Neurobiology of Disease 58 (2013) 258–269

Contents lists available at SciVerse ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

# Peroxisomal multifunctional protein-2 deficiency causes neuroinflammation and degeneration of Purkinje cells independent of very long chain fatty acid accumulation

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ARTICLE INFO

*Article history:*
Received 26 March 2013
Revised 27 May 2013
Accepted 7 June 2013
Available online 15 June 2013

*Keywords:*
X-linked adrenoleukodystrophy
D-bifunctional protein deficiency
Peroxisomal β-oxidation
Multifunctional protein-2
HSD17B4
Mouse model
Cerebellum
Purkinje cell
Oligodendrocyte
Neuroinflammation
Microglia

ABSTRACT

Although peroxisome biogenesis and β-oxidation disorders are well known for their neurodevelopmental defects, patients with these disorders are increasingly diagnosed with neurodegenerative pathologies. In order to investigate the cellular mechanisms of neurodegeneration in these patients, we developed a mouse model lacking multifunctional protein 2 (MFP2, also called D-bifunctional protein), a central enzyme of peroxisomal β-oxidation, in all neural cells (*Nestin-Mfp2*$^{-/-}$) or in oligodendrocytes (*Cnp-Mfp2*$^{-/-}$) and compared these models with an already established general *Mfp2* knockout. *Nestin-Mfp2* but not *Cnp-Mfp2* knockout mice develop motor disabilities and ataxia, similar to the general mutant. Deterioration of motor performance correlates with the demise of Purkinje cell axons in the cerebellum, which precedes loss of Purkinje cells and cerebellar atrophy. This closely mimics spinocerebellar ataxias of patients affected with mild peroxisome β-oxidation disorders. However, general knockouts have a much shorter life span than *Nestin-Mfp2* knockouts which is paralleled by a disparity in activation of the innate immune system. Whereas in general mutants a strong and chronic proinflammatory reaction proceeds throughout the brain, elimination of MFP2 from neural cells results in minor neuroinflammation. Neither the extent of the inflammatory reaction nor the cerebellar degeneration could be correlated with levels of very long chain fatty acids, substrates of peroxisomal β-oxidation. In conclusion, MFP2 has multiple tasks in the adult brain, including the maintenance of Purkinje cells and the prevention of neuroinflammation but this is not mediated by its activity in oligodendrocytes nor by its role in very long chain fatty acid degradation.



*Abbreviations:* AMN, adrenomyeloneuropathy; cALD, cerebral adrenoleukodystrophy; CNS, central nervous system; DBP, D-bifunctional protein (also called MFP2 and HSD17B4); H&E, hematoxylin & eosin; HSD17B4, 17β-hydroxysteroid dehydrogenase type 4 (also called DBP or MFP2); MFP2, Multifunctional protein-2; PBD, peroxisome biogenesis disorders; VLCFA, very long chain fatty acids; X-ALD, X-linked adrenoleukodystrophy.

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**Available online on ScienceDirect (www.sciencedirect.com).**

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http://dx.doi.org/10.1016/j.nbd.2013.06.006

## Introduction

Inherited defects of peroxisomal metabolism cause diverse neurological dysfunctions, ranging from developmental anomalies such as cortical neuronal migration defects to degenerative pathology in the brain and/or spinal cord (Baes and Aubourg, 2009; Powers, 1995; Powers and Moser, 1998). These disease phenotypes also occur in patients with a defect in peroxisomal β-oxidation illustrating that this pathway is one of the key metabolic functions of this organelle. Such dysfunction can be due to mutations either in β-oxidation enzymes or in transporters that shuttle the substrates through the membrane.

MFP2, also called D-bifunctional protein (DBP) and HSD17B4, is a central enzyme of peroxisomal β-oxidation that is necessary for the degradation of very long chain fatty acids (VLCFA), the branched chain fatty acid, pristanic acid, eicosanoids and bile acid intermediates (Huyghe et al., 2006a; Van Veldhoven, 2010). Furthermore, MFP2 participates in the synthesis of docosahexaenoic acid, the most abundant polyunsaturated fatty acid (PUFA) in the brain. The largest cohort of patients with MFP2 deficiency presents with neonatal hypotonia, seizures, psychomotor delays and die before the age of 2 years. Recently, patients were identified with milder mutations resulting in residual enzyme activity (Khan et al., 2010; McMillan et al., 2012; Pierce et al., 2010; Soorani-Lunsing et al., 2005). Their diagnosis is complicated by near-normal values of peroxisomal metabolites such as VLCFA, pristanic acid and bile acid intermediates in serum. They display various symptoms such as polyneuropathy, ataxia, sensorineural deafness and developmental regression (Khan et al., 2010; Pierce et al., 2010; Soorani-Lunsing et al., 2005). X-linked adrenoleukodystrophy, a more common peroxisomal β-oxidation disorder, is caused by a defect in the *ABCD1* gene, which encodes an ATP binding cassette transporter, necessary for the import of VLCFA (Ferrer et al., 2010; van Roermund et al., 2008). In its most aggressive form, boys develop a rapidly progressive fatal inflammatory demyelination of the brain during childhood. Alternatively, males and female heterozygotes can acquire adrenomyeloneuropathy (AMN) a noninflammatory distal axonopathy of the spinal cord. Despite an increased understanding of the genetics of peroxisomal β-oxidation disorders, the molecular mechanistic links between impaired peroxisomal β-oxidation and neurodegeneration are unresolved. To obtain more insight into the molecular mechanisms, several mouse models with peroxisomal β-oxidation defects have been generated. *Abcd1*$^{-/-}$ mice develop a late-onset distal axonopathy in the spinal cord without cerebral neuroinflammation (onset > 15 months) representing the AMN form of X-ALD (Pujol et al., 2002). Accumulation of VLCFA has been implicated as the pathogenic inducer, causing oxidative stress and alterations in energy homeostasis (Fourcade et al., 2008; Galino et al., 2011). However, it remains unclear why the brain is spared from degenerative pathologies. On the other hand, knockout mice lacking MFP2 do develop neuroinflammation in both the brain and spinal cord. These mice present with postnatal growth retardation, early-onset motor problems, severely reduced fertility and die before the age of 6 months (Baes et al., 2000; Huyghe et al., 2006b). They accumulate VLCFA in the brain and bile acid intermediates in the liver, bile and plasma (Baes et al., 2000; Ferdinandusse et al., 2005). At the time of death, massive astrogliosis and microgliosis are present (Huyghe et al., 2006b).

