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Peripheral nervous system defects in a mouse model for peroxisomal biogenesis disorders

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ABSTRACT

Peroxisome biogenesis disorders (PBD) are autosomal recessive disorders in humans characterized by skeletal, eye and brain abnormalities. Despite the fact that neurological deficits, including peripheral nervous system (PNS) defects, can be observed at birth in some PBD patients including those with *PEX10* mutations, the embryological basis of the PNS defects is unclear. Using a forward genetic screen, we identified a mouse model for *Pex10* deficiency that exhibits neurological abnormalities during fetal development. Homozygous *Pex10* mutant mouse embryos display biochemical abnormalities related to a PBD deficiency. During late embryogenesis, *Pex10* homozygous mutant mice experience progressive loss of movement and at birth they become cyanotic and die shortly thereafter. Homozygous *Pex10* mutant fetuses display decreased integrity of axons and synapses, over-extension of axons in the diaphragm and decreased Schwann cell numbers. Our neuropathological, molecular and electrophysiological studies provide new insights into the embryological basis of the PNS deficits in a PBD model. Our findings identify PEX10 function, and likely other PEX proteins, as an essential component of the spinal locomotor circuit.

Introduction

Clinically, peroxisome biogenesis disorders (PBD) are lethal congenital diseases with a spectrum of severity and progressiveness (Braverman et al., 2013; Steinberg et al., 2006). Infants born with Zellweger syndrome (ZS), the most severe disease within the spectrum of PBD, may show a lack of muscle tone and an inability to move, indicating an embryonic origin of the disorder (Wanders and Waterham, 2005, 2006). ZS is lethal within six months and is associated with impaired neuronal migration, abnormal brain development and severe degradation of the white matter. Patients with the PBD, neonatal adrenoleukodystrophy (NALD), display demyelination of white matter tracks in the brain and spinal cord, which results in progressive weakness and mortality within ten years (Steinberg et al., 2006). Ataxia, demyelination and loss of axon integrity are common in patients with peroxisomal dysfunction (Baes and Aubourg, 2009; Steinberg et al., 2006). A common cellular pathology of these diseases is the inability to generate functional peroxisomes to meet the metabolic demands of the cell.

Peroxisomes are single-membrane bound subcellular organelles present in all eukaryotic cells. Mammalian peroxisomes are involved in the breakdown of very long chain fatty acids (VLCFA), amino acids, and polyamines, decomposition of hydrogen peroxide, as well as the biosynthesis of plasmalogens, which are abundant in myelin, bile acids, and polyunsaturated fatty acids. Peroxisome enzyme content, number, and metabolic functions are determined by the cell type and species (Brown et al., 2008). Peroxisomal proteins called peroxins (PEX) control peroxisome assembly, fission, and shuttling of fully folded protein cargo across peroxisomal membranes (Lanyon-Hogg et al., 2010; Van Veldhoven and Baes, 2013; Wanders, 2014). Thus, disruptions in PEX proteins could alter biosynthesis and/or degradation of target substrates leading to PBD in humans.

The zinc RING-finger peroxin, PEX10, is necessary for peroxisome assembly, for import of target substrates, and for recycling or degradation of protein complexes and amino acids (Oeljeklaus et al., 2012; Williams et al., 2008, 2012). For example, PEX10 binds to PEX5 and helps dock PEX5 at the pore to allow cargo import. Moreover, PEX10 and the conserved RING domain peroxisomal

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integral membrane proteins PEX2 and PEX12 act as E3-ubiquitin ligases (Platta et al., 2009; Williams et al., 2012). The RING peroxins serve to mono- or poly-ubiquitinate PEX5 to control PEX5 receptor recycling or degradation. Poly-ubiquitinated PEX5 is sent to the proteasome for degradation whereas mono-ubiquitination of the PEX5 receptor allows it to return to the cytosol where it can bind new protein cargo for import into the peroxisomes (Agne et al., 2003; Chang et al., 1999b; Thoms and Erdmann, 2006).

Mice lacking neural *Pex5* develop postnatal neurological defects with motor dysfunction due to defects in axon integrity, demyelination and neuroinflammatory reactions (Bottelbergs et al., 2012; Hulshagen et al., 2008; Krysko et al., 2007), suggesting a neural basis to the etiology of PBD in patients with mutations in PEX5. Neurological defects such as cerebellar ataxia, spinal ataxia, progressive ataxia and reduced cognitive capacity are diagnoses in patients with mutations in the *PEX10* gene (Steinberg et al., 2004; Warren et al., 2000). However, no published vertebrate model exists with a mutation in *Pex10*. Thus, it is unclear how the loss of vertebrate PEX10 contributes to PBD disease pathology. Here, we identify a mouse model with a mutation in *Pex10* that causes neonatal mortality and defects in embryonic locomotion. Furthermore, we characterize the biochemical defects and pathology of *Pex10* mutant mice. *Pex10* homozygous mutants display prenatal pathology including defects in axonal integrity, decreased Schwann cell number and defects at the neuromuscular interface. Therefore, this *Pex10* model provides new insight into the embryological origin of PBD pathology and highlights the role of peroxisomes in embryonic peripheral nervous system development.

