PEX13 deficiency in mouse brain as a model of Zellweger syndrome: abnormal cerebellum formation, reactive gliosis and oxidative stress

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SUMMARY

Delayed cerebellar development is a hallmark of Zellweger syndrome (ZS), a severe neonatal neurodegenerative disorder. ZS is caused by mutations in PEX genes, such as PEX13, which encodes a protein required for import of proteins into the peroxisome. The molecular basis of ZS pathogenesis is not known. We have created a conditional mouse mutant with brain-restricted deficiency of PEX13 that exhibits cerebellar morphological defects. PEX13 mutant mice exhibit defects in reflex and motor development that correlate with impaired cerebellar fissure and cortical layer formation, granule cell migration and Purkinje cell layer development. Astrogliaosis and microgliosis are prominent features of the mutant cerebellum. At the molecular level, cultured cerebellar neurons from E19 PEX13-null mice exhibit elevated levels of reactive oxygen species and mitochondrial superoxide dismutase-2 (MnSOD), and show enhanced apoptosis together with mitochondrial dysfunction. PEX13 brain mutants show increased levels of MnSOD in cerebellum. Our findings suggest that PEX13 deficiency leads to mitochondria-mediated oxidative stress, neuronal cell death and impairment of cerebellar development. Thus, PEX13-deficient mice provide a valuable animal model for investigating the molecular basis and treatment of ZS cerebellar pathology.

INTRODUCTION

Zellweger syndrome (ZS), the prototypic peroxisomal disorder, is a severe multisystem disorder with significant neurological involvement. In particular, the ZS cerebellum and cerebral cortex feature defects in neuronal migration that lead to pronounced disturbances in motor function (Evrard et al., 1978; Moser, 1996; Volpe and Adams, 1972). The severity of ZS neuropathology underscores the importance of peroxisomes in the maturation of the central nervous system (CNS). Peroxisomes abound during embryonic and early postnatal brain development but decline in abundance into adulthood (Ahlemeyer et al., 2007; Itoh et al., 2000; Nagase et al., 2004; Nardacci et al., 2004). Peroxisomes are abundant at termini of developing neurons and have been implicated in the early determination of neuronal polarity (Bradke and Dotti, 1997; Ishikawa et al., 2001).

The molecular basis of ZS neuropathology is unknown. The inability to form functional peroxisomes in ZS leads to the loss of essential peroxisomal metabolic functions, such as the oxidation of very-long-chain-fatty acids (VLCFA) and the synthesis of bile acids, docosahexaenoic acid and plasmalogens (Baumgartner and Saudubray, 2002; Steinberg et al., 2006; Wanders and Waterham, 2006). However, it is still unclear to what extent the accumulation of peroxisomal substrates and/or deficiency of peroxisomal synthetic products contribute to tissue pathology (Moser, 1996; Moser and Moser, 1996; Powers and Moser, 1998).

ZS arises from mutations in PEX genes, which encode proteins (peroxins) required for the import of proteins into the peroxisomal matrix and membrane. PEX13, a peroxisomal integral membrane protein, is essential for the import of proteins carrying both of the known peroxisome matrix targeting signals, PTS1 and PTS2, a function that has been genetically conserved across species from yeasts to humans (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996; Liu et al., 1999; Maxwell et al., 2003; Shimozawa et al., 1999). In humans, PEX13 gene mutations lead to disease across the Zellweger spectrum (Al-Dirbashi et al., 2009; Krause et al., 2006; Liu et al., 1999; Shimozawa et al., 1999).

To provide insight into the molecular pathogenesis of ZS, mouse gene knockout models of ZS have been generated by targeted disruption of the PEX13 gene (Maxwell et al., 2003), as well as the PEX2 (Faust and Hatten, 1997) and PEX5 (Baes et al., 1997) genes. The latter genes also encode peroxins required for peroxisomal protein import (Dodd et al., 1995; Maxwell et al., 2003; Shimozawa et al., 1992). All three mutants recapitulate the disease phenotype of ZS patients: neonatal lethality, abnormal peroxisomal metabolism and broad tissue pathology, including pronounced neuronal migration defects and associated brain dysmorphology. By contrast, mice deficient in PEX11β (Li et al., 2002), a peroxin...
involved in peroxisome proliferation (Li and Gould, 2002), exhibit the neonatal lethality of the other mutants and a mild defect in neuronal migration, but no significant peroxisomal metabolic dysfunction. These findings appear to challenge the ‘peroxisome metabolic’ hypothesis of ZS pathogenesis.

The two obvious limitations of the PEX knockout animals relate to the neonatal lethality and depletion of peroxisomes in all tissues. These aspects preclude elucidation of postnatal brain development, particularly of the cerebellum, and the contribution of individual tissue to disease pathogenesis. These aspects of ZS pathology have been addressed through the development of longer-surviving PEX2 mutants, generated by back-crossing PEX2 knockout mice onto a different genetic background (Faust, 2003), and of conditional PEX5 mutants (Dirkx et al., 2005; Hulshagen et al., 2008; Janssen et al., 2003; Krysko et al., 2007).

In this paper, we describe the generation and characterization of a novel mouse mutant with brain-restricted PEX13 deficiency, generated using the Cre-loxP recombination technology. These PEX13 brain mutants have enabled us to correlate developmental behavioural abnormalities with morphological indicators of delayed cerebellum formation. We have combined these investigations with an analysis of cultured PEX13-deficient cerebellar neurons to propose a mechanism of ZS cerebellar neuropathogenesis that involves mitochondria and reactive oxygen species (ROS).

RESULTS
Generation of mice with brain-specific PEX13 deficiency
Initially, PEX13^flox/flox animals were mated with PEX13^flox/+ animals carrying the Nestin-Cre transgene. Subsequently, to improve the degree of Nestin-Cre recombinase-mediated exon 2 excision in brain, an alternative strategy was employed whereby PEX13^flox/flox animals were mated instead with PEX13 heterozygotes that carried the Nestin-Cre transgene (i.e. PEX13^+/Δ, Nes-Cre) to generate PEX13^Δ/Δ^flox mutants (or simply PEX13 brain mutants). Identification of potential brain PEX13 mutants was carried out by PCR analysis of tail genomic DNA (Fig. 1A). Semi-quantitative analysis of PEX13 exon 2 excision was carried out initially by Southern blot analysis on brain genomic DNA (Fig. 1B) and subsequently using a quantitative real-time PCR method (Müller et al., 2009). These analyses confirmed PEX13 disruption in brain...
of animals inheriting a PEX13\textsuperscript{lox} allele and the Cre transgene, but not, as expected, in PEX13\textsuperscript{lox}-positive animals lacking the Cre transgene. PEX13 exon 2 excision was confirmed by apparent quantitative loss of PEX13 mRNA transcript in brain of PEX13 brain mutants (Fig. 1C). The specificity of Nestin-Cre-mediated PEX13 disruption was confirmed by Southern blot analysis of brain, liver and kidney, which demonstrated excision only in brain (Fig. 1D).

Consistent with the demonstration of disrupted PEX13 mRNA, full-length (49 kDa) PEX13 protein was not detected in brain tissue (Fig. 1E), although, as previously reported for liver tissue from PEX13-null mice (Maxwell et al., 2003), an immunopositive polypeptide of approximately 26 kDa segregated with the disrupted PEX13 allele. Although PEX13 was deficient in brains of mutant animals, PEX14, a peroxisomal membrane protein, was still present in brain tissue, albeit at reduced levels (Fig. 1E), and still detectable in cellular vesicles (Fig. 1F). In addition, immunofluorescence staining for catalase, a peroxisomal matrix protein, indicated markedly reduced punctate staining (organelle bound) and increased diffuse cellular staining, consistent with mislocalization of catalase to the cytoplasm in most cells (Fig. 1F). These results are consistent with the presence of peroxisomal membrane vesicles (‘peroxisome ghosts’) that are defective in the import of matrix proteins. These findings are similar to those reported for the PEX13-null animals (Maxwell et al., 2003), and are consistent with the established function of PEX13 in peroxisomal matrix, but not in membrane, protein import (Gould et al., 1996; Liu et al., 1999).

**Biochemical analysis of PEX13 brain mutants**

PEX13-null mice were previously shown to display the widespread metabolic abnormalities that are characteristic of ZS and reflect peroxisomal dysfunction (Maxwell et al., 2003). To assess the metabolic effect of selective elimination of peroxisomes in the brain, as well as in a non-target organ (i.e. liver), we measured the levels of selective metabolites and enzymes as indicators of functional peroxisomes, i.e. VLCFA, plasmalogens, and the activity of enzymes involved in plasmalogen synthesis.