In order to decipher the cellular origin of pathologies in general *Mfp2* knockouts and to gain insight into the importance of peroxisomal β-oxidation for the maintenance of the CNS we generated mice with a floxed *Mfp2* gene. *Mfp2-loxP* mice were crossed with *Nestin-Cre* and *Cnp-Cre* mice, to obtain mice with selective elimination of MFP2 from all neural cells or from oligodendrocytes, respectively. We evaluated their macroscopic, microscopic and metabolic features and compared them with general *Mfp2* knockouts.

## Materials & methods

### *Strategy and construction of targeting vector*

MFP2 is also known as D-bifunctional protein and is encoded by the 17β-hydroxysteroid dehydrogenase 4 gene (*Hsd17b4*) but for reasons of clarity, both the protein and gene will be denoted as 'MFP2'. Three genomic DNA fragments corresponding to the left arm (4.86 kb), the right arm (0.86 kb), and the targeted region containing exon 8 (0.57 kb) of the mouse *Mfp2* gene were amplified by PCR from a BAC clone [RP24-94D24 (129S6/SvEvTac)] obtained from Children's Hospital Oakland Research Institute, Oakland, CA. After subcloning in pGEM-T Easy and verification of exons by sequencing, the fragments were cloned in the pCOMTrue vector provided by Dr. K-A Nave (Göttingen, Germany). Besides 2 loxP sites, this vector contains a FRT flanked NEO cassette. After linearization with PmeI, the targeting vector was electroporated into ES cells derived from 129sv/C57Bl6 blastocysts. The cells were subjected to G418 selection and 400 surviving clones were picked, expanded and tested for homologous recombination using Southern blotting with a 5′ and 3′ external probe and respectively EcoRV and BamHI digestion. Three positive clones were recovered, which were further confirmed by PCR using a NEO primer (F2 = CTGGACGAAGAGCATCAGG GGCTCG) and a primer adjacent to the 3′ flank (R2 = AACAAACAAG AGTGGGAGGCAAGCC). One of the clones was amplified and transfected with an FLT encoding plasmid. Deletion of the NEO cassette occurred in 3 out of 220 clones and was shown by PCR with primers flanking the 3′ loxP site (yielding a 256 bp band for the wild type and a 304 bp band for the floxed allele). One clone was used for aggregation with C57BL/6 morulae giving rise to one highly chimeric mouse, which ultimately produced heterozygote mice. Routine genotyping of mice was performed with the primers flanking the 3′ loxP site (F1 = CCCAACGCTGGGTC ACGGATGACG and R1 = GCAACCATAAGTTACACAAAATGCC).

### *Mouse breeding*

The generation of *Mfp2*$^{-/-}$ mice has been described (Baes et al., 2000). *Nestin-Mfp2*$^{-/-}$ and *Cnp-Mfp2*$^{-/-}$ mice were generated by breeding *Mfp2*$^{loxP/loxP}$ with *Nestin-Cre* and *Cnp-Cre* mice, respectively (Lappe-Siefke et al., 2003; Tronche et al., 1999). Mice were bred in the animal housing facility of the KU Leuven, had ad libitum access to water and standard rodent food, and were kept on a 12 hour light and dark cycle. All animal experiments were performed in accordance with the "Guidelines for Care and Use of Experimental Animals" and fully approved by the Research Advisory Committee (Research Ethical committee) of the KU Leuven (#159/2008). As we did not find differences between *Mfp2*$^{+/+}$ and *Mfp2*$^{+/-}$ mice both were used as control mice for the *Mfp2*$^{-/-}$ mice. *Mfp2*$^{loxP/loxP}$ were used as controls for *Nestin-Mfp2*$^{-/-}$ and *Cnp-Mfp2*$^{-/-}$ mice.

### *Rotarod*

An accelerating rotarod (Med Associates, Inc., St. Albans, USA) was used to test neuromotor functions in *Mfp2*$^{-/-}$, *Nestin-Mfp2*$^{-/-}$, *Cnp-Mfp2*$^{-/-}$ and their respective controls. *Mfp2*$^{-/-}$ and *Nestin-Mfp2*$^{-/-}$ were tested every 2 weeks starting at 4 weeks of age until the age of 12 weeks when rotarod testing was impossible due to the severe phenotype. *Cnp-Mfp2*$^{-/-}$ mice were tested at 6 and 12 months. The rotarod test includes an adaptation trial of 2 min at 4 rpm, followed by four test trials with an acceleration of 4–40 rpm over 5 min. Rotarod performance of the last trial was recorded for each animal.

### *Catwalk analysis*

Gait analysis was performed using the Catwalk system (Noldus Information Technology, Wageningen, The Netherlands) as described (Vandeputte et al., 2010). Briefly, each mouse was given three trials during which the animal had to cross a glass plate. Gait was