Developmental and degenerative cerebellar pathologies in peroxisomal β-oxidation deficiency

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Abstract

The integrity of the cerebellum is exquisitely dependent on peroxisomal β-oxidation metabolism. Patients with peroxisomal β-oxidation defects commonly develop malformation, leukodystrophy and/or atrophy of the cerebellum depending on the gene defect and on the severity of the mutation. By analyzing mouse models lacking the central peroxisomal β-oxidation enzyme, Multifunctional protein 2 (MFP2), either globally or in selected cell types, insights into the pathomechanisms could be obtained. All mouse models developed ataxia, but the onset was earlier in global and neural-selective (*Nestin*) *Mfp2*$^{-/-}$ knockout mice as compared to Purkinje cell (PC)-selective *Mfp2* knockouts.

At the histological level, this was associated with developmental anomalies in global and *Nestin-Mfp2*$^{-/-}$ mice, including aberrant wiring of PCs by parallel and climbing fibers and altered electrical properties of PCs. In all mouse models, dystrophy of PC axons with swellings initiating in the deep cerebellar nuclei and evolving to the proximal axon, preceding death of PCs. These degenerative features are in part mediated by deficient peroxisomal β-oxidation within PCs, but are accelerated when MFP2 is also absent from other neural cell types. The metabolic causes of the diverse cerebellar pathologies remain unknown.

In conclusion, peroxisomal β-oxidation is required both for the development and for the maintenance of the cerebellum. This is mediated by PC autonomous and non-autonomous mechanisms.

Introduction

Peroxisomal disorders can be categorized in two major classes based on the molecular defect [29]. Peroxisome biogenesis disorders (PBD) have a widespread dysfunction of the organelle whereas single enzyme/transporter disorders (SED) provoke more restricted metabolic deficiencies. Of the latter group, the most prominent affected pathways are α-oxidation, β-oxidation and ether lipid synthesis, all involved in lipid metabolism [29]. It is quite striking that not only in PBD, but also in the three lipid SED, cerebellar pathology is a common feature [7]. Here, we briefly summarize the cerebellar pathology in these patients and the corresponding mouse models. For a more thorough review the reader is referred to [7].

PBD encompass a spectrum of disease manifestations, ranging from early postnatal death hallmarked by brain malformation and hypotonia, known as Zellweger syndrome, to survival into adulthood [18]. Peroxisome biogenesis in mammals relies on the collaboration of 14 peroxin proteins (PEX) and defects can give rise to Zellweger syndrome or milder diseases depending on the mutation. With regard to cerebellar pathology, the most severely affected patients display developmental defects with Purkinje cell (PC) and granule cell heterotopias which were recapitulated in mouse models [11]. In longer surviving patients, cerebellar atrophy can occur which is often accompanied by regressive white matter changes. Notably, the white matter loss always involves the central cerebellar regions surrounding the dentate nucleus. In many patients, the cerebellar white matter abnormalities precede changes in cerebral white matter [25]. Cerebellar atrophy without white matter changes was reported in a few but not in all patients with the mildest forms of PBD [18]. These patients present with relatively normal development without obvious neurological signs during early childhood. However, between late childhood and adulthood, they develop progressive cerebellar ataxia and show cerebellar atrophy on MRI. To date, patients with mutations in *PEX2* and *PEX10* have been associated with this phenotype but not the larger group of mild ZS patients carrying the common *PEX1G843D* mutation who rather exhibit hearing and vision loss [7].

An isolated disruption of ether lipid synthesis due to mutations in either of the enzymes glyceronephosphate O-acyltransferase (GNPAT), alkylglycerone phosphate synthase (AGPS) or fatty acyl-CoA reductase 1 (FAR) also gives rise to cerebellar atrophy [4]. This is always related to white matter anomalies and linked to the loss of plasmalogens resulting in both developmental and degenerative pathology. Similarly, *Gnpat*$^{-/-}$ mice develop ataxia which may have in part a peripheral and cerebellar origin. In the cerebellum, developmental abnormalities were reported including foliation defects, granule migration and PC anomalies [24].

Impaired degradation of branched chain fatty acids and in particular of phytanic (a 3-methyl fatty acid and α-oxidation substrate) and pristanic (a 2-methyl fatty acid and β-oxidation substrate) acid can be

associated with cerebellar dysfunction. Late onset ataxia, that is partly of cerebellar and of peripheral nervous system origin, is a characteristic of an α-oxidation defect due to mutations in the phytanoyl-CoA hydroxylase (*PHYH)* gene [2]. Avoiding phytanic acid intake rescues the ataxia in patients whereas supplementation of its precursor, phytol to *Phyh*$^{-/-}$ mice induces morphological changes and loss of PC. Also a few patients with either AMACR or SCPx deficiency show ataxic symptoms related to increased levels of pristanic acid [7]. Together, these observations indicate that increased levels of branched chain fatty acids are deleterious for the cerebellum but the underlying mechanisms were not elucidated.

