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# Impaired neurogenesis and associated gliosis in mouse brain with PEX13 deficiency

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ABSTRACT

Zellweger syndrome (ZS), a neonatal lethal disorder arising from defective peroxisome biogenesis, features profound neuroanatomical abnormalities and brain dysfunction. Here we used mice with brain-restricted inactivation of the peroxisome biogenesis gene PEX13 to model the pathophysiological features of ZS, and determine the impact of peroxisome dysfunction on neurogenesis and cell maturation in ZS. In the embryonic and postnatal PEX13 mutant brain, we demonstrate key regions with altered brain anatomy, including enlarged lateral ventricles and aberrant cortical, hippocampal and hypothalamic organization. To characterize the underlying mechanisms, we show a significant reduction in proliferation, migration, differentiation, and maturation of neural progenitors in embryonic E12.5 through to P3 animals. An increasing reactive gliosis in the PEX13 mutant brain started at E14.5 in association with the pathology. Together with impaired neurogenesis and associated gliosis, our data demonstrate increased cell death contributing to the hallmark brain anatomy of ZS. We provide unique data where impaired neurogenesis and migration are shown as critical events underlying the neuropathology and altered brain function of mice with peroxisome deficiency.

## 1. Introduction

Zellweger syndrome (ZS) is a severe neonatal disease caused by mutations in peroxisome biogenesis (*PEX*) genes which lead to loss of peroxisome biogenesis and peroxisomal metabolic function. ZS is a multi-system disorder with a prominent neurological component that features both neurodevelopmental deficits and neurodegeneration (Wilson et al., 1986). Brain abnormalities in ZS subjects, are generally considered to reflect neurodevelopmental defects, include growth retardation during embryonic stages, and neuronal migration defects that result in abnormal cerebellum gyration and cerebral cortex structure (Powers and Moser, 1998). These abnormalities are believed to reflect the importance of peroxisomes in brain development (Faust et al., 2005; Baes et al., 1997). In support of this premise, peroxisome abundance is high at neurite terminals of differentiating neurons during development, suggesting a role in neurite growth and synapse formation (McKenna et al., 1976; Arnold and Holtzman, 1978).

Peroxisomes play a role in the synthesis of plasmalogens, and therefore contribute to membrane stability and second messenger function, as well as being a major component of myelin sheaths (Brites et al., 2004), and docosahexaenoic acid (DHA), which has a significant role in neurotransmission, fine synaptic remodeling, and calcium homeostasis (Salem et al., 2001; Chapkin et al., 2009). These compounds also protect neurons from oxidative damage by scavenging reactive oxygen species (Braverman and Moser, 2012; Yavin et al., 2002). Peroxisomes are proposed as producing anti-inflammatory mediators in the brain (Farooqui, 2012; Farooqui et al., 2007) inferring a role in protecting newly differentiated neurons against oxidative stress and inflammation. Notably, despite these multiple roles for the peroxisome in brain physiology, it remains unclear how peroxisome deficiency causes the neuropathology of ZS.

*PEX* genes are required for the import of matrix and membrane proteins into the developing peroxisome (Distel et al., 1996; Gould and Valle, 2000). Mouse models of ZS have been generated by ubiquitous disruption of genes involved in peroxisomal matrix protein import—*PEX2* (Faust and Hatten, 1997), *PEX5* (Baes et al., 1997), and

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*PEX13* (Maxwell et al., 2003). These mutants have ZS-like phenotypes that feature early neonatal death, neurological dysfunction, and associated peroxisomal metabolic dysfunction.

To address ZS brain pathogenesis specifically, longer surviving PEX2 and PEX5 mutants (Faust, 2003; Faust and Hatten, 1997; Dirkx et al., 2005), and mouse mutants with brain-restricted *PEX* gene deficiency, have been examined. These studies have demonstrated that *PEX* gene deficiency leads to prominent neuronal migration defects, abnormal cerebellum and cerebral cortex cytoarchitecture, demyelination, inflammation, and motor dysfunction (Hulshagen et al., 2008; Krysko et al., 2007; Bottelbergs et al., 2012; Muller et al., 2011; Janssen et al., 2000).

The present study focuses on mouse mutants in which *PEX13* is selectively deleted in of all neuronal lineage cells (PEX13 brain mutant). PEX13 brain mutants have been previously shown to have abnormal neurodevelopmental features that include altered Purkinje cell differentiation and the formation of the granule cell layer in the cerebellum (Muller et al., 2011). More recently we have also demonstrated significant deficiency of the serotonergic system in these mutants (Rahim et al., 2014). Importantly, *PEX13* mutations in humans give rise to clinical phenotypes in the Zellweger spectrum (Shimozawa et al., 1999; Liu et al., 1999), thus PEX13 brain mutants provide a valid animal model to address the neurodevelopmental changes of ZS.

In the present investigations, we used the PEX13 brain mutants to investigate the effect of PEX13 deficiency on neurogenesis, differentiation and migration, and associated pathophysiological and anatomical changes. Our findings indicate significant changes to neurogenesis and neuronal migration, and concomitant gliosis together with increased cell death, which in toto may provide an explanation of the mechanisms underlying the brain pathophysiology of ZS.

## 2. Methods and materials

### 2.1. Generation of PEX13 brain mutants

Animal breeding and experimentation were approved by the Griffith University Institutional Biosafety Committee (NLRD/21/07) and the Griffith University Animal Ethics Committee (BBS/02/09/AEC; ESK/03/13/AEC), respectively.