Unexpectedly, brain tissue VLCFA levels (C26:0/C22:0 ratio) of 3-week-old PEX13 brain mutant mice were not statistically significantly different to levels in normal brain tissue, and the C24:0/C22:0 ratio was reduced by 40%. For liver, C26:0/C22:0 and C24:0/C22:0 ratios were normal, as expected (Fig. 2A,B). These results for brain VLCFAs contrast with those for the PEX13-null mouse, in which the C26:0/C22:0 ratio was elevated 6.5-fold (brain) and ninefold (liver), and the C24:0/C22:0 ratio was elevated twofold in both brain and liver (Maxwell et al., 2003).

Plasmalogen biochemistry was assessed by measuring brain and liver plasmalogen levels and the activities of the peroxisomal enzymes dihydroxyacetone phosphate acyltransferase (DHAP-AT) and alkyl-DHAP synthase. Levels of brain C16:0 and C18:0 plasmalogens were reduced 20-fold and 40-fold, respectively, in PEX13 brain mutants (Fig. 2C). Brain DHAP-AT and DHAP synthase activities were correspondingly markedly reduced (Fig. 2E). These results are similar to those reported for the PEX13-null mice (Maxwell et al., 2003). Surprisingly, C16:0 plasmalogen levels are reduced to 20% of normal, while C18:0 plasmalogen levels are reduced to 40% of normal.

**Fig. 2. Biochemical analysis of PEX13 brain mutants.**

(A,B) VLCFA levels in brain and liver expressed as C26:0/C22:0 and C24:0/C22:0 fatty acid ratios (n=4).
(C,D) Levels of C16:0 and C18:0 plasmalogens in brain and liver, respectively (n=4).
(E) Activities of DHAP-AT and alkyl-DHAP synthase in brain and liver (n=4).
(F) Cholesterol content in brain tissue (n=3). Data are means ± s.e.m. Statistical significance, Student’s t-test: *P<0.05, **P<0.01, ***P<0.001.
in liver of PEX13 brain mutant mice were elevated threefold, and the levels of C18:0 plasmalogens were also slightly, but significantly, increased (Fig. 2D). These changes in liver plasmalogen levels were accompanied by increased liver DHAP-AT activity (fivefold), but not DHAP synthase activity (Fig. 2E).

Given the proposed contribution of peroxisomes to cholesterol metabolism (Kovacs et al., 2002; Kovacs et al., 2007), cholesterol levels were measured on lysates of different brain tissue regions (cerebellum, cortex, remaining brain) relative to protein content (Fig. 2F). Total cholesterol was normal for all three brain regions (cerebellum, cortex, remaining brain) relative to protein content (Kovacs et al., 2002; Kovacs et al., 2007), cholesterol accompanied by increased liver DHAP-AT activity (fivefold), but increased (Fig. 2D). These changes in liver plasmalogen levels were also slightly, but significantly, increased (Fig. 2D). These changes in liver plasmalogen levels were accompanied by increased liver DHAP-AT activity (fivefold), but not DHAP synthase activity (Fig. 2E).

Macroscopic features of PEX13 brain mutants

At birth, PEX13 brain mutants were indistinguishable from their littermate controls. However, clear evidence of growth retardation was observed by P5 (Fig. 3A), and this appeared to ultimately lead to early death. The time of onset of weight loss correlated with survival, effectively enabling classification of mutants into two groups. The majority of mutants (70.5%; designated group 1) exhibited growth retardation during the pre-weaning period and died by 5 weeks of age, with a peak in the post-weaning period (Fig. 3E). By contrast, 29.5% of mutants (group 2) showed post-weaning onset of weight loss (data not shown) and died between 9 and 26 weeks of age (Fig. 3E). Growth retardation affected the weight of organs such as liver and kidney, but not brain, as shown for group 1 animals (Fig. 3B-D). Pup weight at the time of death, as a percentage of that of age-matched littermate controls, was similar for both groups: 31.2±1.3% (mean ± s.e.m.; n=15) for group 1 and 28.8±2.7% (n=9) for group 2.

To ascertain whether the difference in onset and severity of phenotype correlated with efficiency of excision of PEX13 exon 2, the excision efficiency of animals represented in Fig. 3A was determined using quantitative real-time PCR (Müller et al., 2009). A PEX13 excision range of 70.8-89.3% (average 81.1±0.7%; n=40) was established for the PEX13 brain mutant population (Fig. 3F). However, there was no statistically significant difference between group 1 and group 2 animals (Student’s t-test; P=0.5356), suggesting that the level of PEX13 excision did not influence lifespan for the mutant population. This conclusion was supported by further analysis showing a lack of correlation between survival and PEX13 excision (Spearman correlation test, P=0.0811) (Fig. 3G); thus, within this excision range, lifespan and excision level are independent parameters.

Influences on pup survival

Weaning represents a critical time point for survival as pups confront the challenge of independent feeding. Because the majority of PEX13 brain mutants died within the 2-week period following weaning (Fig. 3E), the weight of pups at weaning, as a measure of effective pre-weaning feeding, was assessed. This revealed that group 1 animals (short survival) exhibited significantly lower body weight than those in group 2 (long survival), with mean (± s.e.m.) body weights of 5.0±0.2 g and 5.8±0.2 g, respectively (Student’s t-test; P=0.0318), representing 56.5 and 63.0% of the body weight of littermate controls, respectively (supplementary material Fig. S1A). The difference in body weight between these groups was further emphasized by a positive, statistically significant correlation between survival and weaning-weight (Spearman correlation test; correlation coefficient r=0.4348, P=0.0072; supplementary material Fig. S1B).

Animal development is also dependent on environmental factors such as litter size. A smaller litter size might enable reduced intra-litter competition, potentially allowing feeding-challenged animals to achieve near-normal rates of growth and development (Castellano and Oliverio, 1976; Nagy et al., 1977). To examine this possibility, the litter size was categorized as small (3-5 pups), medium (6-9 pups) or large (10-12 pups) and compared with body weight at time of weaning. For PEX13 brain mutants, a decrease in mean animal body weight of approximately 1 g was observed with increasing litter size category (supplementary material Fig.
Brain PEX13 deficiency in mice

S1C, right panel). This decrease was statistically significant between small and large litters (Student’s t-test; \( P=0.0004 \)), and between medium and large litters (\( P=0.0077 \)), and was not seen for wild-type animals (supplementary material Fig. S1C, left panel). The relationship of body weight to litter size for mutants implies that group 2 animals would not occur in large litters owing to a competitive disadvantage. Indeed, all group 2 animals were from small or medium litters, and large litters contained group 1 animals only (supplementary material Fig. S1D).

These data indicate that both body weight at time of weaning and litter size impact on the survival of mutants, suggesting that ability to suckle and/or competition for food in the period leading up to weaning is a correlate of post-weaning survival.

Reflex and motor development of PEX13 brain mutants

PEX13 brain mutants exhibited a contracted posture that was characterized by an inability to splay hind limbs, and a pronounced hunchback in older animals (Fig. 4A-D). To examine possible deficits in motor development and coordination up to weaning age (P20), animals were subjected to a series of neurobehavioural tests adapted from the ‘Fox Battery’ (Crawley, 2000; Fox, 1965). Mutants exhibited a significant delay in the pattern of acquisition of the majority of tested behaviours (crossed extensor, acceleration righting, hyperkinesias, vibrissae placing and visual placing) (supplementary material Table S1). Furthermore, a proportion of mutants failed to develop a full response for a number of specific reflexes, i.e. those assessed via crossed extensor (5/11 animals failed), negative geotaxis (3/11), cliff avoidance (4/11), bar holding ability (11/11) and rotarod (7/11) (Fig. 4E; supplementary material Table S1). Given the relatively steep improvement in motoric capacity measured between days 18 and 20, rotarod analysis was extended to 30 days for surviving animals. These analyses demonstrated a greater variability of ability at both P25 and P30 (\( n=3 \)) (in part owing to the test response time cut-off of 60 seconds), but nevertheless indicated that motoric deficit measured by this analysis persisted at these later developmental stages (Fig. 4E). These differences in reflex and motor behaviour development indicate severe developmental abnormalities. As most of the affected tests involve motor coordination and performance and balance, these behavioural abnormalities are suggestive of damage to the central region of the brain involved in motor coordination, i.e. the cerebellum.

