Peroxisomal Multifunctional Protein-2 Deficiency Causes Motor Deficits and Glial Lesions in the Adult Central Nervous System

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In humans, mutations inactivating multifunctional protein-2 (MFP-2), and thus peroxisomal β -oxidation, cause neuronal heterotopia and demyelination, which is clinically reflected by hypotonia, seizures, and death within the first year of life. In contrast, our recently generated MFP-2-deficient mice did not show neurodevelopmental abnormalities but exhibited aberrations in bile acid metabolism and one of three of them died early postnatally. In the postweaning period, all survivors developed progressive motor deficits, including abnormal cramping reflexes of the limbs and loss of mobility, with death at 6 months. Motor impairment was not accompanied by lesions of peripheral nerves or muscles. However, in the central nervous system MFP-2-deficient mice overexpressed catalase in glial cells, accumulated lipids in ependymal cells and in the molecular layer of the cerebellum, exhibited severe astrogliosis and reactive microglia predominantly within the gray matter of the brain and the spinal cord, whereas synaptic and myelin markers were not affected. This culminated in degenerative changes of astroglia cells but not in overt neuronal lesions. Neither the motor deficits nor the brain lesions were aggravated by increasing the branched-chain fatty acid concentration through dietary supplementation. These data indicate that MFP-2 deficiency in mice causes a neurological phenotype in adulthood that is manifested primarily by

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During the last decades it has been clearly established that peroxisomes are ubiquitously present in mammalian tissues. These organelles are involved in multiple processes such as the catabolism and synthesis of lipids and sterols and the degradation of hydrogen peroxide. Peroxisomal β -oxidation, in which multifunctional protein-2 (MFP-2) plays a pivotal role, is crucial for the breakdown of very-long-chain fatty acids, branched chain fatty acids, and cholesterol.^{1,2} Synthesis of docosahexaenoic acid (DHA), a major lipid compound of the central nervous system (CNS), and degradation of leukotrienes, important signaling molecules, also need peroxisomal β -oxidation.^{3,4}

Most of the current knowledge on peroxisomal metabolism has been gathered from studying peroxisomal function in liver. In contrast, clinical and pathological observations in patients with various peroxisomal defects point toward an especially important role of peroxisomes in nervous tissue during development and in adulthood.^{5,6} Peroxisomes have been identified in both neurons and glia by immunocytochemical and electron optical methods,^{7–12} but surprisingly little is known about their precise role in the different neural cell types. Generalized impairment of peroxisomal function, as encountered in peroxisome biogenesis disorders such as Zellweger syndrome, causes severe developmental abnormalities including a very characteristic neuronal migration defect, facial dysmorphisms, and severe hypotonia.

It should be kept in mind that besides the neurodevelopmental abnormalities also postdevelopmental degenerative neuronal lesions including demyelination, photoreceptor

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degeneration, cerebellar atrophy, and peripheral neuropathies are well documented in peroxisome biogenesis disorders and/or peroxisomal disorders with a single protein deficiency such as X-linked adrenoleukodystrophy, α -methylacyl-CoA racemase deficiency, or Refsum disease.^{13–17}

Humans with MFP-2 deficiency strongly resemble Zellweger patients.^{13,14} Key constant features are a severe neonatal hypotonia and seizures and absent or at least severely delayed development resulting in death within the first year of life. Neuronal migration defects, giving rise to focal cortical heterotopia and polymicrogyria are documented for ~80% of the cases.

We recently generated mouse models with peroxisome assembly defects (*Pex5* knockout)¹⁸ or with peroxisomal β -oxidation defects (*MFP-2*¹⁹ and *MFP-1/MFP-2* knockout²⁰) to investigate the pathogenesis of peroxisomal disorders and to further explore the role of peroxisomes in brain. In contrast to the situation in humans, a Zellweger-like pathomorphology was only seen in the model with peroxisome assembly defects (*Pex5* knockout)¹⁸ but not in mice with peroxisomal β -oxidation defects. The phenotype of *MFP-2* knockout mice significantly diverged from the clinical presentation and pathology in *MFP-2*-deficient patients^{13,14,18,19}; ie, the *MFP-2* knockout mice were not hypotonic at birth and did not display a neuronal migration defect.¹⁹ However, from day 2 on they were severely growth retarded, which correlated with major abnormalities in bile acid synthesis,²¹ resulting in a generalized failure to thrive and the premature death of approximately one-third of the knockouts within the first 3 weeks. After weaning, the surviving *MFP-2* knockout mice resumed normal weight gain but remained smaller than wild-type (WT) littermates.¹⁹ Metabolic alterations observed in *MFP-2* knockout mice, including accumulations of very-long-chain fatty acids and phytanic and pristanic acids, were similar to the changes seen in patients and correspond to a severe defect in peroxisomal β -oxidation.

