 5-HT) synthesis, dysmorphic 5-HT-positive neurons, abnormal distribution of 5-HT neurons, and dystrophic serotonergic axons. The raphe nuclei region of PEX13 brain mutants also display increased levels of apoptotic cells and reactive, inflammatory gliosis. Given the role of the serotonergic system in brain development and motor control, dysfunction of this system would account in part for the observed neurological changes of PEX13 brain mutants.

Key words: Zellweger syndrome, PEX13 brain mutant, raphe nuclei, serotonin, gliosis, apoptosis.

INTRODUCTION

Zellweger syndrome (ZS) is an autosomal recessive peroxisomal disorder caused by mutations in peroxisome biogenesis (or *PEX*) genes that are required for the formation of the peroxisome (Distel et al., 1996; Gould and Valle, 2000). The PEX13 protein is a component of the peroxisome protein import machinery (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996) and PEX13 mutations in humans lead to disease across the Zellweger phenotypic spectrum (Liu et al., 1999; Shimozawa et al., 1999). ZS is characterized by severe neuropathology and early neonatal death. The neuropathology includes abnormalities in neuronal migration and increased post-developmental neurodegeneration (Volpe and Adams, 1972; Moser, 1996; Powers and Moser, 1998).

The molecular basis of ZS neuropathology is still not completely understood. Early theories implicated accumulated substrates, such as very-long-chain fatty acids, or deficiency of metabolic products, such as plasmalogens and docosahexaenoic acid, that arise due to loss of peroxisomal metabolism (Schutgens et al., 1986; Moser, 1993; Powers and Moser, 1998). Mouse models of ZS have been useful in addressing the cause of ZS neuropathology. In particular, investigations on mice with the ubiquitous deletion of *PEX2*, 5, and 13 (Baes et al., 1997; Faust and Hatten, 1997; Maxwell et al., 2003; Yakunin et al., 2010) or a brain-specific deletion of *PEX5* (Janssen et al., 2000; Krysko et al., 2007; Hulshagen et al., 2008; Bottelbergs et al., 2012) and *PEX13* (Muller et al., 2011) have demonstrated a range of pathological changes similar to those of ZS patients, such as neuronal migration defects, altered cerebellar structure and function and motor dysfunction. Findings from using such models have included axon degeneration, abnormalities in cerebellar morphogenesis, activation of the innate immune system, mitochondrial dysfunction, oxidative stress and protein misfolding.

The serotonergic system is a key modulator of brain physiology, ranging from the regulation of motor control and emotional behavior through to neurogenesis (Lucki, 1998). Neurons that produce serotonin (5-HT) in the central nervous system are predominantly located in the brainstem raphe nuclei, with the rostral group (dorsal and median raphe nucleus) providing innervation of the forebrain. Serotonergic neurons also project extensively to the spinal cord, including the motor neurons in the ventral horn (Lakke, 1997), with an important role in the modulation of motor neurons; indeed, a role for 5-HT as both a facilitator and inhibitor of locomotor activity has been demonstrated (Jacobs and Azmitia, 1992; Jacobs and Fornal, 1993; Schmidt and Jordan, 2000). A lack of brain serotonin in mice has also been shown to affect the postnatal development of neuronal circuitry (Migliarini et al., 2013), suggestive of a role in neurodevelopment.

Here we present the novel finding that PEX13 brain mutant mice exhibit defects in the brain serotonergic

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Abbreviations: 5-HT, 5-hydroxytryptamine; PBS, phosphate-buffered saline; PEX, peroxisome biogenesis; SEM, standard error of the mean; TPH2, tryptophan hydroxylase-2; ZS, Zellweger syndrome.

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system, including deficiency and altered distribution of mid-brain serotonergic neurons and extensive dystrophy of serotonergic neurites. These changes were associated with increased levels of apoptosis and inflammation in the relevant regions of the brain. The findings are consistent with serotonergic disorder contributing to the neurological dysfunctions of ZS.

EXPERIMENTAL PROCEDURES

Animals

Generation and genotyping of PEX13 brain mutants and littermate wild-type mice were carried out at Griffith University as previously reported (Muller et al., 2011). Mouse genetic modification was approved by the Griffith University Institutional Biosafety Committee (NLRD/21/07). Ethics approval for animal experiments was granted by the Griffith University Animal Ethics Committee (BBS/02/09/AEC; ESK/03/13/AEC).

Brain tissue RNA isolation and microarray analysis

Total tissue RNA was extracted using the Qiagen RNeasy lipid tissue mini kit according to the manufacturer's instructions. RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA) and RNA integrity was determined using the Agilent RNA 6000 Nano kit and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Microarray analysis was undertaken at the SRC Microarray Facility at the University of Queensland using Illumina Mouse Sentrix-6 V1.1 Beadchips. Data analysis was carried out using Genespring GX7 software (Agilent Technologies).