This chapter will further focus on the cerebellar pathology in peroxisomal β-oxidation defects, not only because patients can present with cerebellar ataxia but also because several mouse models were generated with the goal to obtain mechanistic insights in the pathogenesis.

Peroxisomal β-oxidation

Peroxisomal β-oxidation is a chain shortening process of diverse substrates which, in contrast to mitochondrial β-oxidation, does not contribute to energy generation. Substrates for peroxisomal β–oxidation are imported into peroxisomes through three ATP-binding cassette type D (ABCD) transporters located in the peroxisomal membrane [26].

Acyl-CoA oxidase (ACOX) mediates the first step of peroxisomal β-oxidation. In mammals, three ACOX enzymes (palmitoyl-CoA oxidase, branched-chain acyl-CoA oxidase and pristanoyl-CoA oxidase) were identified, each with their own substrate specificity [26]. The second and third step of the β-oxidation are catalyzed by two multifunctional proteins that each execute consecutive hydration and dehydrogenation reactions, but with different stereospecificities. The role of multifunctional protein-1 (MFP1 or L-bifunctional protein) in humans and mice seems limited to the degradation of dicarboxylic fatty acids while all other substrates, including VLC-PUFA and branched chain fatty acids are handled by MFP2. During the final step of the β-oxidation, 3-ketoacyl-CoAs are cleaved into shorter acyl-CoAs and acetyl-CoA or propionyl-CoA by three thiolases (thiolase A, B and Sterol Carrier Protein X). Importantly, peroxisomal β-oxidation is not only essential for the degradation of carboxylates, but also for the synthesis of PUFA such as docosahexaenoic acid (DHA, C22:6ω-3) and for the conversion of cholesterol into bile acids [26].

Distribution of peroxisomes and peroxisomal enzymes in the brain

Using PEX14 immunohistochemistry, it was shown that the peroxisome number in the murine brain varies tremendously between developmental and adult stages, with the highest numbers present in the earliest ages [1]. During development, peroxisomes are clearly situated in the external and internal granule cell layers of the cerebellum, the cerebellar nuclei, the cortical plate and the hippocampus. In the adult rodent cerebellum, PCs, Bergmann glia (BG) and the cerebellar nuclei harbor many peroxisomes [1].

Information on the expression pattern of peroxisomal β-oxidation enzymes in the brain is rather scarce. ABCD1 is expressed more highly in non-neuronal cell populations such as oligodendrocytes, microglia and astrocytes than in neurons. ACOX1 was detected to the same extent in neural and glial cells of the human and rodent brain, including in the cerebellum. In humans, the pivotal β-oxidation enzyme, MFP2 was expressed the earliest in PCs and in neurons of the deep cerebellar nuclei (DCN; at 13 – 15 gestational weeks) [16] before being detectable in other brain areas. In the adult human cerebellum, MFP2 was observed in PCs, DCN neurons and glial cells but it was absent from axons and from granule cells. Furthermore, white matter tracts of the cerebellum and cortex, ependymal cells and neurons of the cortex, thalamus and hippocampus were shown to be MFP2 immunoreactive.

Cerebellar pathology in peroxisomal β-oxidation defects

To date, patients were identified that carry defects in any of the different steps of the peroxisomal β-oxidation pathway [29].

The most frequent peroxisomal disorder, X-linked adrenoleukodystrophy (X-ALD) due to a defective ABCD1 transporter, has many clinical presentations [17]. A minority of patients (<2 % worldwide but 9% in Japan) has a cerebello-brainstem dominant phenotype, characterized by cerebellar ataxia, gait and speech disturbances [21]. Histological investigation in one of these patients revealed torpedoes on PC axons and patchy PC loss [21]. The diversity in clinical presentation due to ABCD1 malfunction, which remains linked to increased VLCFA levels as single metabolic disturbance, is still a mystery. In an *Abcd1*$^{-/-}$ mouse model, which only develops a late onset neurological phenotype, PC death was detected at late age. PC atrophy and loss was also observed in *Abcd2*$^{-/-}$ mice, for which no clinical correlate exists. Finally, only one patient was so far identified with a defect in the third ABCD3 transporter, also called PMP70. He died at the age of 4 years from liver disease, which precluded observation of late onset ataxic features. The *Abcd3*$^{-/-}$ mouse model is still poorly characterized.