The present study focuses on a progressive neuromotor phenotype that develops in all *MFP-2*-deficient mice surviving into adulthood and which is lethal at the age of 6 months, ie, at a time schedule quite distinct from that of the initial early postnatal metabolic problems. No abnormalities in the neuromuscular system were found, but we observed lipid accumulations in ependyma and Bergmann glia and a generalized astrogliosis and microglia activation throughout the gray matter, which in sharp contrast to human peroxisomal diseases spared myelinated fiber tracts. Even more surprising, this was not associated with overt neuronal damage, but resulted in the stainability by a degeneration marker, Fluoro-Jade, of astroglial cells, pointing toward an unexpected novel target of *MFP-2* deficiency in the murine brain.

Materials and Methods

Animals

Homozygous *MFP-2*-deficient mice were obtained in the offspring of heterozygous *MFP-2*^{+/-} breeding pairs,

which were in a mixed Swiss [Tac:(Sw)fBR]/sv129 background. Mice were bred in the animal housing facility of the University of Leuven under conventional conditions. They had unlimited access to standard rodent food chow (Muracon-G; Carfil Quality-Pavan Services, Oud-Turnhout, Belgium) and water and were kept on a 12-hour light/dark cycle. All animal experiments were approved by the Institutional Animal Ethical Committee of the University of Leuven.

Rotarod Testing

A rotarod (Ugo Basile Biological Research Apparatus, Varese, Italy) with a constant speed of 17 rpm was used. Two days before the test, mice were trained for two periods of 3 minutes. The test consisted of two trials of 3 minutes with a 1-hour interval. The latency time for the mouse to drop from the rod was recorded.

Light and Electron Microscopical Histology

For light and electron microscopical analysis of plastic-embedded sections, mice were deeply anesthetized with Hypnorm (fentanyl/fluanizone) and midazolam (Janssen Pharmaceutica, Beerse, Belgium). The transcardial perfusion with Ringer's solution containing 0.1% procain and heparin 5 units/ml tissues (2 minutes) was followed by an *in situ* fixation with buffered 2.5% glutaraldehyde and 20 mg/ml sodium cacodylate (10 minutes). Specimens were postfixed for another 24 hours with glutaraldehyde, osmicated, and embedded in Embed 812 (Electron Microscopy Sciences, Fort Washington, PA). Sections 3 μ m thick were stained with *p*-phenylenediamine and studied by light microscopy. Thin sections for electron microscopy were stained with lead citrate and uranyl acetate and investigated in a Philips CM 100 microscope (Eindhoven, The Netherlands). Three or four mice of each genotype, derived from different litters were used for this analysis.

Immunohistochemistry

The procedure for immunohistochemical analysis of paraffin material was described in detail.²² Briefly, Nembutal-anesthetized mice were perfused with modified Bouin's solution. Brain and spinal cord were post-fixed overnight and embedded in paraffin following routine procedures. Three to four different mice of each genotype aged 3 weeks, 5 weeks, 8 weeks, 3 months, and 5 months were used.

Sections were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol and rinsed in 0.1 mol/L phosphate-buffered saline. After antigen retrieval by limited proteolysis or heating in citrate buffer, and blocking of endogenous peroxidase, sections were subsequently incubated for 1 hour with the primary antibody diluted in blocking buffer with normal goat serum (2%), and after washing for 1 hour, with a secondary antibody in the same buffer. All incubations were done in parallel and photograph exposures were equal for control and *MFP-2* knockout mouse sections.

The following primary antibodies were used: polyclonal rabbit antibodies against bovine glial fibrillary acidic protein (GFAP) (DAKO, Heverlee, Belgium) and bovine catalase (Rockland, Gilbertsville, PA), monoclonal rat antibodies against F4/80 (American Type Culture Collection, LGC Promochem, Teddington, UK) and against rat lysosome-associated membrane protein-1 (LAMP-1) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), monoclonal mouse antibodies against bovine Calbindin-D-28K (Sigma-Aldrich, Bornem, Belgium), bovine myelin basic protein (MBP) (Sigma-Aldrich), and porcine GFAP (Sigma-Aldrich).

