Deletion of a single allele of the Pex11β gene is sufficient to cause oxidative stress, delayed differentiation and neuronal death in mouse brain

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SUMMARY

Impaired neuronal migration and cell death are commonly observed in patients with peroxisomal biogenesis disorders (PBDs), and in mouse models of this diseases. In Pex11β-deficient mice, we observed that the deletion of a single allele of the Pex11β gene (Pex11β+/– heterozygous mice) caused cell death in primary neuronal cultures prepared from the neocortex and cerebellum, although to a lesser extent as compared with the homozygous-null animals (Pex11β−/− mice). In corresponding brain sections, cell death was rare, but differences between the genotypes were similar to those found in vitro. Because PEX11β has been implicated in peroxisomal proliferation, we searched for alterations in peroxisomal abundance in the brain of heterozygous and homozygous Pex11β−/− null mice compared with wild-type animals. Deletion of one allele of the Pex11β gene slightly increased the abundance of peroxisomes, whereas the deletion of both alleles caused a 30% reduction in peroxisome number. The size of the peroxisomal compartment did not correlate with neuronal death. Similar to cell death, neuronal development was delayed in Pex11β−/− mice, and to a further extent in Pex11β−/− mice, as measured by a reduced mRNA and protein level of synaptophysin and a reduced protein level of the mature isoform of MAP2. Moreover, a gradual increase in oxidative stress was found in brain sections and primary neuronal cultures from wild-type to heterozygous to homozygous Pex11β−/− deficient mice. SOD2 was upregulated in neurons from Pex11β−/− mice, but not from Pex11β+/− animals, whereas the level of catalase remained unchanged in neurons from Pex11β−/− mice and was reduced in those from Pex11β−/− mice, suggesting a partial compensation of oxidative stress in the heterozygotes, but a failure thereof in the homozygous Pex11β−/− brain. In conclusion, we report the alterations in the brain caused by the deletion of a single allele of the Pex11β gene. Our data might lead to the reconsideration of the clinical treatment of PBDs and the common way of using knockout mouse models for studying autosomal recessive diseases.

INTROXOSOMES

Peroxisomes are dynamic organelles that can arise de novo (membrane formation). They mature by the import of matrix proteins, proliferate and divide, depending on developmental and environmental conditions. This process is called peroxisomal biogenesis and is mediated by more than 30 different proteins, classified as `peroxins.' These proteins are encoded by PEX genes and were numbered according to their date of discovery (Lanyon-Hogg et al., 2010; Wolf et al., 2010). Among them, the PEX11 protein has been implicated in the regulation of peroxisome proliferation and division, because overexpression of PEX11B is sufficient to induce peroxisomal proliferation (Schrader et al., 1998), whereas its disruption reduces the total number of this organelle. It was suggested that PEX11B binds to the peroxisomal membrane of pre-existing organelles and mediates the formation of subdomains, followed by protrusion, extension, segmentation and constriction of the plasma membrane (Delille et al., 2010). In addition, the Pex11p protein has been shown to mediate the transport of medium-chain fatty acids across the peroxisomal membrane, a process that indirectly affects peroxisome number and size in Saccharomyces cerevisiae (van Roermund et al., 2000). Furthermore, the overexpression of PEX11B increases the expression of peroxisome-related genes, such as those encoding PEX5, catalase, PMP70 and PPARα, in Xenopus laevis (Fox et al., 2011).

The importance of peroxisome biogenesis for cell homeostasis is clearly demonstrated by the severe clinical phenotype of peroxisomal biogenesis disorders (PBDs). PBDs are inherited in an autosomal recessive manner and are caused by mutations in at least 12 PEX genes (classified into 12 complementation groups) (Weller et al., 2003). The disease is characterized by disturbances in both developmental and metabolic homeostasis, predominantly in the liver, kidney and brain (Faust et al., 2005). The clinical phenotype varies widely, with Zellweger syndrome (ZS) at the most severe end of the spectrum (survival of less than 1 year) followed by neonatal adrenoleukodystrophy (NALD) and infantile Refsum’s disease (IRD) as milder forms in which the patients survive into the second decade (Gärtnert, 2003). In addition, some features of the disease (renal cysts) are not found in the milder forms. The severity of the symptoms is proposed to depend on the nature of PEX mutation (Brosius and Gärtnert, 2002), e.g. a premature termination codon in the protein will lead to a total loss of function, but in cases in which the mutant protein is misfolded or unable to interact with other peroxins (Geisbrecht et al., 1998; Tamura et al., 1998) or contains a residual import activity (Imamura et al., 2001; Tamura et al., 2001; Walter et al., 2001), peroxisomal...
function is reduced, but not absent. Furthermore, it has been suggested that the full phenotypic range is even wider than we currently appreciate and that diagnosis can easily overlook patients with milder presentations (Weller et al., 2003). Steinberg et al. developed a PEX gene screen for a systematic screening of the PEX1, PEX6, PEX6, PEX10 and PEX2 genes and have identified 25 novel PEX gene mutations and 91 as-yet-unclassified PBDs of the Zellweger syndrome spectrum (ZSS) (Steinberg et al., 2004). They conclude that approximately 20% of patients with a PEX1 or PEX6 defect are not identified owing to uncommon mutations. In addition, all of the mutations identified by the PEX gene screen, 36% of the patients were heterozygous (Steinberg et al., 2004). In a heterozygous patient, the authors were unable to find a second mutation to explain the severe PBD phenotype (ZSS). However, such screening methods might differ with respect to efficacy and correctness, and a detailed analysis of the gene defect would be needed in this case. Another patient, who had sensorineural deafness and retinitis pigmentosa, was first misdiagnosed and was only later – when his son was diagnosed with PBD – found to have one defective PEX6 allele with two missense mutations and a second splice site mutation on another PEX gene (Raa-Rothschild et al., 2002). We hypothesize that disruption of a single allele of a PEX gene might be sufficient to cause milder neurological symptoms. Using the embryonic day 19 (E19) Pex11β-deficient mouse, we searched for differences between homozygous (Pex11β−/−), heterozygous (Pex11β+/−) and wild-type (Pex11β+/+) animals. We especially focused on alterations in the brain, because it is this organ and sensory organs (eye, ear) that seem to be prone to damage in ZS patients; for example, defects in the layer formation of the cerebral and cerebellar cortices, hypomyelination and neurodegeneration have been observed (Powers and Moser, 1998).