Brain-specific disruption of *PEX13* was achieved using *Nestin-Cre* transgenic mice (*Nes-Cre/+*) in which Cre recombinase is expressed in cells of neuronal lineage under the control of the nestin promoter and intron 2 enhancer (Tronche et al., 1999; Zimmerman et al., 1994). To produce sufficient excision, *PEX13* heterozygotes ($PEX13^{\Delta/+}$) were crossed with *Nes-Cre/+* transgenic mice to generate *Nes-Cre*, $PEX13^{\Delta/+}$ mice. These mice were then crossed with $PEX13^{flox/flox}$ mice to generate mice with brain-specific disruption of *PEX13* ($PEX13^{\Delta}/flox^{\Delta}$ mice, or, simply, *PEX13* brain mutants). Because this breeding strategy does not generate wild-type progeny, we have designated 'effective' wild-type (WT) animals as those animals that exhibit no disease phenotype and carry one wild-type allele and one $PEX13^{flox}$ allele (but no Cre transgene); these animals were treated as littermate controls (and designated as *WT* mice) for these studies.

PEX13 gene disruption was assessed using PCR of mouse brain genomic DNA. To support the DNA analyses, PEX13 protein deficiency was confirmed by immunofluorescence staining of brain tissue sections using the antibody to PEX13.

### 2.2. EdU labelling, tissue harvesting and processing

To determine cell proliferation in embryos and postnatal pups, 5-ethynyl-20-deoxyuridine (EdU), dissolved in Dulbecco's phosphate buffered saline (D-PBS), was injected into time-mated pregnant mice at a dose of 50 mg/kg body weight, to label the DNA of dividing cells in the developing brain. For the analysis of neurogenesis, two pregnant dams at each of the embryonic stages E12.5, E14.5 and E18.5 received a single intraperitoneal injection of EdU. The dams were sacrificed 4 h following the EdU injection, as described a terminal dose of a ketamine/xylazine mixture (80 mg/kg body weight ketamine/10 mg/kg body weight xylazine) was administered via intraperitoneal injection, the embryos removed through midline abdominal section, and the embryonic brains drop-fixed in Zambonie's fixative overnight under vacuum. Two litters of mice at postnatal stage P0 similarly received a single intraperitoneal injection and were sacrificed 4 h later.

To evaluate postnatal cell migration, we injected four cohorts of P0 pups with EdU (50 mg/kg i.p.) at birth. Brains from two of these cohorts were harvested after 4 h (designated P0) and the remaining two cohorts after 3 days (designated P3). The pups were perfused transcardially with 10 mM phosphate-buffered saline, pH 7.4 (PBS) containing 0.5% sodium nitrite to flush out the 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 same fixative under vacuum at room temperature.

To calculate serotonergic neurogenesis and cell specification in postnatal stages, we applied three EdU experimental strategies: (1) to label serotonergic progenitors cells: EdU was injected at E9.5–E10.5 stage (in mice, progenitors give rise to serotonergic neurons in the ventral rhombencephalon between E9.5 and E11.5 stages (Deneris and Wyler, 2012)) and harvested at the P0 stage; (2) To label cells that undergo division during serotonergic cell specification:EdU was injected at embryonic stages E12.5 and E14.5 and the brains harvested after 4 h; (3) to label cells at the end of serotonergic neurogenesis: EdU was injected at E12.5–E13.5 (end of serotonergic neurogenesis) and harvested at the P15 stage; the P15 animals were perfusion-fixed as above and the brains harvested.

Brain tissue was collected and processed for cryosectioning using O.C.T. compound (Tissue-Tek; Sakura Finetek, USA, Inc.) embedment using a sucrose gradient as described previously (Norazit et al., 2011; Nguyen et al., 2010). Brains were mounted in 100% OCT and sections were cut on a cryostat at a thickness of 20–40 μm and mounted on superfrost slides. E12.5 to E18.5 embryonic stages for sectioning were selected using a prenatal mouse brain atlas (Schambra, 2008). The Paxinos rodent brain atlas was used to select sections from P0 and P15 stages (Paxinos and Franklin, 2001). Immunofluorescence analysis of neurodevelopmental events—neurogenesis, proliferation, differentiation, migration and cell specification—a minimum 10 sections were selected at periodic intervals from the ventricular zone (VZ) area at embryonic stages E12.5 and E14.5, and from the sub-ventricular zone (SVZ) at E18.5 and P0. For serotonergic cell specification analysis, a minimum of 10 sections were selected from the isthmus region at each stage (E12.5, E14.5, P0 and P15).

### 2.3. EdU click chemistry and immunofluorescence analysis

For the EdU click chemical reaction, brain sections were treated as described earlier (Chehrehasa et al., 2009): brain sections were permeabilized with 0.2% Triton X-100/PBS (wash buffer) for 20 min then incubated with 100% DMSO for 15 min at room temperature, followed by further washes with washing buffer to remove DMSO. Brain sections were then incubated with Click-iT reaction master mix for 45 min in the dark. For immunostaining, Click-iT reaction master mix was removed and the sections washed with buffer for 30 min and incubated in blocking buffer (PBS/0.1% Triton X-100 containing 10% donkey serum (v/v)) for 1 h at room temperature to block non-specific tissue binding sites. Multiple combinations of primary antibodies were used: mouse anti-GFAP, (1:800,Sapphire Biosciences); rabbit anti-GFAP, (1:800, Merck-Millipore); rabbit anti-Iba-1, (1:2000, Wako Chemicals); goat anti-Iba-1, (1:500, Abcam); goat anti-TPH2, (1:750, Everest Biotech); rabbit anti-Tph2, (1:750, ThermoFisher Scientific, Inc. Rockford, IL, USA); rabbit anti-β-III tubulin (or Tuj-1), (1:1000, ThermoFisher Scientific); mouse anti-NeuN, (1:100, Merck-Millipore); rabbit anti-Ki67,