The secondary antibodies (goat anti-rabbit IgGs and goat anti-mouse IgGs, Sigma-Aldrich) were conjugated with peroxidase, and tyramide signal amplification kits (PerkinElmer Life Sciences, Inc., Boston, MA), using tyramide-conjugated fluorochromes, were used for antibody detection. For double immunostainings one of the secondary antibodies was labeled with Alexa-568 or Alexa-546 (Molecular Probes Europe BV, Leiden, The Netherlands). The biotin-tagged lectin *Ricinus communis* agglutinin-1 (RCA-1) (Vector Laboratories, Burlingame, CA) was used as a marker for activated microglia, the Vectastain ABC kit (Vector Laboratories) was used for visualization.

Analysis of Cell Degeneration and Apoptosis

Animals were perfusion fixed with 10% neutral buffered formalin (Prosan, Germany) and embedded in paraffin as described above. Dewaxed sections were stained with Fluoro-Jade B (Chemicon International) to screen for degenerating neurons and glia. Parallel sections were incubated with antibodies against cleaved (activated) caspase 3 to detect apoptotic cells. It should be noted that although the Fluoro-Jade assay was originally developed to identify early (precell death) stages of neuronal degeneration, it has more recently been shown that it also identifies degenerating glial cells.²³

Visualization of Lipids

Free-floating frontal and sagittal vibratome sections of the brain were stained with the lipid-soluble dye Oil Red O (C.I. no. 26125; BDH Laboratory Supplies, UK) 0.24% w/v in isopropanol/water (3:2) for 18 minutes and counterstained with methyl green (Vector Laboratories). A parallel series of sections was additionally double-stained by antibodies against GFAP as described above. Three to four different mice of each genotype aged 4 weeks, 8 weeks, 3 months, and 5 months were analyzed.

Western Blot Analysis

Brain homogenates were analyzed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels as previously described,²¹ using the same antibodies as those used for immunocytochemical staining.

Figure 1. Development of motor deficits in *MFP-2* deficient mice. Beginning at the age of 1 month, the *MFP-2* knockout mice lose the characteristic leg-spreading reflex elicited by being suspended without contact to a supporting surface, as shown for a control mouse in **A**. Instead, mice progressively clasp both fore- and hindlegs (**B**), a phenomenon often observed in supraspinal CNS lesions. **C**: In parallel to the development of pathological reflexes, animals lose grip strength and motor coordination, cutting down their time on a rotarod instrument to <50% of that of control mice. Mean $\pm$ SEM of three control and three *MFP2*^{-/-} 8-week-old mice is shown. * $P = 0.05$, ** $P < 0.005$.

Denervation Experiments

The mice were anesthetized with Hypnorm (fentanyl/fluanizone) and midazolam and the sciatic nerve was crushed for 2 minutes with a microsurgical needle holder with smooth branches. The wound was closed with one suture. Nerve regeneration was monitored by testing the toe-spreading reflex.

Electrophysiology

The mice were kept in superficial anesthesia [Hypnorm (fentanyl/fluanizone) and midazolam]. The sensory conduction velocity was measured along the tail nerve with two proximal stimulating and two distal recording electrodes. A ground electrode was placed between these two sets. The gastrocnemius muscle was stimulated with the cathode at the sciatic notch and the anode in the skin.

of the lateral abdomen. Stimulus strength was supramaximal. The different recording electrode was placed inside the gastrocnemius muscle and the indifferent one in the skin of the hind paw.