RESULTS

Deletion of a single allele of the Pex11β gene causes neuronal death

Impaired neuronal migration and focal areas of enhanced neuronal apoptosis are the typical pathological alterations in the medial neocortex of Pex11β−/− mice and other ZS mouse models (Baes et al., 1997; Baes et al., 2002; Faust and Hatten, 1997; Li et al., 2002a; Li et al., 2002b; Maxwell et al., 2003). Because this group of diseases is inherited in an autosomal recessive manner, it was generally thought that the phenotype of heterozygous animals is identical to the wild-type ones, so studies on ZS included only wild-type and homozygous animals. When we prepared primary neuronal cultures from the neocortex and cerebellum of Pex11β−/−, Pex11β+/− and Pex11β−/− mice, we measured a higher basal level of TUNEL (terminal desoxynucleotidyl transferase-mediated dUTP nick end labeling)-positive cells in cultures from homozygous mice compared with those from wild-type mice – neuronal damage was even more enhanced in those from the homozygous-null animals (Fig. 1A,C). Cell death in cortical cultures was further characterized by immunofluorescence preparations to reveal caspase-3 activation (Fig. 1B,C) and by propidium iodide staining to detect membrane leakage (Fig. 1C). Nuclear staining using Hoechst 33342 was performed in parallel. Evaluation of different parameters in the same cells revealed that 85% of all neurons with an apoptotic nuclear morphology were positively stained for TUNEL, but only a proportion of these contained activated caspase-3 (38%) or were leaky for propidium iodide (37%; Fig. 1C). The caspase inhibitor zVAD.fmk (100 μM) partially protected neurons against death in all three genotypes (Fig. 1E). Thus, cell death in Pex11β-deficient neurons showed typical features of apoptosis, a partial involvement of caspase-3 activation and membrane leakage (also known to occur in later stages of apoptosis). Differences in cell death between all three Pex11β genotypes were highly reproducible, as shown for four different litters in Fig. 1D. In addition, we determined neuronal death in parallel in cultures from two different areas of the neocortex – the lateral and medial part – as well as from the cerebellum of the same animal (Fig. 1F), and at different time points in culture (Fig. 1G).

Next, we examined cell death in the brain of these mice to verify the results that we obtained in primary neuronal cultures. Heterozygous animals are the same size and weight as wild-type mice and no differences are observed macroscopically (Li et al., 2002b). Careful analysis of the medial neocortex and the cerebellum revealed a higher number of TUNEL-positive neurons in heterozygotes compared with wild-type animals – cell death was again more pronounced in homozygous animals (Fig. 2, Table 1). Similar results were obtained when we compared active caspase-3 immunoreactivity in the respective brain areas (Table 1), suggesting apoptotic cell death of the neurons. We would like to emphasize that cell death in brain tissue was rare compared with that observed in neuronal cultures (apoptotic neurons were removed in vivo), and thus differences between heterozygous and wild-type animals might have been overlooked in previous studies. Analysis of brain sections confirmed our previous observation of neuronal death being present in animals with one deficient Pex11β allele and to a further extent in those with defects in both alleles.

Neuronal death does not correlate with peroxisome abundance

Pex11β has been implicated in peroxisome proliferation, and a reduced abundance of peroxisomes can be found in cultured mouse Pex11β−/− fibroblasts, as shown in our previous studies (Li et al., 2002b). No information, however, was given for alterations in heterozygous Pex11β mice. In brain sections from E19 fetuses, the abundance (number of peroxisomes/area) was reduced by 50% in the medial and lateral neocortex (Fig. 3A,D) and by 30% in the cerebellum of homozygous animals (Fig. 3B,F) compared with wild-type littermates. By contrast, no change and a slight increase of 15% was noted in the cerebellum and medial neocortex of heterozygous mouse, respectively (Fig. 3A,D). Semi-quantification of PEX14 immunoreactivity in heterozygous Pex11β mice revealed increased (Fig. 3C, neocortex) or unchanged (Fig. 3E, cerebellum) levels, whereas, in neurons from homozygous Pex11β mice, the PEX14 level was only slightly reduced (Fig. 3F). Similarly to as found in vivo, the abundance of peroxisomes in cultured neurons (95-98% of all cells in culture) and astrocytes (2-5% of all cells in culture) was reduced by half when prepared from Pex11β−/− mice compared with those from their heterozygous and wild-type littermates, as analyzed by PEX14 immunofluorescence (Fig. 4A,B) and western blot analysis (Fig. 4C). Thus, in cortical neurons, the abundance of peroxisomes was reduced in the same manner as the overall protein level of PEX14, suggesting no change in the size of the peroxisomes as found for the cortical tissue. A comparison of
Damage in \( \text{Pex11}^{\beta+/} \) and \( \text{Pex11}^{\beta-/-} \) brain