Brain PEX13 deficiency impairs cerebellum formation

We next investigated whether the behavioural abnormalities of the PEX13 brain mutants correlated with cerebellum defects. At P5, both PEX13 brain mutants and their littermate controls exhibited normal cerebellar folia characteristics (Fig. 5A,B; supplementary material Table S2), but at P10 additional fissures (i.e. decidual, intercural, uvular) that had developed for control mice were lacking in some mutants (declival, absent in 2/5 animals; intercral, 1/5; uvular, 2/5) (Fig. 5C,D). Quantitative measurements revealed significantly shallower intercral, precentral, primary and prepyramidal fissures for mutants. Precentral and intercral fissures were still significantly shallower when normalized for cerebellar size (supplementary material Table S2). At P15, the decival, intercral and uvular fissures had still not formed in all mutants (declival, absent in 3/5 animals; intercral, absent 1/5; uvular, absent 2/5) (Fig. 5E,F). The decival and prepyramidal fissures were significantly shallower in absolute dimensions at this time point, but near-normal when normalized to cerebellum size (supplementary material Table S2). By the expected end of cerebellum development (P20) (Fujita, 1967; Goldowitz and Hamre, 1998), littermate control mice had developed a mature foliation pattern, with characteristic folia separated by well-defined fissures (Fig. 5G,H), whereas mutants showed at best partial development of the declival, intercral and uvular fissures. This abnormality in development was again reflected in the depth of fissures at P20, with the declival, intercral, uvular and posterolateral fissures significantly shallower, even when normalized to cerebellum size (supplementary material Table S2). The mid-saggital cerebellum dimensions were similar at P5, P10 and P15 for mutants and littermate controls. Although a statistically significant difference was observed at P20 (supplementary material Table S2), this might be attributable to a smaller cerebellum size of mutants at this stage. Importantly, some impairment of cerebellum foliation persisted in mutants beyond P20, until at least P30 (declival, absent in 1/3 animals; intercral, absent 0/3; uvular, absent 0/3) (Fig. 5I,J; supplementary material Table S2). Indeed, a long-
surviving mutant (P162) still showed cerebellar foliation defects (especially intercrural fissure; supplementary material Fig. S2). These data are consistent with a persistent defect, not just a delay, in cerebellar development.

The formation of cerebellar layers is delayed in PEX13 brain mutants and implicates impaired migration of granule cells

The rapid increase in cerebellum mass during postnatal development is closely linked to the proliferation of granule cells in the external granule layer (EGL) and their migration through the molecular layer (ML) towards the internal granule layer (IGL) (Altman and Bayer, 1997). We assessed the dynamics of the granule cell population to enable a comparison with the foliation abnormalities of the PEX13 brain mutants (supplementary material Table S3). By P15, the migration of granule cells in the mutants appeared to be delayed; the EGL was significantly thicker than for littermate controls (7.4±0.7 vs 4.7±0.5 μm; P=0.05), and still more evident at P20. Consistent with this observation, P20 mutants exhibited significantly thinner IGL (113.0±4.6 vs 136.4±4.4 μm for controls; P=0.01) and ML (114.9±5.0 vs 131.8 ±9.3 μm for controls; P=0.05). This trend was still evident at P30, with the mutant ML layer still significantly thinner (131.9±3.0 vs 155.0±4.2 μm for controls; P=0.01). The delayed disappearance of the EGL together with the delayed formation of the IGL is consistent with a defect in migration of granule cells in the mutant animals. To assess this directly, animals were administered bromodeoxyuridine (BrdU) at P6 and sacrificed 1-4 days later in order to monitor granule cell migration. Overall, the results of these analyses (Fig. 6A) are consistent with a delay in granule cell migration from the EGL through the ML to the IGL during the 4-day test period, and correlate with the observed defect in cerebellar layer formation.

Abnormal Purkinje cell differentiation in PEX13 brain mutants

The reduced thickness of the ML in PEX13 brain mutants suggests impaired differentiation and expansion of Purkinje cells, because this layer enlarges in concert with Purkinje cell expansion. At P5, control Purkinje cells (selectively stained using anti-calbindin immunofluorescence) were polarized, as evident by the presence of small apical dendritic processes, and cells were aligned in a monolayer (Fig. 6B). By contrast, mutants did not exhibit a distinct Purkinje cell monolayer, and displayed only small dendritic processes with little evidence of a forming main dendrite. By the second postnatal week (P10), long and parallel dendritic processes were formed in controls and were apparent as a primary dendrite arborising into secondary and tertiary dendrites. By contrast, many mutant Purkinje cells exhibited two main dendritic processes, the degree of branching was irregular and less complex, and dendrites were not in parallel alignment. Furthermore, somata were not aligned in a strict monolayer, and small dendritic processes and spines arose randomly. These abnormalities persisted during subsequent stages when differentiation normally occurs (P15-P20) and beyond (to P30). The total length of Purkinje cells (cell body plus dendritic tree) was significantly less in mutants at P15 (controls 132.1±3.2 μm, mutants 112.9±4.8 μm; P=0.0103), P20 (controls 141.1±8.9 μm, mutants 118.4±4.8 μm; P=0.0489) and P30 (controls 161.4±4.1 μm, mutants 132.8±3.4 μm; P=0.01) (mean ± s.e.m.; n=3-5). The long-surviving mutant (P162) exhibited similar abnormalities, including pronounced axonal swelling, indicating a persistent developmental defect (supplementary material Fig. S2).

Overall, and in keeping with the delayed granule cell migration, the defect in Purkinje cell differentiation in PEX13 brain mutants parallels the observed defect in cerebellar development.
Abnormal cerebellum development in PEX13 brain mutants is associated with reactive gliosis

Reactive gliosis describes the rapid response to neuronal injury of two classes of glial cell in the CNS, astrocytes and microglia (Streit et al., 1999). To assess whether the morphological changes observed in the cerebellum of PEX13 brain mutants were associated with reactive gliosis, cell-specific immunofluorescence was carried out at P20, which correlates with an advanced stage of cerebellum dysmorphology. Compared with littermate controls, mutants exhibited significantly increased staining for glial fibrillary acidic protein (GFAP) (Fig. 7A,B), an established marker of astrogliosis (Eng et al., 2000). Intense GFAP staining was predominantly localized to Bergmann glial cells in the molecular layer and was associated with hypertrophy of this cell population, another characteristic of astrogliosis. Western blot analysis of cerebellum tissue (supplementary material Fig. S3) and microarray data of whole brain (not shown) demonstrated an approximately twofold increase over controls for both mutant GFAP protein and mRNA expression, respectively. Similarly, an examination of microglia abundance revealed increased staining of the microglia-specific protein ionized calcium binding adapter protein 1 (Iba1) in the cerebellum of mutants (Fig. 7C-F). This increase was predominantly in the molecular layer, and to a lesser extent in the granule layer, and was accompanied by a significant change in cell morphology (Fig. 7D,F). Microglial cells in control animals displayed long, thin processes, which are characteristic of resting microglia (Fig. 7E), whereas the majority of microglial cells in mutants exhibited signs of hypertrophy, with short and thick processes, both morphological characteristics of hyper-ramified and activated microglia (Fig. 7F).

Cultured cerebellar neurons from PEX13-null mice display increased oxidative stress and apoptosis

Reactive gliosis and neurodegeneration are commonly associated with increased production of ROS. To obtain direct experimental evidence for the involvement of oxidative stress in the observed cerebellar pathogenesis, we used 7-day-old cultured cerebellar neurons isolated from E19 PEX13-null (knockout) mice. Immunofluorescence microscopic analysis of these neurons demonstrated loss of punctate catalase fluorescence but the presence of less abundant, larger, PEX14-containing vesicles, consistent with the expected isolated defect in PTS1 matrix protein import (Fig. 8A). Direct assessment of the cellular ROS level using the superoxide (O$_2^-$)-detecting probe hydroethidine (HEt) demonstrated ROS levels for PEX13-null cerebellar neurons that were almost twice those of littermate controls (Fig. 8B). By contrast, measurement of ROS levels using 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA), which is more selective for intracellular hydrogen peroxide and peroxyl and hydroxyl radicals, showed no
statistically significant difference between wild-type and PEX13-null neuronal cultures (data not shown). Because elevated ROS might lead to cell death, we measured levels of neuronal apoptosis, using three different approaches: evaluation of neuronal damage by counting the number of cells with an apoptotic nuclear morphology (Hoechst stain), immunofluorescence detection of active caspase-3, and TUNEL stain. All three analyses demonstrated a two- to threefold increase in the percentage of damaged neurons in 7-day PEX13-null mouse cultures (Fig. 8C1-C4). Similar results for neuronal damage (wild-type, 7.4±2.5%; heterozygous, 14.9±3.1%; homozygous knockout, 21.8±3.6%) and ROS levels (wild-type, 48.6±2.2%; heterozygous, 42.2±3.1%; homozygous knockout, 81.0±2.3%) were found for 1-day-old cerebellar cultures. Given the implication of mitochondrial involvement in both oxidative stress and apoptosis, we also quantified mitochondrial dehydrogenase activity, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Fig. 8D, PEX13-null neurons exhibited a 40% decrease in mitochondrial dehydrogenase activity, a change that is consistent with the identified increase in apoptosis (Lobner, 2000). To determine possible cellular responses to the identified ROS changes, we measured the levels of specific antioxidant enzymes. The protein level of mitochondrial superoxide dismutase-2 (MnSOD; also known as SOD2) was increased approximately twofold, whereas catalase and superoxide dismutase-1 (Cu/ZnSOD, also known as SOD1) levels were normal (Fig. 8E).