Material and methods

Forward genetic screen and identification of the Pex10 mutation

ENU mutagenesis was performed as described (Kasarskis et al., 1998) on males of C57BL/6J background and then outcrossed onto 129S1/Svlmj background to score G3 embryos at embryonic day 18.5 for recessive mutations that affect embryonic locomotion. Through meiotic mapping which followed linkage between the non-motile phenotype and C57BL/6J markers, the genetic region containing the mutation that affected locomotion was first mapped to the proximal third of chromosome 4 using a panel of 96 MIT and SKI SSLP markers and then narrowed to a 7 Mb region by the use of additional MIT SSLP markers on the telomeric end of chromosome 4. DNA from phenotypic mutant embryos ($n=4$) was sent to the Broad Institute to identify all C57BL/6J regions, which confirmed localization to a 3 Mb interval of the telomeric region of chromosome 4. DNA from phenotypic embryos was sent for whole exome enrichment followed by next generation sequencing (Otogenetics, Inc) and this identified only 1 candidate homozygous variant in the *Pex10* gene within the surrounding 20 Mb region of chromosome 4. This variant was a single base substitution (G to A) that introduces a C294Y non-synonymous amino acid change. Using Ensembl Genome Browser (ensembl.org) combined with whole exome capture data analysis, PEX genes and genes involved in peroxisome function (ABC transporter family and PPAR family) were subsequently examined yet no homozygous variants in exon sequences were found, other than the *Pex10* mutation. To confirm the mutation in *Pex10*, genomic DNA in overlapping segments of the *Pex10* gene was amplified by PCR from phenotypic E18.5 embryos and compared with control E18.5C57Bl/6J DNA. Subsequently, embryos were genotyped as follows: Tissue was placed in tail lysis buffer (100 mM Tris × Cl pH8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) overnight. DNA was amplified using Taqman Gold (Applied Biosystems) with primer set to *Pex10* (forward primer: AGAACCCTCATCCATTTGCCTGGT; reverse primer: AAAGTACCTCAAGCTCCCTGCACA. PCR amplification was performed for 35 cycles at 55 °C. PCR product was sent to Barbara Davis Center Molecular Biology Service Center at the University of Colorado Denver for sequencing. The official nomenclature of this mutant allele is *Pex10*^{m1Nisw}, but for simplicity we will use *Pex10*^{CY} throughout the manuscript. All of the data presented here were obtained after outcrossing >12 generations onto 129S1/Svlmj background.

Mouse embryonic touch assay

This touch assay was designed to examine the spinal locomotor response from the activation of the muscle spindles that carry signals to the dorsal root ganglion, to the interneuron relays between the dorsal root ganglion and motoneurons, and the motoneuron activation of the muscle to induce a contraction. Embryos were dissected from timed pregnant dams and placed in room temperature oxygenated mouse Tyrode's solution. To induce limb movement, the foot-pads were pinched with tweezers. For example in E18.5 wildtype embryos, pinching induces paw retraction and cross-extensor reflexes. Both forelimb and hindlimb were assayed and retraction of the limb was scored as 1, no retraction of limb was scored as 0 and slow or modest retraction of limb was scored as 0.5. We also scored for S-shaped movements in axial muscles by touching the forceps to the dorsal rib cage. Touch assay was performed on each litter to identify phenotypic embryos. The genotype of all embryos was confirmed as above.

Biochemical analysis

Bloodspots were collected from E18.5 embryos and from adult mice (>2 months old) on filter paper (Whatman 903, GE Lifesciences). Markers of PBD were analyzed by the Peroxisomal Diseases Laboratory within the Moser Center for Leukodystrophies at the Kennedy Krieger Institute (Baltimore, MD). Human samples from normal control and individuals with PBD and rhizomelic chondrodysplasia punctacta (RCDP) were collected and analyzed by the Kennedy Krieger Institute and only the data without identifying information was provided. Lyso-phosphatidylcholines, (lyso-PCs), including C26:0 lyso-PC, peroxisomal bile acid intermediates and plasmalogens were analyzed by combined liquid chromatography–tandem mass spectrometric (LC–MS/MS) method (Hubbard et al., 2009, 2006; Johnson et al., 2001; Zemski Berry and Murphy, 2004).

Cell culture

Mouse embryonic fibroblasts (MEFs) were isolated from E18.5 limb dermis. Dermis was removed from limbs and cut into small fragments and dissociated in 0.25% trypsin-EDTA (Gibco) for 15 min at 37 °C. Fragments were sheared by 5 passes through a 1000 µl pipette in DMEM/F12 (Gibco) with10% FCS (Gibco). Suspension was centrifuged and re-suspended in DMEM/F12 medium consisting of 10% FCS, penicillin/streptavidin (Gibco) and L-glutamine (Gibco). Cells were re-plated at least once before experimentation.

Peroxisomal localization

For peroxisomal localization studies, a 'LQSKL' PTS1 signal sequence from acyl-CoA oxidase was appended to the C-terminus of an EGFP reporter construct (in pCDNA3.1) using PCR. MEFs from wild-type or homozygous mutant *Pex10*^{CY} embryos were plated on 18-mm glass coverslips in DMEM/F12 (Gibco) with