**Fig. 1.** Deletion of one allele of the \( \text{Pex11}^{\beta} \) gene caused an increase in neuronal death in primary cultures of the mouse neocortex and cerebellum. (A) Double fluorescence staining of cortical cultures from one litter, including all three \( \text{Pex11}^{\beta} \) genotypes, using the TUNEL assay (red; Aa,Ac,Ae) and Hoechst 33342 (blue; Ab,Ad,Af). Positive control cultures were treated with DNase (Ag,Ah). (B) Double fluorescence staining of neuronal cultures from \( \text{Pex11}^{\beta+/} \) and \( \text{Pex11}^{\beta-/-} \) mice using an antibody against the active form of caspase-3 (green) and the neuronal marker MAP2 (red) to characterize neuronal death. (C) Quantitative characterization of neuronal death using Hoechst 33342 combined with either propidium iodide (PI) or active caspase-3, or TUNEL staining. (D) Quantification of neuronal death using Hoechst 33342 in cortical cultures of four different litters showing the reproducibility of the results. (E) The broad-spectrum caspase-inhibitor z-VAD (100 \( \mu \text{M} \)) partially reduced neuronal death independent of the genotype. (F,G) Increased neuronal death was found in cultures derived from the lateral and medial neocortex and cerebellum (F) and as well as at different time points during cultivation (G) of heterozygous \( \text{Pex11}^{\beta} \) mice and – to a further extent – in those from homozygous mice. (C-G) Neuronal death was determined according to the nuclear morphology in three areas in each of four different cultures (800-1200 cells) for each genotype derived from the same (C,E-G) or from different (D) litters. Mean values ± s.d. are shown; (C-F) differences between the indicated groups: ***\( P<0.001 \); (G) differences in comparison to cultures from wild-type \( \text{Pex11}^{\beta} \) mice: ***\( P<0.001 \); difference between cultures from heterozygous and homozygous \( \text{Pex11}^{\beta} \) mice: #\( P<0.05 \).
the cell volume – both in primary neuronal cultures and in brain sections of the neocortex – revealed no difference between the three Pex11β genotypes. However, a detailed analysis of changes in the size and form of peroxisomes in Pex11β-deficient neurons requires electron microscopy and will be the subject of future studies. For the investigation of adaptive responses to the impaired ability of peroxisome division, we analyzed the expression of other PEX11 protein family members as well as of FIS-1 and DLP1 genes (Kobayashi et al., 2007). Interestingly, an increase in Pex11γ mRNA was noted in cultures from Pex11β–/–, but not in those from Pex11β+/–, mice (Fig. 4D, Table 2). We suggest that this increase could reflect an adaptive response to the peroxisome division defect. However, in comparison to the high expression level of Pex11β mRNA in the brain, the expression level of Pex11γ is 64-fold lower and the amount of protein is below detectable levels (Li et al., 2002a). Therefore, upregulation of Pex11γ might not adequately substitute for the Pex11β defect, but prevent the total absence of peroxisomes. In addition, we cannot exclude that the presence of peroxisomes in the Pex11β knockout mouse is due to a residual function of the exon-4-encoded PEX11β protein. The mRNA levels of Fis-1 and Dlp1, whose proteins mediate peroxisomal fission, were not affected (Fig. 4E).

Deletion of a single allele of the Pex11β gene causes a delay in neuronal differentiation

The neuronal marker protein microtubule-associated protein 2 (MAP2) is only expressed in neurons that have reached their final destination, and its level is progressively increased during the first postnatal week (Ahlemeyer et al., 2007; Ferreira et al., 1987). Thus, MAP2 was undetectable in the E19 brain, but we have analyzed the expression of synaptophysin, a marker for early synaptogenesis that is present at a sufficiently high level at birth (Li et al., 2010; Shimohama et al., 1998). In comparison to the neocortex and cerebellum of wild-type animals, synaptophysin immunoreactivity was reduced in heterozygous Pex11β animals and this decrease was even more pronounced in the homozygous ones (Fig. 5A,B). The delay in neural development can be reproduced in cortical neurons from Pex11β+/– and Pex11β–/– mice, demonstrated by the reduced

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<th>Genotype</th>
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<td>+/+</td>
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<td>–/–</td>
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+++ very high; +++, high; +, moderate; ø, weak.

Table 1. The abundance of TUNEL- and active-caspase-3-positive cells, and the signal intensity of synaptophysin, 8-OHdG, catalase and SOD2 immunoreactivity in the neocortex and cerebellum from wild-type, heterozygous and homozygous Pex11β mice
formation and branching of the neural network after 7 days in culture (Fig. 5C) and by reduced mRNA levels of synaptophysin (Fig. 5Da, Table 2) as well as by the reduced protein levels of MAP2 and synaptophysin (Fig. 5Db). Interestingly, the higher molecular weight form of MAP2 (MAP2a/b at 280 kDa), which normally increases in level in the postnatal period at day 10 (Przyborski and Cambray-Deakin, 1995), was not detectable in cultures from Pex11β+/– or Pex11β−/− mice, but was clearly present in the more mature wild-type cultures (Fig. 5Db). The level of the low molecular weight form of MAP2 (MAP2c), known to be expressed at early developmental stages but to decrease postnatally after 2 weeks (Crandall and Fischer, 1989), was not different between the genotypes. Moreover, MAP2a/b is largely located in dendrites and axons, whereas MAP2c is widely located in every neuronal compartment (Tucker et al., 1988).

Increased astrogliosis has been shown in adult CNP-Cre/Pex5loxP- and nestin-Cre/PEX13loxP-knockout mice (Kassmann et al., 2007; Müller et al., 2011). However, at early stages of neuronal development such as E19, glial fibrillary acidic protein (GFAP)-immunopositive reactions were only found in the membrana glia limitans and the subventricular zone (Ahlemeyer et al., 2007), and primary neuronal cultures contained only very few clustered astrocytes (less than 2%) with the same GFAP mRNA and protein level (Fig. 5E) independent of the Pex11β genotype.

Deletion of a single allele of the Pex11β gene causes oxidative stress
A deficiency in peroxisomal function has been suggested to cause oxidative stress (Baumgart et al., 2001; Bonekamp et al., 2009). In E19 sections of the mouse neocortex and cerebellum, an elevated 8-hydroxy-2'-deoxyguanosine (8-OHdG) immunoreactivity was found in heterozygotes with a further increase in homozygous animals (Fig. 6A,B, Table 1). Consistent findings were obtained by Dot blot analyses of 8-OHdG immunoreactivity in nuclear extracts from the neocortex of Pex11β−/− mice (Fig. 6C). With respect to antioxidant enzymes, we found a selective increase in manganese...
Superoxide dismutase (superoxide dismutase 2; SOD2) immunoreactivity in the neocortex and cerebellum of heterozygous Pex11β mice (Fig. 6D, Table 1), and a decrease in catalase immunoreactivity in the neocortex of homozygous Pex11β mice (Fig. 6E, Table 1). Primary neuronal cultures allow a direct measurement of the cellular reactive oxygen species (ROS) levels. Compared with neurons from wild-type mice, ROS levels in those from heterozygous and homozygous Pex11β animals were indeed increased at day 2, 4, 7, 10 and 14 in culture, in the latter ones to a higher extent (Fig. 7A,C,D). Data from four different litters at day 7 in culture is shown in Fig. 7E. We suggest oxidative stress as the mediator of cell death because tocopherol, when added 4 hours after seeding, not only reduced the increased ROS levels (Fig. 7C), but also reduced cell death (Fig. 7B) in cultured neurons from both heterozygous and homozygous Pex11β animals to the level seen in the wild-type controls. Similar to our in vivo findings, the mRNA and protein levels of SOD2 were selectively higher in cultures from heterozygotes and those of catalase were lower in cultures from homozygous mice (Fig. 7F,G,H, Table 2) when compared with those from wild-type littermates. Further studies are needed to find out why neurons from Pex11β knockout mice are compromised in upregulating antioxidative survival pathways.