**Oxidative changes in brains of PEX13 brain mutant mice**

Given the findings from cultured neurons, we expanded the investigation of oxidative stress and mitochondrial involvement to tissue immunofluorescence of cerebellum sections from PEX13 brain mutants. As shown in Fig. 8F, levels of MnSOD were markedly increased in Purkinje cells and in the Purkinje cell layer generally for PEX13 brain mutants at P10, a developmental stage at which cerebellar morphological changes are first evident. These findings are consistent with the changes seen in PEX13-null cultured neurons.

**DISCUSSION**

In this study, we created and characterized a conditional mouse mutant that models specific aspects of ZS neuropathology. Specifically, we have shown that brain-restricted deficiency of PEX13 leads to defects in cerebellum morphology and motor development that correlate with impaired cerebellar fissure and cortical layer formation, granule cell migration and Purkinje cell layer development; these changes were also accompanied by astrogliosis and microgliosis. In pursuing the molecular basis of these changes, we have shown that PEX13 brain mutants exhibit increased levels of cerebellar MnSOD, and that cultured cerebellar neurons from embryonic PEX13-null mice exhibit mitochondrial dysfunction, elevated levels of ROS and MnSOD, and enhanced apoptosis. From these findings we suggest a sequence of events in which PEX13 deficiency leads to mitochondria-mediated oxidative stress, neuronal cell death and impairment of cerebellar development.

PEX13 brain mutants exhibit only a subset of the tissue biochemical changes associated with ZS peroxisomal metabolic dysfunction. Specifically, brain plasmalogon content was significantly reduced in the brain mutants, comparable to that seen for PEX13-null mice (Maxwell et al., 2003), whereas liver (a non-target organ) showed increased plasmalogon content. A possible explanation for this latter observation is compensatory synthetic activity by liver as a result of depleted brain plasmalogens. In support of this explanation, whereas brain activities of alkyl-DHAP synthase and DHAP-AT were significantly reduced, liver DHAP-AT was increased (albeit liver alkyl-DHAP synthase levels were normal). However, VLCFA levels in brain tissue of PEX13 brain mutants, when expressed as a C26:0/C22:0 ratio, were normal, and the C24:0/C22:0 ratio was significantly reduced. These parameters differ from those documented for PEX13-null mice, which, for example, showed a brain C26:0/C22:0 ratio that was elevated 6.5-fold (Maxwell et al., 2003), and for Nestin-Cre PEX5 mutant mice, which displayed a 2.8-fold increase in C26:0 levels in brain at E18.5 (Krysko et al., 2007). The reason for the lack of VLCFA accumulation in the PEX13 brain mutants is not immediately apparent, and is seemingly at odds with the observed depletion of plasmalogens when viewed as an independent measure of deficient peroxisomal metabolic function.

Overall, the metabolic profile of the PEX13 brain mutants suggests that (accumulated) VLCFA is not a major cause of disease pathology in these animals, although measurement at earlier developmental time-points would be needed to convincingly...
Fig. 8. Increased oxidative stress as a potential cause of cerebellar pathology of PEX13-null mice. (A) Presence of PEX14-positive peroxisome 'ghosts' (A3) and cytoplasmic mis-localization of catalase (A9) in cultured cerebellar granule neurons (MAP2-immunopositive) from PEX13-null mouse. Scale bar: 20 μm. (B) Levels of cellular ROS detected by hydroethidine in neurons from PEX13-null mice and littermate controls. B1: quantitative data presented as means ± s.e.m. (n=200-300 cells in three different Petri dishes from one animal for each point). B2: representative fluorescence images of cellular ethidine (Et). Scale bar: 100 μm. (C) Increased neuronal apoptosis in cerebellar cultures from PEX13-null mice. Neuronal damage was quantified by counting cells with an apoptotic nuclear morphology (C1), and cells immunopositive for active caspase-3 (C2) or TUNEL (C3). Representative TUNEL staining is indicated in C4. (D) Mitochondrial dehydrogenase activity of cultured neurons as measured by MTT reduction. Data are from 7-15 Petri dishes derived from 3-5 animals for each genotype from two different litters. Stau, wild-type cultures treated with staurosporine. (E) Levels of selected proteins in cerebellar granule neurons from PEX13-null mice. E1: representative image from one litter; E2: quantification (mean ± s.d.) of relative protein levels normalized to α-tubulin from two or three different litters. PEX13 genotype: +/+ wild-type; +/- heterozygote; ∆/∆ homozygous knockout. (F) Confocal immunofluorescence of cerebellum sections from P10 PEX13 brain mutants (BM) and littermate controls (Con) for levels of MnSOD (SOD2). Merge, overlaid images. Arrows delineate the Purkinje cell layer. Scale bar: 50 μm. Statistical significance, Student’s t-test: *P<0.05, **P<0.01, ***P<0.001.
postnatal formation of fissures does not appear to be a result of a developmental delay, because the defects persist throughout later developmental stages. Similar deficiencies in cerebellum development have been reported for the postnatal-surviving PEX2-null mutant (Faust, 2003), the PEX5 liver mutant and, to a lesser extent, the PEX5 brain mutant (Krysko et al., 2007). With regard to cerebellum maturation, the phenotype of the PEX13 brain mutants compares favourably with that of the longer-surviving PEX2-null mice, which were generated by breeding onto a different background strain (Faust, 2003). However, there is a discrepancy between our findings and those described for the Nestin-Cre conditional PEX5 (brain) mutant: PEX13 mutants exhibit significant defects in granule cell migration and foliation that persist to at least P30, whereas Nestin-Cre PEX5 mutants exhibit comparatively mild changes, with foliation normalized by P10-P11 (Krysko et al., 2007). The reason for the milder phenotype of the Nestin-Cre PEX5 mutants is not immediately clear, given the use in both cases of the same Nestin-Cre transgenic animals and the similar phenotypes of the PEX5 and PEX13 knockout mice (Baes et al., 1997; Maxwell et al., 2003); however, one plausible explanation is a difference in the efficiency of gene disruption achieved in each case. The comparison of PEX5 and PEX13 mutants is important in terms of the projected roles of the brain and extra-neuronal tissues in cerebellum development. Thus, whereas the analyses of conditional PEX5 mice suggested a minor role for brain factors per se in cerebellum maturation (and a greater contribution from liver) (Janssen et al., 2003; Krysko et al., 2007), our findings infer an essential and primary role for intrinsic brain factors.

A plausible explanation for the defects in cerebellar foliation of the PEX13 brain mutants is the impaired migration of granule cells and abnormal Purkinje cell development. The impaired migration of granule cells might be secondary to the abnormal differentiation of Purkinje cells, because granule cell precursors require interaction with Purkinje cells for proliferation and differentiation prior to migration of the mature granule cells (Corrales et al., 2006; Lewis et al., 2004). Impaired interaction between Purkinje cells and granule cells might also result in enhanced granule cell apoptosis. Conversely, impaired migration of granule cells might lead to reduced outgrowth and irregular alignment of Purkinje cell bodies, because migrating granule cells facilitate these developmental milestones in Purkinje cells (Baptista et al., 1994; Salinas et al., 1994). Similar effects of developmental abnormalities arising from impaired cell type interaction have been reported for numerous cerebellar mutants (Lalonde and Strazielle, 2007). A further possible cause of the developmental abnormalities of Purkinje cells and granule cells is a defect in Bergmann glia, which provide neurotrophic factors and essential structural substrates and scaffolding for Purkinje cell dendritic growth and granule cell migration (Hatten and Mason, 1990; Lordkipanidze and Dunaevsky, 2005). Indeed, cerebellar defects owing to impaired Purkinje cell development have previously been attributed to abnormal Bergmann glial cell function in mice (Colucci-Guyon et al., 1999), and Bergmann glial cells were distinctive components of the observed reactive gliosis of the PEX13 brain mutant cerebellum. Finally, in keeping with the conclusions from investigations on a number of cerebellar migration disorders (Laure-Kamionowska and Maslinska, 2007), the reactive gliosis observed for the PEX13 brain mutants might be associated with another process arising from...
delayed neuronal migration, namely the elimination of heterotopic neurons. Such a function would contribute another dimension to the cerebellar pathology as well as provide an explanation for the persistent cerebellar effects of the PEX13 brain mutants beyond P30. Reactive gliosis was also described as a feature of Nestin-Cre PEX5 conditional mutants (Hulshagen et al., 2008).