Patients with a peroxisomal β-oxidation defect due to inactivity of ACOX1 or MFP2 deficiency [12, 13] exhibit strong similarity with the PBD patients, both with respect to the severe, intermediate and mild end of the spectrum [25]. Two mildly affected ACOX1 siblings developed cerebellar atrophy leading to ataxia in their teens, and survived into their fifties. Likewise, genetic screening of a few ataxic patients

led to the diagnosis of MFP2 deficiency, which failed to be made based on metabolic parameters (reviewed in [7]). Still, of all children diagnosed with MFP2 deficiency, the majority displays the more severe phenotype with neuronal migration defects of the cortex. In the cerebellum, ectopic PCs, hypoplasia and demyelination are frequently observed [12]. As in the PBD, an infantile onset variant exists with a dominant leukodystrophy starting in the hilus of the dentate nucleus and superior cerebellar peduncles followed by the cerebellar white matter, brainstem tracts and the cerebrum [25]. These patients achieve some developmental milestones, but then deteriorate, sometimes rapidly [10]. Notably, auto-antibodies to MFP2 can be the cause of Stiff-man syndrome, indicating the importance of MFP2 for cerebellar functioning.

Cerebellar phenotype of mouse models with peroxisomal β-oxidation defects

Although knockout mouse models were generated for nearly every protein active in the peroxisomal β-oxidation pathway, the neuropathological investigations of the cerebellum were limited. As already mentioned, a late onset cerebellar pathology was reported in the *Abcd1*$^{-/-}$ and *Abcd2*$^{-/-}$ models besides their spinal cord and peripheral nerve anomalies. In what follows, we will elaborate on the prominent cerebellar phenotype of MFP2 deficient mice generated in our lab [3]. Besides global knockouts in which the phenotype is influenced by peripheral sources [15], also a neural selective MFP2 knockout was generated (*Nestin-Mfp2*$^{-/-}$)[27]. In the latter mouse model, all neural cells (neurons, astrocytes and oligodendrocytes) are devoid of MFP2. However, it should be taken into account that the *Nestin* promotor driving CRE expression is less selective than originally expected. It indeed also gives rise to gene deletion in peripheral organs such as kidney and pancreas. Finally, a PC restricted MFP2 knockout (*L7-Mfp2*$^{-/-}$) was generated which was instrumental to distinguish between cell autonomous and non-autonomous and between developmental and degenerative mechanisms [9]. The cerebellar phenotypes of these mouse models are summarized in Table 1.

CNS deletion of MFP2 induces ataxia

For unknown reasons, total ablation of MFP2 in mice does not cause severe neurodevelopmental abnormalities and *Mfp2*$^{-/-}$ mice are indistinguishable at birth from wild type mice [15]. This is in striking contrast to the severe pathology in neonates with MFP2 deficiency [12]. However, already from the age of 3 weeks, *Mfp2*$^{-/-}$ mice display limb dyskinesia rapidly evolving in an unsteady gait with coordination problems [15]. From the age of 4 months, their motor phenotype rapidly deteriorates, mutants become immobile and lethargic, and they always die before the age of 6 months. The absence of lesions in the peripheral nervous system and skeletal muscles of *Mfp2*$^{-/-}$ mice pointed to MFP2

deficiency in the CNS as the origin of their motor problems [15]. Likewise, no lesions were observed in muscle biopsies of MFP2 deficient patients.

It is, however, striking that the phenotype of neural specific *Nestin-Mfp2*$^{-/-}$ mice [27] strongly deviates from global *Mfp2*$^{-/-}$ mice. At the histological level, global *Mfp2*$^{-/-}$ show a delay in foliation of the cerebellum, impaired GC migration and PC maturation in the first postnatal weeks, associated with a significant increase in apoptotic cell death, confirming the importance of peroxisomal β-oxidation for early cerebellar development [19]. In contrast, these anomalies were not observed in *Nestin-Mfp2*$^{-/-}$ mice. The difference may stem from normal MFP2 function in the liver, where it is essential for several chain shortening processes of carboxylates including the formation of mature bile acids. This is in agreement with studies in *Pex2*$^{-/-}$ mice in which bile acid feeding partially corrected the cerebellar developmental defects [11]. A second divergence is that in *Mfp2*$^{-/-}$ mice an extensive neuroinflammation spreads over the brain with age, although the cerebellum is relatively spared [27, 28]. This is much less pronounced in *Nestin-Mfp2*$^{-/-}$mice, likely leading to their increased survival (> 10 months)[5]. Hence, the latter mouse model was better suited to perform a longitudinal study of the cerebellar pathology [8].