**DISCUSSION**

Two aspects regarding PBDs can be drawn from our experiments using brain tissue and cultured neurons from E19 Pex11β-deficient mice: (1) this study provides direct evidence for oxidative stress as a result of Pex11β deficiency together with a defective regulation of the antioxidant response in the brain of Pex11β−/− compared with Pex11β+/− animals; and (2) the observation of a neural phenotype in Pex11β heterozygous mice, although with less severity than in the homozygous animals.

**Oxidative stress in Pex11β+/− and Pex11β−/− brains, and a defective regulation of the antioxidative response in Pex11β−/− brain**

To date, only limited data of the mechanism or role of oxidative stress in the neuropathology observed in ZS is available, although it is an observed phenomenon in other disorders of the neonatal human brain (Robertson et al., 2009; Taylor et al., 1999). Previous morphological studies in general Pex5 knockout mice revealed ultra-structural alterations of mitochondria, indicative of oxidative stress, together with reduced activities of mitochondrial respiratory chain complexes and an increase in SOD2 in cardiomyocytes and hepatocytes, but the brain was not examined (Baumgart et al., 2001). Similar mitochondrial alterations have been observed in the liver of mice lacking Pex13 (Maxwell et al., 2003) and Pex2 (Keane et al., 2007) as well as in patients with Hsd17b4 gene mutations (Ferdinandusse et al., 2003). However, hepatocyte-specific Pex5
knockout mice do not show oxidative damage of proteins or lipids (Dirks et al., 2005). By contrast, but similar to our findings, ABCD3 knockdown in primary fibroblast cultures was found to increase the production of superoxide in combination with an upregulation of SOD2 and a decrease in catalase levels (Di Benedetto et al., 2009). Oxidative stress has also been discussed as a pathogenic factor in X-linked adrenoleukodystrophy (X-ALD), because patients exhibit increased levels of very long chain fatty acids and develop inflammatory demyelination and neurodegeneration similar to that found in patients with PBDs (Berger and Gärtner, 2006). In addition, we know from our mouse models that the animals carrying just one defective Pex11β allele is sufficient to induce a neural phenotype.

Deletion of a single allele of the Pex11β gene is sufficient to induce a neural phenotype

Mutations in PEX genes in human PBDs and their respective knockout mouse models are inherited in an autosomal recessive manner. However, we here describe a phenotype in the brain of animals carrying just one defective Pex11β allele. The severity of the symptoms is less in Pex11β heterozygous than in homozygous mice. This is of relevance: (1) for mouse models and in individuals with PBDs (although the loss of one Pex11β allele does not necessarily follow the same pattern as the other PBD models); (2) for mouse models of recessive diseases in general, because sometimes heterozygous animals were used as controls owing to the limited availability of wild-type animals in a litter and were compared with the knockout ones; and (3) because our data might contribute to the discussion about heterozygous advantages and disadvantages.

As mentioned in the Introduction, the genotype-phenotype relationship and especially the severity of symptoms in human patients suffering from PBDs is very complex, whereas all PBD mouse models exhibit severe symptoms with varying biochemical parameters. The discrepancy between mice and humans might be because, in mice, one [Pex2 (Faust and Hatten, 1997) and Pex7 (Brites et al., 2003)] to three [Pex5 (Baes et al., 1997), Pex11β (Li et al., 2002b) and Pex13 (Maxwell et al., 2003)] exons of the respective genes were deleted, whereas humans with PBDs often exhibit only a single point mutation in a PEX gene, with severe or less severe consequences for the function of the corresponding protein. In addition, we know from our mouse models that the severity of the symptoms depends on the genetic background of the animals. For example, Pex mutations generated in C57Bl/6J mice lead to a severe outcome and the mutant mice die directly

Table 2. The sequence and efficiency coefficient of forward and reverse primers and a comparative analysis of the mRNA expression of defined genes after quantitative RT-PCR

| Target gene | Accession number | Sequence (3' to 5') | E   | Fold change | Pex11α
dhet/wt | Pex11β
dhet/wt | Pex11γ
dhet/wt | Pex14
dhet/wt | Sod1
dhet/wt | Sod2
dhet/wt | Catalase
(Cat)
dhet/wt | Mtap2
dhet/wt | Syp
dhet/wt | Gapdh
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<td>NM_011068.1</td>
<td>F: ACTGCGCGTAAATGGTTTGCAGA</td>
<td>1.90</td>
<td>1.28±0.21</td>
<td>n.s.</td>
<td>0.95±0.19 n.s.</td>
<td>0.95±0.15 n.s.</td>
<td>1.51±0.31 n.s.</td>
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<td>F: CGCCTATTTGATGAAACAAGAAGCT</td>
<td>1.98</td>
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<td>Pex11γ</td>
<td>NM_026951.2</td>
<td>F: GACTCTGTCCTGGTGGGGA</td>
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<td>1.02±0.14 n.s.</td>
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<td>0.86±0.17</td>
<td>n.s.</td>
<td>0.83±0.14 n.s.</td>
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<td>1.77±0.36</td>
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<td>F: GGAGGGGGAACCACTACAG</td>
<td>1.91</td>
<td>0.93±0.21</td>
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<td>0.66±0.06 *</td>
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<td>1.16±0.22</td>
<td>n.s.</td>
<td>0.96±0.14 n.s.</td>
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<td>R: AAGCTCTGGGGAGAGGAGACAG</td>
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<td>n.s.</td>
<td>0.75±0.06 *</td>
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<td>R: AGCCTGCTTCCCTGAAACACG</td>
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<td>ufn</td>
<td>ufn</td>
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</table>
after birth (Baes et al., 1997; Brites et al., 2003; Faust and Hatten, 1997; Li et al., 2002a; Li et al., 2002b; Maxwell et al., 2003), but the survival could be prolonged up to postnatal day 18 (P18) when the mutation was placed on a Swiss Webster genetic background (Faust, 2003). In X-ALD, a peroxisomal single-enzyme defect, the opposite was observed: all three Abcd1 knockout mouse models (Forss-Petter et al., 1997; Kobayashi et al., 1997; Lu et al., 1997) did not develop a detectable phenotype up to 6 months, in contrast to humans with X-ALD, who show severe symptoms in the adrenal gland and brain. However, older X-ALD mice (starting from 15 months of age) exhibited slower nerve conduction as well as myelin and axonal abnormalities in the spinal cord and the sciatic nerve (Dumser et al., 2007; Pujol et al., 2002). Interestingly, X-ALD is inherited in an X-linked manner and thus the severity of the symptoms is sex dependent. Males who inherit the mutation will be variably affected with an unpredictable phenotypic expression and prognosis. Females who inherit the mutation are carriers, but also 50% are usually mildly affected (Berger and Gärtner, 2006). Thus, even when both alleles of genes are mutated, the severity of symptoms can vary widely depending on the type of mutation, the individual’s genetic background, sex and the species.