Mouse brain perfusion and fixation

For transcardial perfusion mice were anesthetized with 80 mg/kg ketamine and 10 mg/kg xylazine. Mice were perfused through the left ventricle (0.5 mL/min for P20 adult mice) with 10 mM phosphate-buffered saline, pH 7.4 (PBS) containing 0.5% sodium nitrite to flush out blood, followed by perfusion with modified Zambonie's fixative reagent (2% paraformaldehyde, 0.2% picric acid, 0.1 M PBS, pH 7.2). The brains were subsequently removed and placed overnight in the fixative *in vacuo*. The brains were then washed three times for 30 min in PBS, followed by three 15-min washes with PBS containing 0.1% w/v sodium azide (PBS/azide), and stored in PBS/azide at 4 °C until further processed.

Tissue immunofluorescence analysis

For cryosectioning, brains were rinsed twice with PBS for 30 min and placed overnight at 4 °C in PBS/azide containing 30% sucrose. The brains were then embedded using a series of graded OCT (Tissue-Tek, Sakura Finetek, Leiden, NL) solutions (20%, 30%, 50%, 70% OCT prepared in PBS/azide containing 30% sucrose) for 1 h each, placed in cryomolds (25 cm × 22 cm) with 100% OCT solution and stored at −80 °C until sectioned. Coronal or sagittal or tissue cryo-sections (40 μm), matched using a mouse brain atlas (Paxinos and Franklin, 2001), were prepared using a Leica CM 3050s cryostat (Leica Microsystems, Wetzlar, Germany), and placed either free-floating in a 24-well plate containing PBS/azide or on supra-frost slides as adherent sections, and stored at 4 and −80 °C respectively, until further processing.

Brain sections were washed in PBS containing 0.1% Triton X-100 (PBS/Triton X-100) and permeabilized using 100% DMSO (Sigma Aldrich Pty Ltd, St Louis, Mo, USA) for 20 min. After three washes in PBS/Triton X-100, nonspecific binding sites were blocked with PBS/Triton X-100 containing 10% donkey serum (v/v) for 1 h at room temperature. A primary antibody cocktail containing combinations of mouse anti-GFAP, at a dilution of 1:800 (Sapphire Biosciences, Waterloo, NSW, Australia); rabbit anti-IBA-1, 1:2000 (Wako Chemicals, Richmond, VA, USA); Goat anti-TPH2 (Everest Biotech Ltd, Oxfordshire, UK), rabbit anti-TPH2 (ThermoFisher Scientific Inc, Rockford, IL, USA), rabbit anti-5-HT (Sigma Aldrich), Goat anti-5-HT (Life Research, Immunostar Inc, Hudson, WI, USA), rabbit cleaved caspase-3, 1:500 (Cell Signalling, Danvers, MA, USA), rabbit anti-catalase, 1:1000 (Maxwell et al., 2003), mouse anti-PMP70, 1:600 (Maxwell et al., 2003) were diluted in the same blocking buffer and incubated with sections overnight at 4 °C. Sections were washed thoroughly in PBS/TritonX-100 and incubated with relevant Alexa Fluor-conjugated secondary antibodies – donkey anti-rabbit 488, 594 (1:400; Life Technologies, Carlsbad, CA, USA); donkey anti-goat 594, 488 (1:400, Life Technologies); donkey anti-goat 650 (1:50, Abcam), donkey anti-mouse 594 (1:400; Life Technologies) – diluted in PBS/TritonX-100, for 3 h at room temperature. To reduce non-specific background staining from secondary antibodies, numerous washing steps (up to 10 times depending on the antibody) in PBS/TritonX-100 were essential at the end of the staining procedure.

Image acquisition

Images were captured using a Zeiss Axio imager™ Z1, upright epi-fluorescence microscope, with ApoTome attachment (Carl Zeiss Microscopy GmbH, Gottingen, Germany), using 20× and 40× (dry) and 63× (oil immersion) Plan-Apochromatic objectives (numerical apertures of 0.75 and 1.40, respectively). Immunofluorescence images of P20 brain sections were also captured using an Olympus FV1000 laser scanning confocal microscope (Olympus America Inc, Center valley, PA, USA). Images were further processed using Adobe Illustrator 14 (Adobe Systems Incorporated), ImageJ and Inkscape software.

To analyze the serotonergic system, i.e. rostral, middle and caudal regions of the raphe nuclei, 40-μm thickness sections from 3 wild-type and 3 PEX13 brain mutants (6 sections per mouse) were selected using the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2001). Selected sagittal and coronal sections were labeled using immunofluorescence with a TPH2 antibody and appropriate secondary antibodies. Images were captured at 4× magnification at a constant exposure time using an Olympus BX50 microscope and SPOT camera, or using an Olympus FV1000 confocal microscope (Olympus America Inc).