The PC selective MFP2 knockout (*L7-Mfp2*$^{-/-}$) mice, allowed to demonstrate that, in addition to developmental anomalies, loss of MFP2 causes a purely PC degenerative phenotype. In this PC restricted knockout model signs of ataxia did not occur until the age of 6 months [9]. The discordance between the motor phenotypes in *L7-Mfp2*$^{-/-}$ and *Nestin-Mfp2*$^{-/-}$ mice might have several origins that will be explained below.

Cerebellar wiring is affected in Nestin-Mfp2*$^{-/-}$ *mice

Although the anatomy of the cerebellum is not overtly disturbed in *Nestin-Mfp2*$^{-/-}$ mice, there are remarkable impairments in PC excitatory innervation i.e. both climbing fibers (CFs) and parallel fibers (PFs) synapses are reduced [8]. In addition, the presence of multiple CF synapses on PC somata of adult *Nestin-Mfp2*$^{-/-}$ mice instead of a single CF innervation at the proximal dendritic part of a PC is a sign of inadequate maturation of afferent connections. In contrast, inhibitory GABA-ergic synaptic contacts on the PC dendritic tree were unaffected [8]. The actual consequences of this impaired PC wiring pattern, and the relative contribution of CFs and PFs to the cerebellar phenotype of *Nestin-Mfp2*$^{-/-}$ mice could be further investigated by studying their synaptic strength by means of electrophysiological measurements. These mild alterations in PC excitatory innervation in MFP2 deficient mice may reflect what has been observed in MFP2 deficient patients namely, malformations of the inferior olivary nucleus and granule neuron migration and maturation defects which in turn can influence the normal extension of CF and PF axons.

Electrophysiological properties of Purkinje cells are altered in Nestin-Mfp2$^{-/-}$ mice

Another novel insight that was unveiled by studying *Nestin-Mfp2$^{-/-}$* cerebellum was the altered electrophysiological functioning of PCs. MFP2 deficient PCs exhibit a reduced firing rate and excitability [8]. This can in part be caused by an increase in membrane capacitance, although we cannot exclude that additional mechanisms might play a role. The clearcut increase in PC membrane capacitance was not associated with an increased size of the cell soma, nor with a more elaborate dendritic arborization [8]. Whether the increased spine surface could account for this effect remains unsure. As an alternative explanation, we hypothesize that impaired peroxisomal β-oxidation might affect the membrane capacitance through altering the lipid composition of the membrane. At present, the accumulation of VLCFA is the only known metabolic change in the brain of patients and mice with peroxisomal β-oxidation defects.

Detailed analysis of the action potentials of *Nestin-Mfp2$^{-/-}$* PCs did not reveal alterations in action potential amplitude and shape, suggesting that Na^+, K^+ or Ca^{++} channels operate properly. Not unimportant is the fact that peroxisomes were shown to be more crowded in the PC axon hillock, where the action potential is initiated. Of note, the aberrant development of the PC afferent innervation (CF and PF) in *Nestin-Mfp2$^{-/-}$* mice could be a consequence of the reduced PC electrical activity. Indeed, it was reported that decreasing PC excitability by specifically expressing a chloride channel in PCs impaired the redistribution of CF synapses from PC somata to the proximal dendrites. Importantly, this was the first demonstration that inactive peroxisomal β-oxidation affects the electrical activity of a neuron.

Purkinje cell axons degenerate in MFP2 deficiency

It is well known that axonal dystrophy precedes neurodegeneration in several disorders. It is remarkable that spheroids (also called torpedoes and swellings) on PC axons develop both in global *Mfp2$^{-/-}$*[27], in *Nestin-Mfp2$^{-/-}$* [8] and in the PC selective *L7-Mfp2$^{-/-}$* mice [9]. The latter observation is crucial and proves that PC axonal dystrophy is in part a cell autonomous event. In the *L7- Mfp2$^{-/-}$* mice, the first torpedoes were observed on the terminal domains of these axons at the age of 6 months and this coincided with the onset of motor problems (Figure 1)[9]. Since cerebellar ataxia in *L7-Mfp2$^{-/-}$* mice was observed well before extensive PC death [9], the motor disabilities of these mutants are due to defects in PC functioning.