Interestingly, we previously observed a similar phenomenon of a heterozygous phenotype in cerebellar cultures prepared from Pex13 knockout mice (Müller et al., 2011). However, this observation was not analyzed in more detail. It is likely that the brain is especially sensitive to damaging conditions, and one may ask whether heterozygous Pex11β mice might also show defects in other organ systems. In general, heterozygous Pex11β animals are indistinguishable from and are the same size and weight as their wild-type littermates, and are fertile (Li et al., 2002b). We did not see obvious histopathological alterations or defects in the lung (development of the alveolar compartment) and bone (desmal and enchondral ossification), but the mRNA and protein levels of a number of genes were different in heterozygous compared with wild-type animals (e.g. peroxins, peroxisomal membrane
matrix proteins, antioxidant enzymes, and transcription factors; data not shown). Thus, even though there was no obvious phenotype, heterozygotes are different and not comparable to wild-type animals, at least on the molecular level.

Next, we investigated whether there are other examples in the literature describing alterations in the brain of heterozygous animals or in patients with autosomal recessive diseases. In the early-onset autosomal recessive form of Parkinson’s disease (PD), PARK2 is mutated. Sun et al. showed that individuals with homozygous, but also heterozygous, mutated PARK2 showed symptoms that differed in the onset as well as in severity compared with healthy PD individuals with an intact PARK2 gene (Sun et al., 2006). Similarly, clinical studies on PINK1 gene mutations, which predispose individuals to PD, showed that, although none of the young heterozygous affected family members were aware of symptoms, they already exhibited unilaterally reduced or absent arm movement or rigidity (Hedrich et al., 2006; van Nuenen et al., 2009). For amyotrophic lateral sclerosis, the D90A...
Damage in Pex11β+/– and Pex11β–/– brain

Fig. 7. Deletion of one allele of the Pex11β gene caused oxidative stress and changes in the level of antioxidant enzymes in primary neuronal cultures from the neocortex. (A–D) Representative micrographs of ethidine fluorescence in neurons from Pex11β+/+, Pex11β+/– and Pex11β–/– mice in the absence (Aa,Ab,Ac) or presence (Ad) of 10 μM α-tocopherol (toco). (B–E) Quantification of the mean fluorescence intensity (MFI) of ethidine in single cells in a cortical culture from all three genotypes from four distinct litters (E), at different time points during the cultivation (D) as well as in one litter in the absence or presence of tocopherol (C) together with evaluation of the cell death (B) were shown. For C–E, 200–350 cells were analyzed in ten areas in each of three cultures for each genotype derived from the same litter. (F) Representative micrographs showing a selective increase in SOD2 immunoreactivity in neurons from Pex11β+/– mice (Fb) compared with those from Pex11β+/+ (Fa) and Pex11β–/– (Fc) mice. (G,H) RT-PCR (G) and western blot analysis (H) of different antioxidant enzymes, showing increased mRNA (G) and protein (Ha) levels of SOD2 in neurons from Pex11β–/– mice and a decreased protein level of catalase in neurons from Pex11β–/– mice. For Ha, neurons were treated with vehicle (vh) or tocopherol (toco). Semi-quantification of catalase and SOD2 protein levels normalized to α-tubulin in vehicle-treated neuronal cultures from Pex11β+/+, Pex11β+/– and Pex11β–/– mice of four different litters is given in Hb. (B–E,Hb) Mean values ± s.d. are shown. Differences either between the indicated groups or in comparison to cultures from wild-type Pex11β mice: *P<0.05, **P<0.01, ***P<0.001 (B,C,E,Hb); difference between cultures from heterozygous and homozygous Pex11β mice: ###P<0.001 (D).
mutation of the copper-zinc superoxide dismutase (superoxide dismutase 1; SOD1) gene is inherited in an autosomal recessive manner, but slight motor symptoms have also been found in heterozygous relatives (Mezei et al., 1999). In ataxia telangiectasia, a typical autosomal recessive disease, heterozygous individuals have an increased radiosensitivity and risk of cancer as well as changes in the baseline expression of many genes compared with normal control individuals (Watts et al., 2002). Thus, one might speculate that the less severe symptoms in heterozygotes are often overlooked and that these patients were incorrectly considered as asymptomatic. Hedrich et al. hypothesized that re-evaluation of the role of single gene mutations might have a major implication for gene counseling. However, also the opposite might be possible: the existence of the so-called ‘heterozygote advantage’. In this theory, the heterozygote phenotype was supposed to have a higher fitness because this selection should be one of the mechanisms to maintain polymorphism in evolution (Gillespie, 2004; Otto and Yong, 2002). As examples for the heterozygote advantage, one can mention sickle cell anemia, in which the carriers are resistant to malaria infection (Williams, 2006), as well as hemochromatosis, in which the low iron content renders macrophages resistant to pathogens (Weinberg, 2008), or cystic fibrosis in which model mice are resistant to cholera (Gabriel et al., 1994). However, for the latter disease, the results could not be reproduced in humans. Another phenomenon is so-called over-dominance, in which the phenotype of the heterozygote lies outside the range of homozygous individuals (Gillespie, 2004).

In summary, our results indicate that, when analyzing knockout mice as models for autosomal recessive diseases, all three genotypes (at best from one litter) should be compared with each other in order to fully understand the pathophysiology of the gene defect. Heterozygous animals – even when they seem to be asymptomatic – might differ in the expression of many genes and the investigation thereof could help to understand (and to mimic) endogenous adaptation to gene defects.