Foliation defects in the PEX13 brain mutant were not evident until P10 and were most pronounced in the posterior forming fissures, possibly reflecting a correlation between the onset of phenotypic abnormalities around P10 and a regional difference in the maturation of granule cells and Purkinje cells. These cell populations develop latest in the posterior folia VI-VIII (Altman and Bayer, 1997; Vastagh et al., 2005), and the monolayer alignment of Purkinje cells in this region is not complete before P15-P16 (Vastagh et al., 2005). This suggests that late-developing cell populations, such as granule cells and Purkinje cells in the posterior part of the cerebellum, would be most affected as a result of the cumulative effects of disease progression. The impairment of cerebellum layer formation, as reported here for the PEX13 brain mutants, is comparable in severity to that seen for postnatally surviving PEX2-null mice (Faust, 2003), once again suggesting a major involvement of intrinsic brain factors in this process.

In addressing the molecular basis of the neuropathogenesis of PEX13 brain mutants, we set aside the longstanding, but ill-supported, view of a disturbance of peroxisomal intermediary PEX13 brain mutants, we set aside the longstanding, but ill-supported, view of a disturbance of peroxisomal intermediary metabolism being the primary cause of ZS pathogenesis. Instead, we focused on the reported link between peroxisomal disorders and mitochondrial damage that is characteristic of ZS patients (Goldfischer et al., 1973) and of the ZS-like PEX5 (Baes et al., 1997) and PEX13 (Maxwell et al., 2003) knockout mice, as well as on the evidence for prominent reactive gliosis. In the case of PEX5-null mice, mitochondrial abnormalities in liver have been shown to correlate with increased levels of ROS and elevated levels of MnSOD in this tissue; a parallel analysis of brain tissue was not undertaken (Baumgart et al., 2001). When mitochondria are damaged, superoxide generation by the electron transport chain complexes I and III is enhanced, leading to the production of more ROS and thereby setting up a cycle of cellular damage (Finkel and Holbrook, 2000). The results of our targeted analysis of cultured PEX13-null cerebellar neurons are compatible with such a scenario in demonstrating elevated levels of ROS [specifically superoxide, O$_2^-$, generated primarily via the mitochondrial electron transport chain (Keating, 2008)], elevated mitochondrial MnSOD (a predictable mitochondrial response for scavenging highly reactive mitochondrial O$_2^-$) and increased neuronal apoptosis. In keeping with a central role of mitochondria in this cellular process, we demonstrated that mitochondrial dehydrogenase activity was significantly reduced, a finding that is consistent with mitochondrial dysfunction as a consequence of the oxidative conditions. Furthermore, mitochondrial dehydrogenase activity (MTT reduction) has been shown to correlate with, and thus determine, apoptotic death of neurons in culture (Lobner, 2000). Thus, we propose that the mitochondria-mediated redox disturbance is the primary cause of the neuronal apoptosis. Parallel analysis of cerebellum sections from P10 PEX13 brain mutants demonstrated increased levels of MnSOD, consistent with the findings from the PEX13-null cultured neurons, and further supporting an involvement of mitochondria-mediated oxidative stress in pathogenesis.

Whether plasmalogen deficiency, a consistent feature of this and other mouse ZS models, might contribute to such an oxidative stress pathway is not known. It has been proposed that plasmalogens act as ROS scavengers in vitro (Zoeller et al., 1988), but the recent analysis of mice deficient in peroxisomal DHAP-AT did not specifically address oxidative stress or mitochondrial function (Teigler et al., 2009). We propose a link between elevated cerebellar ROS and the observed reactive gliosis of PEX13 brain mutants as being consistent with the recently established function of Sirt1, which under oxidative conditions favours astrocyte proliferation over neurogenesis (Prozorovski et al., 2008). In summary, although the link between peroxin loss, peroxisome dysfunction and mitochondrial damage is yet to be established, our findings support a novel paradigm of ZS neuropathology that involves mitochondrial damage and ROS generation in a process that leads to neuronal degeneration and, consequently, undermines cerebellum formation.

In establishing and validating the PEX13-deficient mutants as animal models of ZS cerebellar pathogenesis, we thus have means by which to elucidate the cellular and molecular processes of this and mechanistically related, neurodegenerative disorders, as well a model to investigate the impact of peroxisome deficiency during stress response in neuronal disease.

**METHODS**

**Generation of PEX13-null and conditional brain PEX13 mutants**

Ethics approval for animal use was granted by the Griffith University Animal Ethics Committee (approval no. BBS/10/06/aec). The PEX13 $loxp$-modified strain (exon 2 $loxp$-flanked, or floxed; PEX13$^{lox}$ allele), the targeting construct used to generate this strain, and the generation of the PEX13-null mouse have been described previously (Bjorkman et al., 2002; Maxwell et al., 2003). PEX13 heterozygotes (PEX13$^{+/-}$) are defined as animals that carry one inherited disrupted (exon 2 excised) allele in all tissues. For cell culture experiments, heterozygous PEX13$^{+/-}$ C57BL/6J mice (background >F10 generation) were mated to generate PEX13$^{+/-}$ (wild-type), PEX13$^{3/3}$ and PEX13$^{3/3}$ (PEX13-null) pups within one litter. Brain-specific disruption of PEX13 was achieved using Nestin-Cre transgenic mice (Nes-Cre/+ in which Cre recombines in 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 heterozygous mice (PEX13$^{3/3}$) were crossed with Nes-Cre/+ transgenic mice to generate Nes-Cre, PEX13$^{3/3}$ mice. These mice were then crossed with PEX13$^{lox}$ mice to generate mice with brain-specific disruption of PEX13 (PEX13$^{3/3}$ mice, or, simply, PEX13 brain mutants). Note that we use the designations PEX13$^{3}$ and PEX13$^{3}$ to distinguish disrupted alleles that are inherited or result from Nes-Cre excision, respectively (see Fig. 1). Because this breeding strategy does not generate wild-type progeny, we have designated ‘effective’ wild-type animals as those animals that exhibit no disease phenotype and carry one wild-type allele and one PEX13$^{lox}$ allele (but no Cre transgene); these animals were treated as littermate controls for these studies. We have also included ‘effective’ heterozygotes as littermate controls, i.e. animals with one PEX13$^{3}$ allele and one PEX13$^{lox}$ allele (but no Cre transgene), because these animals also exhibit a wild-type phenotype that is similar to true PEX13 heterozygotes (Maxwell et al., 2003).
Animal genotyping

Genotyping of PEX13 homozygous knockout mice by PCR analysis of tail genomic DNA was carried out as previously described (Maxwell et al., 2003). For PEX13 brain mutants, genotyping of tail genomic DNA to determine the presence of the Cre transgene, the disrupted allele (inherited PEX13\(\Delta\) allele and Nes-Cre-generated PEX13\(^{\text{flox}}\\Delta\) allele) and the PEX13\(^{\text{flox}}\) allele, and Southern blot analysis and quantitative real-time PCR for determination and quantification of PEX13 exon 2 excision in brain, were performed as recently described (Müller et al., 2009).

Northern blot analysis

Northern blot analysis was carried out as previously described (Maxwell et al., 2003), but on brain tissue RNA that had been extracted using the Qiagen RNeasy lipid tissue mini-kit according to the manufacturer’s instructions.

Tissue biochemical analyses

Mice were euthanized by cervical dislocation, and the organs of interest removed and snap-frozen in liquid nitrogen. Tissue levels of VLCFAs (expressed as C26:0/C22:0 and C24:0/C22:0 ratios), plasmalogens (C16:0, C18:0) and the enzymes DHAP synthase and DHAP-AT were measured as previously described (Maxwell et al., 2003). Cholesterol was measured using the Amplex Red Cholesterol Assay Kit (Invitrogen) based on the manufacturer’s protocol. For measurement of plasma bile acids, 1 ml of high performance liquid chromatography (HPLC)-grade methanol was added to plasma containing an internal standard mixture and the solution heated at 55°C for 20 minutes. The solution was cooled and centrifuged at 900 g for 5 minutes, the supernatant transferred to fresh glass tubes, and the solvent evaporated to dryness under a nitrogen stream. Acetonitrile (0.5 ml) was added, and the remaining procedure for analysis was performed as described (Johnson et al., 2001).