However, in *Mfp2$^{-/-}$* and in *Nestin-Mfp2$^{-/-}$* mice, axonal swellings were observed at a much earlier age [8], indicating that loss of MFP2 from other cell types may accelerate axonal dystrophy. In *Nestin-Mfp2$^{-/-}$* mice for example, swellings were observed as early as postnatal day 14, where after the number of swellings progressively increased. Amongst others, defects in myelin formation or oligodendrocyte

function can affect axonal integrity. For example, in multiple sclerosis, chronic loss of myelin is associated with axonal degeneration. When myelin insulation is lost, axons face an imbalance in ion homeostasis which can lead to Ca^{2+}-induced axonal degeneration. However, in *Mfp2*$^{-/-}$ and *Nestin-Mfp2*$^{-/-}$ mice loss of myelin is only seen at later ages, after the first signs of axonal deterioration. Determination of the g-ratio in *Nestin-Mfp2*$^{-/-}$ mice also proved that axonal swellings on PCs existed without myelin loss. Furthermore, oligodendrocytes are known to provide direct support to axons independent of their role in myelin-formation [20]. Hence, compromised oligodendrocyte function can lead to axonal degeneration in the presence of normal myelin. We could however exclude that deterioration of PC axons is due to MFP2 inactivity in oligodendrocytes as selective elimination of MFP2 from these cells does not induce the neurological phenotype observed in *Mfp2*$^{-/-}$ and *Nestin-Mfp2*$^{-/-}$ mice [27]. Motor dyscoordination and cerebellar neurodegeneration have been described in both glia specific as well as BG specific knockout mouse models mainly linked to defects in glutamate transport. However, a role for BG in peroxisomal cerebellar pathology is questionable as selective deletion of peroxisomes from the glial cell population (*Gfap-Pex5*$^{-/-}$) did not provoke a neurological phenotype. It remains possible that the PC-non-autonomous contribution to axonal atrophy is not related to the absence of MFP2 in a particular cell type (cerebellar neurons, astrocytes, oligodendrocytes) but that it is due to the inability to maintain lipid homeostasis in the PC environment.

For both the early non-PC-autonomous and the late PC-autonomous defects in axonal transport, the exact link with peroxisomal β-oxiation dysfunction remains elusive to date. Further investigations on the factors provoking PC axonal torpedoes are of high importance given the simultaneous onset of motor problems in MFP2 deficiency in mice. The pronounced deterioration of the axonal compartment preceding the degeneration of PC bodies in both *Nestin-Mfp2*$^{-/-}$ and *L7-Mfp2*$^{-/-}$ mice suggests that MFP2 deficiency induces a dying-back pathology of PCs. It is interesting to note that axonal torpedoes were also reported in an ABCD1 patient [21]. Additional pathological studies in peroxisomal β-oxidation patients are warranted to validate the findings in mice.

The spines but not the dendritic tree complexity are altered in the absence of MFP2

In contrast to the axonal compartment, no differences were found in the complexity of the PC dendritic tree in 4-week-old *Nestin-Mfp2*$^{-/-}$ mice but morphological changes were present at the level of the spines [8]. Studies on organotypic slice cultures of the mouse cerebellum revealed that the development of an elaborate PC dendritic tree occurs independent of glutamate-mediated excitatory neurotransmission, although a role of these extrinsic signals in spine formation, spine shape and synaptogenesis was not refuted. Therefore, the altered interaction of PCs with their stimulatory input, CFs and PFs in *Nestin-Mfp2*$^{-/-}$ mice may not affect the territory covered by the dendritic tree but may

cause subtle changes in spine density and length. This hypothesis is also reinforced by the absence of abnormalities in dendritic thickness and spine number and density in *L7-Mfp2*$^{-/-}$ mice, in which cerebellar development is unaltered and PCs are likely well-innervated by axons originating from granule and inferior olivary neurons [9].