METHODS

Chemicals and reagents

Neurobasal medium, B27 supplement, glutamine, penicillin-streptomycin, (di)hydroethidium, DNase I (Cat. 180668-015), SuperScript II First-Strand Synthesis System (Cat. 1806-022), RNaseOut (Cat. 10777-019) and TOTO-3 iodide were purchased from Invitrogen (Karlsruhe, Germany). Poly-L-lysine, papain, trypsin inhibitor, dimethylsulfoxide (DMSO), Hoechst 33342, Triton X-100, Tween 20, bovine serum albumin (BSA), propidium iodide, (±)-α-tocopherol, Ponceau S and z-Val-Ala-Asp fluoromethyl ketone (z-VAD.fmk) were obtained from Sigma-Aldrich (Deisenhofen, Germany). The ApoTag Red In Situ Detection Apoptosis kit (S7165) was purchased from Chemicon (Schwalbach, Germany). Details on all primary antibodies used in this study, containing immunogen, host and source are given in Table 3. The following secondary antibodies were used for indirect immunofluorescence: goat anti-rabbit IgG Alexa Fluor 488 (1:300; Invitrogen), goat anti-chicken Alexa Fluor 633 (1:300; Invitrogen) and goat anti-mouse IgG Texas Red (1:100; Vector Laboratories, Burlingham, CA), and for western blot analysis: goat anti-rabbit and goat anti-mouse IgG coupled to alkaline phosphatase (1: 20,000; Sigma-Aldrich).

Animals

Animals had free access to food and water and were kept under standardized environmental conditions (12-hour light-dark cycle, 23±1°C and 55±1% relative humidity). Experiments with laboratory mice were approved by the Government Commission of Animal Care, Germany. For all experiments, heterozygous Pex11βC57BL/6j mice (genetic background >F8 generation) were mated overnight. In some series of experiments all E19 fetuses from one litter were perfused with paraformaldehyde to obtain tissue sections, or the medial neocortices were homogenized to obtain nuclear extracts. In other series of experiments, all E19 fetuses were used for the preparation of primary neocortical and cerebellar cultures.

Genotyping of mouse tail DNA

Mouse tails were digested overnight and 0.2 μg of the isolated DNA was used for PCR analysis the following day as previously described (Li et al., 2002b). The following primers were used for the genotyping: primer 8: 5’-GTCTAGGACAGGTTCTGTGTTC-3’, primer 9: 5’- GTTTCCCCATCTTTCCCTTGA-3’ and neo primer 5’- ATATTGCTAAGAGCTTGGCGCGGCG-3’. The wild-type Pex11β allele was amplified using primers 8 and 9 (590 bp), whereas the recombinant knockout allele was amplified by primer 8 and neo primer (980 bp). The PCR reaction was performed using TaqDNA polymerase (Eppendorf, Hamburg, Germany) in a Bio-Rad iCycler (Bio-Rad, München, Germany) using the following parameters: denaturation at 95°C, 5 minutes; followed by 35 cycles of denaturation at 95°C, 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute; and a final extension at 72°C for 10 minutes.

Perfusion fixation of mice and processing of brain tissue

Pregnant dames of heterozygous Pex11βC57BL/6j mouse matings including all E19 fetuses were anesthetized with isoflurane and subsequent intraperitoneal injection of a mixture of ketamine/xylazine. After a short rinse with 0.9% NaCl to remove blood cells, perfusion fixation was carried out for 5 minutes via the left ventricle of the heart using freshly prepared 4% depolymerized paraformaldehyde in Ca2+- and Mg2+-free phosphate-buffered saline (PBS). Whole brains were carefully dissected out of the skull and additionally immersion fixed overnight in the same fixative. The following morning, the complete brains were transferred to an automated vacuum infiltration tissue processor (Leica TP 1020) and processed for paraffin embedding. The following dehydration and infiltration steps were used: (1) 70% ethanol, 80% ethanol, 90% ethanol, 4× 100% ethanol, 4× xylene, each step 1.5 hours, and (2) 2× paraffin (Paraplast plus containing 0.8% DMSO), each step 2 hours. After vertical embedding, 2-μm paraffin sections of total brain (coronal orientation) were cut on a Leica RM 2135 microtome and mounted on Superfrost Plus slides. We examined brain sections containing the medial neocortex (comparable to Bregma 0.98 mm of the adult mouse) and cerebellum (comparable to Bregma –5.8 mm of the adult mouse) of each genotype from the same litter.

Indirect immunofluorescence and TUNEL stain on paraffin-embedded brain sections

For optimal retrieval of peroxisomal antigens and accessibility of epitopes, deparaffinized and rehydrated brain sections were subjected to digestion with 0.01% trypsin for 10 minutes at 37°C.
followed by microwaving in 10 mM citrate buffer at pH 6.0 for 3 × 5 minutes at 800 W in a conventional household microwave oven (Baumgart et al., 2003; Grabenbauer et al., 2001). Nonspecific binding sites were blocked with 4% BSA and 0.05% Tween 20 in PBS and thereafter incubated with the secondary antibodies for 2 hours at room temperature and sections were incubated with primary antibodies (for details see Table 3) overnight at 4°C. On the following morning, the sections were rinsed carefully with PBS and the secondary antibodies were removed by Cesarean section from the uterus of the pregnant dame previously (Ahlemeyer and Baumgart-Vogt, 2005). We dissected and processed the cortices or cerebellum of individual mice separately and in parallel, because immediate preparation prior to PCR genotyping was necessary for obtaining optimal neuronal cultures. Cortical and cerebellar neurons were seeded at a density of 3 × 10^5 cells and 2 × 10^6 cells onto 35-mm poly-L-lysine-coated Petri dishes, respectively. In one series of experiments, neuronal death and ROS level were determined at different time points during culture. All other experiments were performed on day 7 in culture. At that time, primary neocortical and cerebellar cultures contained approximately 95-98% and 90% neurons, respectively. The low amount of astrocytes is mainly due to neuronal growth-promoting culture conditions such as poly-L-lysine coating and the use of neurobasal medium as well as to the fact that cultures were prepared from E19 fetuses. Drug treatment and some techniques were applied solely to primary neuronal cultures either because some of these techniques need living cells (ROS detection, propidium iodide stain) or because of the advantage to detect changes in neurons only [reverse transcriptase (RT)-PCR, western blot analysis], whereas tissue homogenates contain all other cell types, including blood vessels, meninges and matrix proteins.