Brain morphological and immunofluorescence analyses

Mice were anesthetized with ketamine-xylazene (4:1) and perfused via the left ventricle with 4% paraformaldehyde (Merck) in 10 mM phosphate-buffered saline (PBS) at pH 7.4. Brain tissue was removed and immersed in fixative overnight in vacuo. Primary antibodies consisted of: rabbit anti-GFAP 1:400 (Sapphire Biosciences); rabbit anti-Iba1 1:1000 (Wako Chemicals); mouse anti-calbindin 1:3000 (Sigma-Aldrich); rabbit anti-catalase 1:1000 and rabbit anti-PEX14 1:1000 (Nguyen et al., 2006); rabbit anti-SOD2 1:1000 (RDI Systems). Sections were washed thoroughly in PBS containing 0.1% Triton X-100 and incubated for 3 hours at room temperature with Alexa Fluor-conjugated secondary antibodies [goat anti-mouse 488 and 568, 1:200; goat anti-rabbit 488 and 568, 1:200; donkey anti-rabbit 488, 1:200 (Invitrogen)] and 4',6-diamidino-2-phenylindole (DAPI) (0.1 µg/ml; Sigma) or Hoechst 33342 (10 µg/ml), both diluted in blocking buffer. Sections were subsequently washed several times in PBS containing 0.1% Triton X-100 and viewed using a fluorescence microscope (AxioImager.Z1; Zeiss).

Images were routinely captured with the AxioCam camera (Zeiss), and resolution in the apical dimension was enhanced where necessary using the ApoTome module (Zeiss). In order to image large areas, such as whole cerebellum, a mosaic scan was performed and individual images subsequently merged. All processing of images was performed using AxioVision 4.6.3 digital image editing software (Zeiss). Confocal images of P10 cerebellum sections for MnSOD immunofluorescence were captured using an Olympus FV1000 confocal laser scanning microscope (60× objective lens).

Quantification of cerebellum morphology was carried out using the methodology described by Faust (Faust, 2003), with minor modifications. The thickness of the external granule layer, molecular layer and internal granule layer, and the length of the Purkinje cells (Purkinje cell body plus dendritic tree) were measured at the midpoint of folia IV-V along the preculminate and primary fissure. ‘Virtual’ measuring boxes with the following dimensions were applied in this area for different stage mice: 400×250 µm for P5; 550×350 µm for P10; 700×600 µm for P15; and 900×550 µm for P20. For both the anterior and posterior regions, two measurements were taken for the thickness of individual layers and Purkinje cell length, to obtain a total of four (pooled) data points per section. In order to measure the depth of individual fissures, a straight line was taken between the top points of the two adjacent folia, with the midpoint defined as the top of the fissure. The depth of individual fissures was defined by taking a line from the base of the fissure along its course to the top of the fissure. In order to account for the varying size of the cerebellum between PEX13 brain mutants and control littermates, the fissure depth was normalized to the square root of the section area. The area of the cerebellum was measured by taking a line around the outline of the cerebellum and determining the enclosed area. For each animal, four sections within a region of 200 µm of the midpoint of the cerebellum were measured and data pooled to obtain a mean value per animal.

BrdU analysis of neuronal migration

Cells were labelled with BrdU (Sigma) by administering pups a single intraperitoneal injection of BrdU (75 µg/g body weight in PBS) at P6. Brains were harvested after perfusion-fixation at P7, P8, P9 and P10 followed by PEG embedding and sectioning. For detection of BrdU-labelled cells, double-stranded DNA was denatured by incubating sections for 30 minutes in 2 M HCl at 37°C followed by a treatment in 0.2% pepsin (Sigma) for 20 minutes. Sections were washed in PBS containing 0.1% Triton X-100 and incubated in 5× SSC medium (0.75 M NaCl, 75 mM sodium citrate buffer, pH 7.0) twice for 5 minutes. Sections were
incubated in hybridization solution [45% (v/v) formamide, 25% (v/v) 20× SCC, 2.5% (w/v) chondroitin sulphate] for 5 minutes at 95°C, washed twice in 2× SCC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) for 5 minutes and twice in PBS containing Triton X-100 for 5 minutes. Sections were then and immunostained using mouse anti-BrdU 1:200 (DakoCytomation).

**Postnatal neurobehavioral development**
Mice were assessed using a range of tests adapted from the ‘Fox Battery’ (Fox, 1965). Tests were performed on a daily basis from P1 through P20 (weaning) to P30 between 4 pm and 8 pm, with each mouse tested once for a given response. Scores were allocated according to Fox (Fox, 1965): 0 = no response, 1 = weak response, 5 = moderate response, 9 = full response. The day of first appearance of individual scores was recorded. If a response did not develop to its full extent, day 20 (latest time point examined) was recorded as an arbitrary day of first appearance (Crusio and Schmitt, 1996). Individual litters were treated as one sample by pooling data points obtained from control mice and mutant mice of one litter, as proposed previously (Spear and File, 1996).

**Preparation of primary cerebellar cultures**
Heterozygous PEX13 C57BL/6J mice (background >F10 generation) were mated overnight and all foetuses removed by Caesarean section from the uterus of the pregnant dam at E19. Because immediate preparation prior to PCR genotyping was necessary for yielding optimal neuronal cultures, the cerebellum of individual mice from one litter were dissected and processed separately and in parallel. The preparation of cerebellar granule neurons from E19 animals was carried out as previously described (Ahlemeyer and Baumgart-Vogt, 2005). Experiments were performed, if not otherwise mentioned, on cells cultured for 7 days, at which time primary neuronal cultures contained less than 5% astrocytes. In one experiment, neuronal viability and cellular ROS levels were measured just 1 day after seeding. In this case, cerebellar cultures contained less than 1% astrocytes. All comparative analyses were carried out on cultures of all three different genotypes from the same litter.

**Fluorescence microscopic analysis of cultured neurons**
Cerebellar granule neurons grown on poly-L-lysine-coated glass coverslips were fixed with 4% paraformaldehyde, 2% saccharose in PBS for 20 minutes and permeabilized with 0.1% (v/v) Triton X-100 for 10 minutes. After blocking nonspecific binding sites with 1% BSA in PBS for 30 minutes, cells were incubated with a set of primary antibodies (rabbit anti-PEX14 1:1000 (Nguyen et al., 2006), rabbit anti-active caspase-3 (1:100; Cell Signalling) or rabbit anti- catalase 1:1000 (Nguyen et al., 2006); combined with chicken anti-MAP2 1:500 (Sigma-Aldrich) overnight at 4°C in blocking buffer. A set of secondary antibodies was then added for 1 hour at room temperature (goat anti-chicken Alexa Fluor 633, goat anti-rabbit Alexa Fluor 488; both diluted 1:300 and obtained from Invitrogen). Images were captured with a Leica confocal microscope (TCS SP2) using the digital image editing Leica Confocal Software Program.

Neuronal apoptosis was quantified by three different approaches: (i) cells were incubated with the DNA-binding fluorochrome Hoechst 33342 (10 µg/ml) for 10 minutes prior to fixation with 4% paraformaldehyde. Neurons with shrunkened and fragmented nuclei or condensed chromatin were counted as damaged cells. Apoptotic neurons were also counted as those that positively stained for (ii) active caspase-3 or (iii) terminal transferase dUTP nick-end labelling (TUNEL ApopTag Red in situ detection kit; Chemicon). For TUNEL staining, DNase-treated wild-type neurons were used as a positive control and images were captured using a Leica DM-RD fluorescence microscope.