Postdevelopmental PC degeneration in MFP2 deficiency

The *L7-Mp2*$^{-/-}$ mice not only clarified that lack of peroxisomal β-oxidation in PCs elicits axonal dystrophy in a cell autonomous way, they also allowed to conclude that the cerebellar atrophy is in part a mere degenerative process. In contrast to *Nestin-Mfp2*$^{-/-}$ mice, the formation and wiring of the cerebellum in *L7-Mfp2* mice appeared normal. This could be expected as in the latter mice, CRE expression in PCs occurs from postnatal day 6 onwards , and is not fully established until 2 to 3 weeks after birth, a time point at which the major phases of cerebellar development are finalized in mice. Conversely, CRE expression driven by the *Nestin* promotor occurs in virtually all cells of the CNS at embryonic day 15.5. Histological follow-up studies of *L7-Mfp2*$^{-/-}$ mice proved that PC death is progressive and occurs in a non-random manner since PCs in the caudal cerebellum survive longer [9]. The fact that PCs in the anterior cerebellar lobules are more affected in MFP2 deficiency suggests a link between the accumulations of toxic substrates and the pattern of PC degeneration. Moreover, the patterned degeneration of PCs in elder *L7-Mfp2*$^{-/-}$ mice suggests a differential vulnerability of PCs to deviations in the peroxisomal β-oxidation. This characteristic 'banded pattern' of PC degeneration has been described in several other mouse models and is often related to the expression of zebrin II, also known as aldolase C, a key enzyme involved in glycolysis [14]. Zebrin II is only expressed by a subset of PCs that are generally positioned adjacent to zebrin^{-} neurons in many areas of the cerebellar cortex. Depending on the mutation, zebrin II^{+} or zebrin II^{-} PCs appear more vulnerable and degenerate first. *Nervous* and *pcd* mutations, for example, primarily affect the zebrin II^{+} neurons, while zebrin II^{-} PCs seem more vulnerable in mouse models of *Leaner*, NPC and *Slc9a6*$^{-/-}$ (Angelman syndrome). In some cases, this patterned PC loss is eventually followed by widespread degeneration of all neurons, independent of their zebrin II expression. However, not only the presence or absence of zebrin determines the specific vulnerability of PCs to degeneration since other biological molecules are also expressed in longitudinal stripes within the cerebellum. Zebrin II^{+} PCs express phospholipase Cβ3 (PLCβ3), excitatory amino acid transporter 4 (EAAT4), GABA B receptor subtype 2 (GABABR2) and neural calcium sensor 1 (NCS1), while zebrin II^{-} cells co-localize with PLCβ4, metabotropic glutamate receptor 1β (mGluR1β), microtubule-associated protein (MAP1A), neuroplastin and neurogranin (Figure 1) [6].

Investigations on what PC population is more vulnerable in mouse models with MFP2 deficiency are of high importance as it might provide useful insights on how peroxisomal β-oxidation affects other metabolic processes, such as glycolysis, in PCs and how it is related to these molecular markers.

Multiple cell types establish the motor phenotype of MFP2 deficient mice

The relatively mild and late onset motor signs in *L7-Mfp2*$^{-/-}$ mice as compared to *Nestin-Mfp2*$^{-/-}$ mice may have additional reasons besides the longer preservation of PC axons. In *L7-Mfp2*$^{-/-}$ mice the enzymatic activity of MFP2 is unaffected in the PC outflow pathways namely the vestibular nuclei and the DCN. Either of these neurons may restore the neurotransmitter equilibrium by increasing their activity.

We hypothesize that in juvenile *Nestin-Mfp2*$^{-/-}$ mice a chain reaction takes place starting with abnormalities in cerebellar formation and PC dysfunction, whereas in *L7-Mfp2*$^{-/-}$ mice the innervation of the cerebellum is normal sparing them from early-onset motor problems. This may also explain why motor learning is affected in *Nestin-Mfp2*$^{-/-}$ but not in *L7-Mfp2*$^{-/-}$ mice. Indeed, the two determinants of motor learning - CF input to the cerebellar cortex and adequate PC functioning - are unaffected in 4-months-old *L7-Mfp2*$^{-/-}$ mice, the age at which motor learning was assessed.

Lack of insight into the metabolic origin

After adipose tissue, the CNS contains the highest lipid concentration in the body. Deregulation of lipid metabolism is therefore of particular interest for CNS disorders and the lipid field has gained importance in the field of neuroscience. Regarding the widely accepted role of peroxisomes in lipid metabolism, many previous (and possibly future) studies concentrate on causal links between lipid-induced toxicity and CNS pathology in peroxisomal disorders.