Preparation of primary cultures of the medial neocortex and cerebellum of E19 mice

The fetuses of a heterozygous Pex11β C57BL/6j mating were removed by Cesarean section from the uterus of the pregnant dam at E19. Primary cultures were prepared from the medial neocortices or the cerebellum of all fetuses from the same litter as described previously (Ahlemeyer and Baumgart-Vogt, 2005). We dissected and processed the cortices or cerebellum of individual mice separately and in parallel, because immediate preparation prior to PCR genotyping was necessary for obtaining optimal neuronal cultures. Cortical and cerebellar neurons were seeded at a density of 3 × 10^5 cells and 2 × 10^6 cells onto 35-mm poly-L-lysine-coated Petri dishes, respectively. In one series of experiments, neuronal death and ROS level were determined at different time points during culture. All other experiments were performed on day 7 in culture. At that time, primary neocortical and cerebellar cultures contained approximately 95-98% and 90% neurons, respectively. The low amount of astrocytes is mainly due to neuronal growth-promoting culture conditions such as poly-L-lysine coating and the use of neurobasal medium as well as to the fact that cultures were prepared from E19 fetuses. Drug treatment and some techniques were applied solely to primary neuronal cultures either because some of these techniques need living cells (ROS detection, propidium iodide stain) or because of the advantage to detect changes in neurons only [reverse transcriptase (RT)-PCR, western blot analysis], whereas tissue homogenates contain all other cell types, including blood vessels, meninges and matrix proteins.

Indirect immunofluorescence on primary neuronal cultures

Cells on coverslips were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. After fixation, cells were washed three times with PBS. Subsequently, they were incubated for 10 minutes in PBS containing 1% glycine and 0.3% Triton X-100 for permeabilization, and for an additional period of 10 minutes in PBS containing 1% glycine. After washing with PBS, cells were incubated for 30 minutes in PBS containing 1% BSA and 0.05% Tween 20 for blocking of nonspecific protein-binding

Table 3. Primary antibodies used in this study

<table>
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<tr>
<th>Target</th>
<th>Source; catalog number</th>
<th>Dilution WB</th>
<th>Dilution IC, brain sections</th>
<th>Dilution IC, neuronal cultures</th>
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<td>Sigma-Aldrich, Deisenhofen, Germany; T5168</td>
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<td>1:1000</td>
<td>1:2000</td>
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<td>GFAP</td>
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<td>1:1000</td>
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<td>1:400</td>
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WB, western blotting; IC, immunofluorescence staining; Ms, mouse; Rb, rabbit; Ch, chicken.
sites. Indirect immunofluorescence was performed as previously described (Ahlemeyer et al., 2007) by one or two subsequent incubations with different sets of primary antibodies plus secondary antibody incubations separated by extensive washing steps (for details see Table 3). Images of immunofluorescence preparations were taken with a confocal laser scanning microscope (Leica TCS SP2, Leica, Bensheim, Germany). The number of PEX14-positive peroxisomes/100 μm² was determined as described above in four areas in each of the six different Petri dishes in each of the three Pex11β genotypes. Values are expressed as number of peroxisomes/100 μm².

Characterization of neuronal damage
Neuronal damage was characterized by four different approaches using either Hoechst 33342 (nuclear stain), the membrane-impermeable dye propidium iodide (to detect membrane damage), an antibody against the active (cleaved) form of caspase-3 or the TUNEL assay. In three different sets of experiments, cells were stained with either propidium iodide or anti-active-caspase-3 antibodies or TUNEL combined with Hoechst 33342 (Fig. 1A-K). The numbers of propidium-iodide-, active-caspase-3- or TUNEL-positive cells were counted in relation to the number of neurons with apoptotic nuclear morphology as detected by Hoechst 33342 in the same areas within the same cultures. In all experiments, three distinct areas in each of the four different Petri dishes (corresponding to 800-1200 cells) were determined for each Pex11β genotype all derived from the same litter. Positive and negative controls for TUNEL staining were performed as described above for the brain sections. Images were taken with a Leica DM RD fluorescence microscope equipped with a Leica DC480 camera (Leica, Bensheim, Germany). Data are presented as percentage of neuronal death (cells with an apoptotic nuclear morphology or propidium-iodide-, activated-caspase-3- or TUNEL-positive cells).

Drug treatment
To get further insight into the pathomechanism of neuronal death in peroxisome deficiency, cortical cultures were treated with the antioxidant α-tocopherol (10 μM) or the broad spectrum caspase inhibitor z-VAD.fmk (100 μM), which were both added 4 hours after seeding to half of the Petri dishes prepared from one animal. All drugs remained continuously in the culture medium up to day 7. Control cultures received vehicle only.

RT-PCR analyses
Total RNA from cultured cortical neurons of distinct Pex11β genotypes was prepared using the RNAeasy Mini kit (Qiagen, Hilden, Germany). cDNA was synthesized from DNase-I-treated total RNA using the SuperScript II First-Strand Synthesis System plus RNaseOut. For semi-quantitative analysis, specific primers for each gene were designed using the PRIMER3 program (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and synthesized by Eurofins/MWG/Operon (Ebersberg, Germany; Table 4). PCR reaction was performed containing 50 ng cDNA, 100 nmol forward and reverse primers and 5PRIME TaqDNA polymerase in a final volume of 25 μl 5PRIME Master Mix (5PRIME, Hamburg, Germany), with the following parameters: denaturation at 95°C for 2 minutes; followed by 32-45 cycles of denaturation at 95°C for 30

Table 4. Primers used in this study for semiquantitative RT-PCR

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<th>Cyc</th>
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F, forward primer; R, reverse primer; °C, annealing temperature; Cyc, number of cycles.
Clinical issue
Peroxisomal biogenesis disorders (PBDs) are inherited in an autosomal recessive manner and can be caused by mutations in PEX genes, of which there are at least 12 in humans. PEX genes encode peroxin proteins, which are required for the normal biogenesis or maintenance of peroxisomes (intracellular organelles with a range of essential biochemical functions). PBDs are characterized by disturbances in developmental and metabolic homeostasis, predominantly affecting the liver, kidney and brain. The clinical phenotype varies widely; Zellweger syndrome is the most severe form (survival rate is less than 1 year), followed by neonatal adrenoleukodystrophy and a milder form called infantile Refsum's disease, where patients survive into the second decade. Obtaining further insight into the molecular pathogenesis and pathologies of these devastating disorders is of high interest.