Two independent methods were used to measure cellular ROS levels. The cell-permeable probe (di)hydroethidine (HET; Invitrogen) is preferentially oxidized by superoxide (O2•–) to a fluorescent product, ethidine (Et). Et is retained intracellularly, thus allowing quantitative estimations of the cellular ROS level (Ahlemeyer et al., 2001; Bindokas et al., 1996). For measurement of the intracellular hydrogen peroxide, peroxyl and hydroxyl radical levels, cells were loaded with DCHF-DA (Invitrogen) (Russell et al., 2002). Either HET or DCHF-DA (as 5 mM or 10 mM stock in DMSO) were added for 20 minutes to the culture medium of neurons grown on coverslips to reach a final concentration of 5 µM and 10 µM, respectively. Thereafter, cells were washed with PBS, fixed with 4% depolymerized paraformaldehyde in PBS for 20 minutes and the coverslips mounted on slides for measuring cellular Et or 2’,7’-dichlorofluorescin (DCF) fluorescence under a confocal laser scanning microscope (Leica TCS SP2). Images were captured with a 40× fluorescence objective under fixed settings for each series of experiments with respect to laser energy and pinhole size (1.02 airy units) using the 488 nm (DCF) or 519 nm (Et) laser line of an argon/krypton laser. Signal detection (PMT, offset) was adjusted until the border of each cell in wild-type control cultures was clearly visible. Ten regions in each of the three different Petri dishes of the same genotype from the same litter were chosen and scanned in the single scan mode. Fluorescence intensity values of the scanned regions were digitized as 512×512 pixel images and Et or DCF fluorescence (mean pixel value) was quantified individually in all cells using the Leica Confocal software. Values were expressed as mean fluorescence intensity (MFI) of DCF or Et per cell.

**MTT assay**
Disturbance of mitochondrial function was quantified by measurement of the reduction of MTT by mitochondrial dehydrogenases to produce a purple formazan dye. MTT was added to culture media at a final concentration of 5 mg/ml. After 20 minutes of incubation, media was removed and cells dissolved in DMSO. The formation of the formazan product was measured spectrophotometrically and normalized to protein content determined in corresponding sister cultures using the Bradford method (Bradford, 1976). Results were normalized to wild-type cultures, which were set to 100%. Wild-type cultures treated for 8 hours with 200 nM staurosporine were used as positive controls.

**Western blot analysis**
Mouse whole-brain tissue was pulverized under liquid nitrogen and resuspended in extraction buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% SDS) containing protease inhibitors (50 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml chymostatin, 200 µM phenylmethylsulphonyl fluoride). The mix was passed ten times through a Hamilton syringe and centrifuged at 18,000 g for 20
**Clinical issue**

Zellweger syndrome (ZS) is an incurable fatal autosomal recessive disorder with widespread tissue pathology. Sufferers have severe motor dysfunction as a result of defective neuronal migration and associated neurodegeneration in the neocortex and cerebellum. ZS is caused by mutations in PEX genes, which encode proteins required for the biogenesis of peroxisomes, organelles that break down toxic substances in the cells of the liver, kidneys and brain. Despite the evident importance of peroxisomes in brain development and function, the relationship between peroxisomes and neurological dysfunction is far from clear.

**Results**

Previous mouse knockout models of ZS have generated equivocal data regarding the link between specific peroxosomal metabolic dysfunction and disease pathogenesis. To resolve this question, this paper describes a mouse model of ZS cerebellum dysfunction generated by conditional disruption of the PEX13 gene in the brain. Mutant mice survive to weaning, but display motor abnormalities and defects in cerebellum development characterized by abnormal foliation and cell migration, and have accompanying reactive gliosis (excessive proliferation of astrocytes). The molecular basis of these changes was examined using PEX13-mutant brain in combination with cultured cerebellar neurons from embryonic day 19 (E19) PEX13-null mice. The authors demonstrate that PEX13 loss is associated with mitochondrial dysfunction, and increased levels of reactive oxygen species and cell apoptosis.

**Implications and future directions**

PEX13-deficient mice are an important model of the neurological changes that occur in ZS. The data presented in the paper support a new theory of ZS neuropathology, in which mitochondria and reactive oxygen species play a role in disease pathogenesis. The mice should be useful models in which to test therapeutic approaches that target mitochondria and oxidative stress.

**REFERENCES**


**SUPPLEMENTARY MATERIAL**

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.004622/-/DC1

**AUTHOR CONTRIBUTIONS**

D.I.C., C.C.M., B.A. and E.B.-V. wrote the paper. The authors declare no competing financial interests.

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**COMPETING INTERESTS**

The authors declare no competing financial interests.

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A. Relative GFAP protein expression in Cerebellum

B. Bar graph showing relative GFAP protein expression with BM and AM treatments.
Table S1. Statistical analysis of reflex and motor development of PEX13 brain mutants

<table>
<thead>
<tr>
<th>Tested behaviour</th>
<th>Tested degree of responsea</th>
<th>Day of first appearanceb</th>
<th>Differencec</th>
<th>P-valuec</th>
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<td>Controls</td>
<td>Mutants</td>
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<td></td>
<td>4.6 ± 0.1</td>
<td>6.2 ± 0.3</td>
<td>1.6</td>
<td>0.0018</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>6.8 ± 0.3</td>
<td>9.2 ± 0.2</td>
<td>2.4</td>
<td>0.0001</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>10.5 ± 0.5</td>
<td>12.4 ± 0.5</td>
<td>1.9</td>
<td>0.04</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>12.6 ± 0.3</td>
<td>14.5 ± 0.6</td>
<td>1.9</td>
<td>0.027</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>14.8 ± 0.5</td>
<td>16.9 ± 0.7</td>
<td>2.1</td>
<td>0.0288</td>
<td>*</td>
</tr>
<tr>
<td>Crossed extensor</td>
<td>1.2 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>0.1</td>
<td>0.8267</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>2.9 ± 0.9</td>
<td>12.4 ± 3.5</td>
<td>9.5</td>
<td>0.0267</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>4.8 ± 0.6</td>
<td>5.9 ± 0.6</td>
<td>1.1</td>
<td>0.2061</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>6.8 ± 0.4</td>
<td>7.5 ± 0.9</td>
<td>0.7</td>
<td>0.4826</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>8.5 ± 0.7</td>
<td>10.7 ± 0.5</td>
<td>2.2</td>
<td>0.0314</td>
<td>*</td>
</tr>
<tr>
<td>Acceleration righting</td>
<td>13.1 ± 0.3</td>
<td>14.9 ± 0.4</td>
<td>1.8</td>
<td>0.0113</td>
<td>*</td>
</tr>
<tr>
<td>Bar holding ability</td>
<td>6.4 ± 0.6</td>
<td>8.2 ± 1.0</td>
<td>1.8</td>
<td>0.1456</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>10.6 ± 0.6</td>
<td>16.8 ± 0.8</td>
<td>6.2</td>
<td>0.0003</td>
<td>***</td>
</tr>
<tr>
<td>Straight line walking</td>
<td>8.6 ± 0.2</td>
<td>9.2 ± 0.3</td>
<td>0.6</td>
<td>0.17</td>
<td>ns</td>
</tr>
</tbody>
</table>

aScores for first appearance of a response were assigned as follows: 0 = no response, 1 = weak response, 5 = moderate response, 9 = full response.
bAll data are presented as mean ± s.e.m.; n = 5 per group.
cDifference represents the difference in time (days) of first appearance of the tested behaviour between mutants and littermate controls.
dDifference was tested for significance using the Student’s t-test: ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.
eTests intensify during early development, then weaken, and finally disappear before the end of postnatal development.
fSeveral mutants failed to develop a full response to this reflex; time difference is partly due to the arbitrary score of 20 given to animals not developing a full response.
Table S2. Postnatal cerebellum fissure development of PEX13 brain mutants