In BG of *Mfp2*$^{-/-}$ cerebella, lipid droplets harboring neutral lipids such as triglycerides and cholesterylesters accumulate extensively, but this seemed harmless as it was not accompanied by BG degeneration [15]. As mentioned in the introduction, branched chain fatty acid elevations are supposedly toxic for the cerebellum. However, the cerebellum of *Mfp2*$^{-/-}$ and *Nestin-Mfp2*$^{-/-}$ mice is free of phytanic or pristanic acid accumulation and the ataxic behavior of *Mfp2*$^{-/-}$ mice fed with pellets enriched in branched-chain fatty acids (phytol) did not differ from MFP2 knockout mice on a normal diet [15]. The most likely other metabolic culprit in brain due to MFP2 deficiency is the accumulation of VLCFA. The adverse effects of C26:0 accumulation on neurons were extensively demonstrated in several in vitro studies [30]. Lipid analyses on cerebellar homogenates of *Mfp2*$^{-/-}$ mice revealed a

significant increase in the levels of C26:0 in cholesterylesters and in the phospholipid fraction compared to wild type littermates, whereas no changes in DHA concentrations were found. However, the absence of a clinical phenotype in *Cnp-Mfp2*$^{-/-}$ mice, in which MFP2 was selectively deleted from oligodendrocytes, despite highly elevated C26:0 levels in cerebellar homogenates, argues against VLCFA accumulation as the cause of cerebellar pathology [27]. An important drawback of lipid analyses on homogenates of tissues in which many cell types are represented is that the cellular origin of the changes is lost and/or that mild changes are leveled out and not detectable. Imaging mass spectrometry is a relatively new technique that is used to analyze and image the distribution of any type of molecule in a biological tissue. Recent studies have shown its particular value for lipidomic analysis in the brain as it allows the visualization of spatial differences in lipid distribution in an unbiased way [23]. Imaging mass spectrometry was recently applied on the hindbrains of *Mfp2*$^{-/-}$ and *Nestin-Mfp2*$^{-/-}$ mice to study whether a peroxisome-dependent metabolite may account for their cerebellar phenotype and to assess whether other metabolites may be deregulated. At this point, no significant changes were observed in the cerebellar layers but the sensitivity of the instrument used was likely insufficient to detect minor lipid species such as VLCFA [22]. It should also be emphasized that in mild MFP2 patients, no or borderline metabolic changes are detectable in plasma. It is interesting to note that cerebellar pathology was reported in ELOVL4 deficiency, leading to reduced synthesis of VLCFA, possibly indicating that VLCFA levels should be well balanced in cerebellar tissue. It remains also unexplained why among adult X-ALD patients who all have increased levels of VLCFA, only few present with a cerebellar phenotype.

Conclusions and future perspectives

The cerebellum exquisitely depends on an intact peroxisomal β-oxidation. Complete inactivity of this pathway causes cerebellar malformation and early onset dysfunction. More recently, patients who presented with ataxia in adulthood as the primary pathology were found to have mutations in the gene *HSD17B4*, encoding the peroxisomal β-oxidation enzyme MFP2.

Developmental and degenerative pathologies were recapitulated in different mouse models with MFP2 deficiency. Whole body deletion of MFP2 affects the formation of cerebellar folia which likely has a systemic origin, as it was not seen in *Nestin-Mfp2*$^{-/-}$ mice. In the latter, absence of MFP2 from all neural cells affected the electrophysiological properties of PCs and, impaired PC functioning and innervation, and induced axonal degeneration. The *L7-Mfp2*$^{-/-}$ model proved that peroxisomal β-oxidation within PCs is required to preserve axons but that some PCs are more vulnerable than others.

The ataxic phenotype in all mouse models, coincided with the occurrence of axonal swellings on PCs and not with the loss of PCs. The fact that the early-onset motor phenotype of *Nestin-Mfp2*$^{-/-}$ mice is not present in the *L7-Mfp2*$^{-/-}$ model strengthens the notion that an intact peroxisomal β-oxidation system is necessary in the neural circuit innervating PCs and/or in the neighboring cells for a normal cerebellar formation.

These findings are relevant for all patients with peroxisomal disorders presenting with cerebellar pathologies. Whereas MRI of the cerebellum is regularly performed to document cerebellar atrophy, histological and ultrastructural studies in these patients are needed to better document the cellular abnormalities. The lack of insight in the metabolic origin of the cerebellar demise is another hiatus in our understanding.