Results
This study addresses the effects in mice of homozygous versus heterozygous deletion of Pex11β, a gene previously implicated in the regulation of peroxosomal proliferation. In their analyses of primary neuronal cultures and of brain samples from Pex11β+/−, Pex11β−/− and Pex11β−/+ mice, the authors find that the proportion of cell death in homozygous mice is higher than in wild-type mice, but less than in homozygous knockout mice. Moreover, the extent of cell death correlates with a decrease in peroxisome number in homozygotes, but not in heterozygotes. Heterozygotes also show delayed neuronal differentiation and increased levels of oxidative stress, which are both, however, more pronounced in homozygotes. Importantly, the authors carefully analyze brain alterations not only in homozygous and wild-type mice, but also in heterozygous animals, in which a phenotype has not previously been reported.

Implications and future directions
The finding that loss of function of a single Pex11β allele can cause neurological symptoms in mice might have important implications for the diagnosis and future research of PBDs – as well as other recessive disorders – in humans. In addition, these new data on the role of oxidative stress in the pathology of PBDs might help to develop new antioxidant-based strategies for treating the disorders in humans.

Determination of the cellular ROS level
The cell-permeable probe (di)hydroethidine is preferentially oxidized by superoxide to its fluorescent product, ethidine. Ethidine is retained intracellularly, thus allowing quantitative estimations of the cellular ROS level (Bindokas et al., 1996; Ahlemeyer et al., 2001). Dihydroethidine was prepared as a stock of 5 mM in DMSO and was added to the culture medium to a final concentration of 5 μM. After incubation for 20 minutes, cortical neurons from distinct Pex11β genotypes, grown on coverslips, were washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes. The coverslips were mounted on slides for measuring cellular ethidine fluorescence under a confocal laser scanning microscope (Leica TCS SP2, Leica, Germany). Images were taken with a 40× fluorescence objective (HCxPL Apo CS 40× 1.25 Oil) using the 519 nm laser line (argon/krypton laser) under fixed settings with respect to laser energy, signal detection (gain, offset, pinhole size) for each series of experiments. Ten regions in each of the three different Petri dishes for each genotype (corresponding to 2500-3000 cells) were chosen, scanned in the single scan mode and the fluorescent images were saved and digitalized in a 512×512 pixel format. Ethidine fluorescence was quantified individually in all cells using the Leica Confocal software program (Leica, Bensheim, Germany). The values are expressed as mean fluorescence intensity (MFI) of ethidine per cell.

Western blot analysis
Neuronal cultures from different Pex11β genotypes (3×10^5 cells) were homogenized in 50 μl of a homogenization buffer containing 50 mM MOPS, pH 7.4, 250 mM sucrose, 1 mM EDTA, 0.1% ethanol (v/v) and 10% protease inhibitor mix M (Serva, Heidelberg, Germany) using a 2 ml Potter-Elvehjem homogenizer. The homogenate was centrifuged at 400 g for 10 minutes at 4°C to remove nuclei and non-homogenized cell debris. The protein amounts of the nuclear fraction and supernatants were determined according to Bradford (Bradford, 1976) using BSA as standard. Protein samples (4-8 μg) were separated on 12-15% SDS-polyacrylamide gels (depending on the expected band size of the antigen to be detected) and transferred onto a polyvinylidene difluoride membrane by semi-dry blotting (Transcell Blot SD, BioRad, München, Germany). In one series of experiments (Fig. 6C), nuclear extracts were spotted onto the membranes for the preparation of a Dot blot to semi-quantify 8-OHdG immunoreactivity. Nonspecific protein binding on the membranes was blocked using Tris-buffered saline with 0.05% Tween 20 (TBST) and 10% non-fat dry milk (blocking buffer). The blots were incubated for 1 hour at room temperature with the primary antibodies in TBST with 5% non-fat dry milk, washed intensively and incubated for 1 hour at room temperature with the secondary antibodies in TBST (for details see Table 3). Alkaline phosphatase activity was detected using Immun-Star AP substrate from BioRad (München, Germany). The blots were exposed to Kodak Bio-Max MR-1 films (Sigma-Aldrich, Deisenhofen, Germany), scanned and the integrated optical densities of the signals were analyzed semiquantitatively using QuantityOne (BioRad, München, Germany). We calculated the ratio of the integrated optical densities of each tested protein versus tubulin. Data are given as relative percentage of a distinct protein in comparison to that of the wild-type cultures, which was set to 100%.
Statistical analysis
In some series of experiments, data were derived from three or
four litters (Fig. 1D, Fig. 4B, Fig. 7E,Hb, Table 2) and statistical
differences between the genotypes were evaluated by ANOVA-1
followed by post-hoc Scheffé test. Data from four litters were further
analyzed by Levene’s test followed by general linear model analysis
(univariat mode) and Scheffé, Tukey and Sidak multicomparison
post-hoc analysis showing that data evaluation is independent of
the litter. In addition, the normality of the values was ensured by a
Shapiro-Wilk test and we excluded residual influencing parameters by a Q-Q-Plot analysis. Therefore, when data were derived from several culture dishes and/or areas, but from the same litter, the univariat (Fig. 1C,F, Fig. 3C-F, Fig. 4B) or multivariat (Fig. 1G, Fig. 7D) mode of the general linear model for repeated experiments followed by post-hoc Scheffé test was used to evaluate statistical differences between the genotypes. In case of a drug treatment (Fig. 1E, Fig. 7B,C), differences between non-treated and treated groups were analyzed by nonparametrical test for multiple related samples followed by Friedman test.

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COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS
E.B.-V. and B.A. conceived and designed the experiments. E.B.-V. provided advice, gave suggestions and financial support. M.G. performed tissue sectioning and immunofluorescence staining on brain sections, B.A. performed all other experiments and data analysis. B.A. and E.B.-V. wrote the paper.

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