<table>
<thead>
<tr>
<th>FISSURE</th>
<th>Controls P5</th>
<th>Mutants P5</th>
<th>Controls P10</th>
<th>Mutants P10</th>
<th>Controls P15</th>
<th>Mutants P15</th>
<th>Controls P20</th>
<th>Mutants P20</th>
<th>P30</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>P5</td>
<td>P10</td>
<td>P15</td>
<td>P20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>370.3 ± 73.1</td>
<td>474.7 ± 48.8</td>
<td>634.5 ± 48.3</td>
<td>428.8 ± 57.5**</td>
<td>917.7 ± 48.6</td>
<td>935.5 ± 46.3</td>
<td>902.9 ± 71.4</td>
<td>938.1 ± 97.7</td>
<td>968.7 ± 67.3</td>
</tr>
<tr>
<td>Precentral</td>
<td>0.28 ± 0.04</td>
<td>0.34 ± 0.05</td>
<td>0.33 ± 0.01</td>
<td>0.24 ± 0.02**</td>
<td>0.38 ± 0.01</td>
<td>0.42 ± 0.02</td>
<td>0.37 ± 0.03</td>
<td>0.41 ± 0.05</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>Preculminate</td>
<td>704.8 ± 32.0</td>
<td>666.7 ± 50.3</td>
<td>1098.0 ± 98.8</td>
<td>941.9 ± 89.6</td>
<td>1544.0 ± 52.1</td>
<td>1339.0 ± 89.8</td>
<td>1624.0 ± 67.3</td>
<td>1239.0 ± 142.1</td>
<td>1804 ± 63.8</td>
</tr>
<tr>
<td></td>
<td>0.51 ± 0.05</td>
<td>0.43 ± 0.07</td>
<td>0.58 ± 0.03</td>
<td>0.56 ± 0.04</td>
<td>0.64 ± 0.01</td>
<td>0.59 ± 0.03</td>
<td>0.67 ± 0.01</td>
<td>0.54 ± 0.06</td>
<td>0.67 ± 0.04</td>
</tr>
<tr>
<td>Primary</td>
<td>791.7 ± 24.2</td>
<td>830.6 ± 12.6</td>
<td>1326.0 ± 57.5</td>
<td>1152.0 ± 43.0**</td>
<td>1693.0 ± 23.6</td>
<td>1588.0 ± 47.3</td>
<td>1658.0 ± 75.2</td>
<td>1600.0 ± 54.9</td>
<td>1797 ± 45.32</td>
</tr>
<tr>
<td></td>
<td>0.55 ± 0.038</td>
<td>0.64 ± 0.016</td>
<td>0.69 ± 0.059</td>
<td>0.69 ± 0.001</td>
<td>0.71 ± 0.016</td>
<td>0.71 ± 0.031</td>
<td>0.69 ± 0.018</td>
<td>0.69 ± 0.035</td>
<td>0.68 ± 0.03</td>
</tr>
<tr>
<td>Declival</td>
<td>-</td>
<td>-</td>
<td>15.8 ± 4.5</td>
<td>5.9 ± 3.7</td>
<td>12.6 ± 2.9</td>
<td>3.2 ± 2.3**</td>
<td>11.8 ± 4.0</td>
<td>0.7 ± 0.7**</td>
<td>13.3 ± 4.9</td>
</tr>
<tr>
<td>Interccrural</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>104.6 ± 21.1</td>
<td>20.7 ± 4.9**</td>
<td>79.2 ± 40.8</td>
<td>12.5 ± 6.3</td>
<td>174.6 ± 52.6</td>
<td>9.4 ± 2.3**</td>
</tr>
<tr>
<td>Prepyramidal</td>
<td>322.9 ± 36.0</td>
<td>248.9 ± 10.3</td>
<td>483.4 ± 18.3</td>
<td>380.3 ± 20.3**</td>
<td>567.2 ± 24.8</td>
<td>480.0 ± 26.2*</td>
<td>604.7 ± 54.1</td>
<td>476.5 ± 42.1</td>
<td>625.6 ± 11.6</td>
</tr>
<tr>
<td></td>
<td>0.23 ± 0.033</td>
<td>0.19 ± 0.008</td>
<td>0.26 ± 0.011</td>
<td>0.23 ± 0.012</td>
<td>0.24 ± 0.007</td>
<td>0.21 ± 0.011</td>
<td>0.24 ± 0.015</td>
<td>0.21 ± 0.018</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>Secondary</td>
<td>530.8 ± 25.9</td>
<td>452.4 ± 20.8</td>
<td>832.1 ± 40.5</td>
<td>736.2 ± 28.4</td>
<td>908.1 ± 96.2</td>
<td>882.6 ± 17.8</td>
<td>958.0 ± 65.2</td>
<td>837.6 ± 35.9</td>
<td>1101 ± 11.8</td>
</tr>
<tr>
<td></td>
<td>0.37 ± 0.038</td>
<td>0.36 ± 0.029</td>
<td>0.45 ± 0.017</td>
<td>0.44 ± 0.009</td>
<td>0.40 ± 0.026</td>
<td>0.39 ± 0.007</td>
<td>0.40 ± 0.020</td>
<td>0.37 ± 0.024</td>
<td>0.41 ± 0.006</td>
</tr>
<tr>
<td>Uvular</td>
<td>-</td>
<td>-</td>
<td>88.2 ± 25.1</td>
<td>25.6 ± 15.4</td>
<td>110.0 ± 29.1</td>
<td>52.7 ± 21.9</td>
<td>174.8 ± 57.9</td>
<td>43.6 ± 15.3*</td>
<td>155.6 ± 32.4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.05 ± 0.014</td>
<td>0.01 ± 0.009</td>
<td>0.04 ± 0.013</td>
<td>0.02 ± 0.01</td>
<td>0.07 ± 0.023</td>
<td>0.020 ± 0.006*</td>
<td>0.061 ± 0.02</td>
<td>0.008 ± 0.005</td>
</tr>
<tr>
<td>Posterolateral</td>
<td>306.3 ± 20.5</td>
<td>264.0 ± 18.7</td>
<td>429.7 ± 26.2</td>
<td>429.5 ± 45.8</td>
<td>708.7 ± 38.0</td>
<td>636.9 ± 23.7</td>
<td>731.3 ± 6.5</td>
<td>561.2 ± 28.9*</td>
<td>856.2 ± 16.1</td>
</tr>
<tr>
<td></td>
<td>0.220 ± 0.025</td>
<td>0.190 ± 0.019</td>
<td>0.250 ± 0.013</td>
<td>0.250 ± 0.027</td>
<td>0.300 ± 0.011</td>
<td>0.280 ± 0.006</td>
<td>0.310 ± 0.004</td>
<td>0.220 ± 0.026*</td>
<td>0.32 ± 0.005</td>
</tr>
<tr>
<td>Cerebellum area (mm²)</td>
<td>1.80 ± 0.06</td>
<td>1.69 ± 0.02</td>
<td>3.05 ± 0.22</td>
<td>2.83 ± 0.27</td>
<td>5.68 ± 0.24</td>
<td>5.05 ± 0.21</td>
<td>5.81 ± 0.35</td>
<td>4.78 ± 0.18*</td>
<td>7.10 ± 0.20</td>
</tr>
</tbody>
</table>

Difference in measurements between controls and mutants was tested for significance using the Student’s t-test: ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001. Upper values in each row represent absolute length of fissures (µm), lower values represent length of fissure normalised to square root of cerebellum area. P5–P30, postnatal days 5–30. 

a Data are presented as mean ± s.e.m.; n = 5 per group, except for P30 where 5 controls and 3 mutants were analysed.
Table S3. Cerebellum layer formation in PEX13 brain mutants

<table>
<thead>
<tr>
<th>Layer thickness (μm)</th>
<th>Control</th>
<th>Mutant</th>
<th>Control</th>
<th>Mutant</th>
<th>Control</th>
<th>Mutant</th>
<th>Control</th>
<th>Mutant</th>
<th>Control</th>
<th>Mutant</th>
<th>Control</th>
<th>Mutant</th>
<th>Control</th>
<th>Mutant</th>
<th>Control</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P5</td>
<td></td>
<td>P10</td>
<td></td>
<td>P15</td>
<td></td>
<td>P20</td>
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<td>P30</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>External granule layer</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>56.2 ± 2.0</td>
<td>55.4 ± 1.1</td>
<td>30.7 ± 1.9</td>
<td>31.8 ± 1.0</td>
<td>4.7 ± 0.5</td>
<td>7.4 ± 0.7*</td>
<td>0.9 ± 0.3</td>
<td>3.5 ± 0.8*</td>
<td>-</td>
<td>-</td>
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<td></td>
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</tr>
<tr>
<td>Molecular layer</td>
<td>25.0 ± 1.4</td>
<td>23.5 ± 0.3</td>
<td>55.6 ± 3.9</td>
<td>53.3 ± 7.9</td>
<td>125.3 ± 3.1</td>
<td>110.5 ± 5.4</td>
<td>131.8 ± 9.3</td>
<td>114.9 ± 5.0*</td>
<td>155.0 ± 4.2</td>
<td>131.9 ± 3.0**</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Internal granule layer</td>
<td>71.5 ± 3.2</td>
<td>63.8 ± 2.7</td>
<td>119.9 ± 10.1</td>
<td>109.7 ± 5.3</td>
<td>126.6 ± 3.9</td>
<td>119.1 ± 5.7</td>
<td>136.4 ± 4.4</td>
<td>113.0 ± 4.6**</td>
<td>140.9 ± 3.8</td>
<td>130.6 ± 5.9</td>
<td></td>
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</table>

Difference between controls and mutants was tested for significance using the Student's t-test. ns, not significant; *P < 0.05; **P < 0.01.

Layer measurements (in μm) are presented as mean ± s.e.m.; n = 5 per group.