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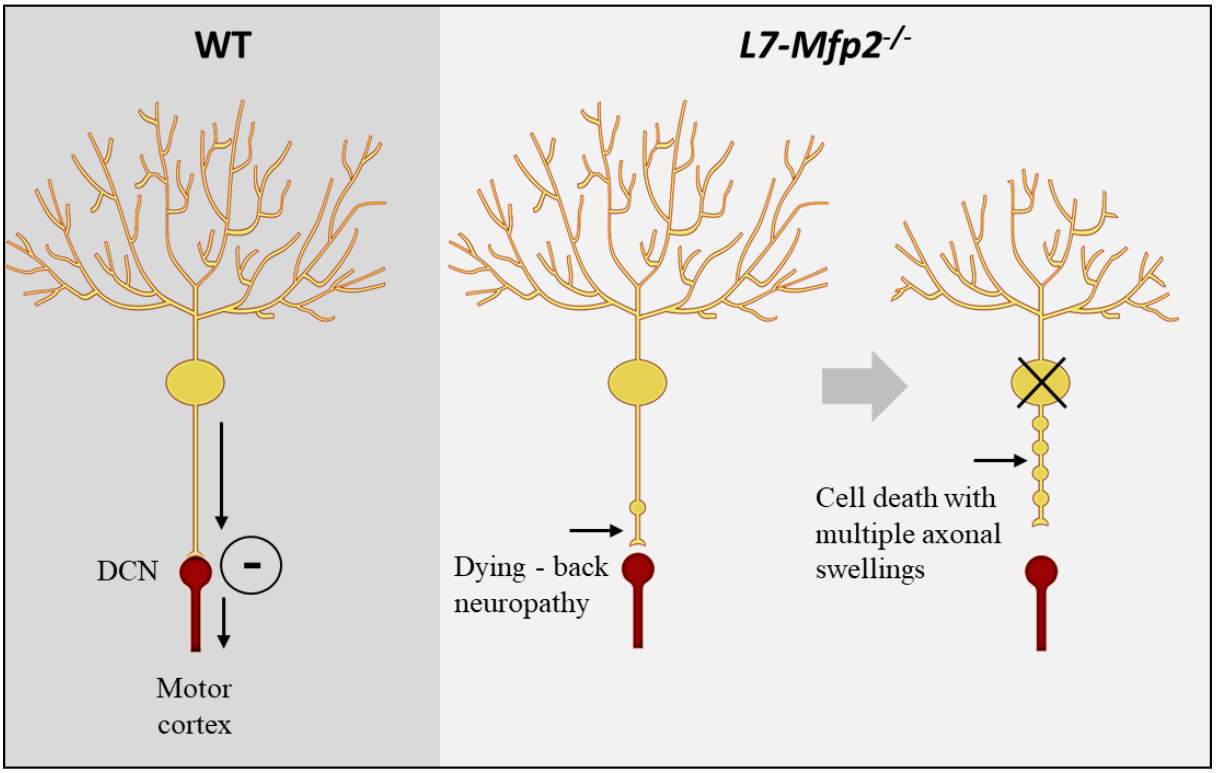
Table 1. **Comparison of the phenotype and cerebellar histology of mouse models with elimination of MFP2 from all cells (*Mfp2*$^{-/-}$), all neural cells (*Nestin-Mfp2*$^{-/-}$) or PCs (*L7-Mfp2*$^{-/-}$).**

	Mfp2$^{-/-}$	*Nestin-Mfp2*$^{-/-}$	*L7-Mfp2*$^{-/-}$
MFP2 loss in	*All cells*	**All neural cells**	**PCs**
Phenotype			
Lifespan	4 – 6 months	10 – 12 months	> 12 months
Onset motor impairment	< 4 weeks	< 4 weeks	6 months
Impaired motor learning	Not determined	4 weeks	Not at 4 months
Cerebellar histology			
Altered development	Yes	Yes	No
Onset axonal swellings	2 months $^{\delta}$	2 weeks	6 months
PC death	Not at time of death	8 – 12 months	9 months
Onset cerebellar atrophy	Not at time of death	10 – 12 months	Not determined
Myelin loss	Yes	Yes	Not determined

$^{\delta}$ Earliest age investigated. Altered cerebellar development in the *Mfp2*$^{-/-}$ model refers to a delay in cerebellar foliation (91). In *Nestin-Mfp2*$^{-/-}$ mice, we found mild disturbances in PC afferent innervation and spine morphology (32). Since MFP2 was deleted at a postnatal age in *L7-Mfp2*$^{-/-}$ mice, no developmental problems are expected. Myelin loss in cerebellar branches of (*Nestin-*) *Mfp2*$^{-/-}$ mice occurs after the appearance of PC axonal swellings (34).

Figure 1



Pathological events in *L7-Mfp2*$^{-/-}$ mice. The occurrence of torpedoes on PC axons is the first pathological sign that coincides with ataxic features, starting at the age of 6- to 8-months and worsening with time. This is followed by degeneration and loss of PCs. Reproduced from (9) with permission from John Wiley and Sons.