Lipid Analysis

All solvents were of the highest quality commercially available (Biosolve, Valkenswaard, The Netherlands). Butylated hydroxytoluene (0.05%, w/v) was added at all stages of the extraction to minimize auto-oxidation of polyunsaturated fatty acids (PUFAs). Lipids were extracted from tissues, homogenized in 3.8 ml of $\text{CH}_3\text{OH}/\text{CHCl}_3/\text{H}_2\text{O}$ (2:1:0.8), using a Polytron tissue homogenizer,²⁴ and separated into neutral lipids, fatty acids, and phospholipids by solid phase extraction (Bond Elut NH_2 column, 500 mg; Varian Benelux, Sint-Katelijne-Waver, Belgium).^{20,25} Cholesterol,²⁶ cholesteryl esters,²⁶ neutral glycerolipids,²⁷ and phosphorus content of the phospholipid fraction²⁸ were determined as previously described. The content of DHA in the phospholipid fraction was determined by GC analysis as previously described.²⁹ Phytanic, pristanic acid, and $\text{C}_{26:0}$ were quantified by GC-MS analysis in phospholipids and neutral lipids.²⁰

Results

MFP-2 Knockout Mice Develop a Severe Neurological Phenotype Lethal in Early Adulthood

MFP-2-deficient mice developed a dyskinesia of the limbs, the first signs of which were visible at the age of 3 weeks. On lifting mice by their tail they either over-stretched and cramped the hind legs or contracted them to the trunk often together with the front legs whereas WT mice spread their limbs and struggled (Figure 1, A and B). These abnormalities became much more prominent after 3 months. The walking pattern of *MFP-2* knockout mice was characterized by an unsteady gait. Rotarod testing revealed that motor coordination and equilibrium were already affected at the age of 8 weeks (Figure 1C). Instead of walking on the rotating rod, *MFP-2*-deficient mice often gripped the rod and made passive rotations before dropping off during the 3-minute test period.

During the fifth to sixth month the mobility of the *MFP-2* knockout mice was severely reduced. Because they had

difficulties in standing up and reaching the chow, food pellets were placed into the cage. Nevertheless, they progressively lost weight down to only 35 to 40% of the body weight of their WT or heterozygous littermates. The mice died at the age of 5 to 6 months after dramatic wasting, so that in the final stage of their disease they lay immobile on their side. At the time of death, the major organs appeared macroscopically normal, except for a severe shrinkage of the testicles (Huyghe et al, unpublished observations) and the absence of white adipose tissue.

Lipids Accumulate in Specific Regions of Brain and Spinal Cord

The morphology of the CNS of *MFP-2* knockout mice in preterminal stage (5 months old, as compared to a life-span of 5 to 6 months) appeared normal on hematoxylin and eosin-stained sections (data not shown). However, Oil Red O-positive lipid droplets were seen in specific regions of the CNS whereas no lipid droplets were found in any region of the CNS of the WT or heterozygous littermates.

Lipid storage was most impressive within ependymal cells along the entire ventricular system and within selected regions of the gray matter. The ependymal lining of the four ventricles and the spinal central canal (Figure 2, A–D) showed very prominent fatty inclusions that unambiguously distinguished sections from knockout and WT mice. It was thereby puzzling to observe that in the lateral ventricles the size of the inclusions consistently correlated to the position within the medial, dorsal, or lateral walls, respectively, pointing toward functional differences in lipid turnover between ependymal cells in these different locations (Figure 2A, insets). These inclusions were globular or near globular, membrane-bound, and heavily osmiophilic except oblong osmiophobic inclusions suggestive of remnants of ciliae (Figure 2D). However, scanning electron microscopical analysis of the ependymal cells of the lateral ventricle revealed no abnormal ciliae (data not shown). No lipid inclusions were found in the choroid plexus epithelium, despite its close developmental and topographic relationship to ependymal cells. Strikingly, development of lipid inclusions abruptly ceases at the juncture between ependyma and plexus epithelium (Figure 2B, d and e, arrows in insets). Next to the ventricular system, lipid inclusions were especially prominent in the molecular layer of the cerebellar cortex,

Figure 2. Lipid accumulations in the CNS. Oil Red O-positive lipid inclusions are present in the ependymal cells of the entire ventricular system (A–D) and in cerebellar Bergmann glia fibers (E–K) of 5-month-old mice. Within the lateral ventricles (overview in A and B, the labels D, L, M, V indicating the dorsal, lateral, medial, and ventral direction, respectively) the size of the inclusions strikingly correlates with the position of the ependymal cells. They remain small and dust-like in the medial wall (inset a in A) and progressively increase in the dorsal (b) and lateral wall (c). Lipid inclusions are likewise present in the lining of the third ventricle (C) and the central canal of the spinal cord (not shown). On the TEM level, the inclusions appear as round, membrane-bound structures filled with coarse, highly contrasted material in a translucent matrix (D). Note that the choroid plexus epithelium is not affected by lipid storage, which abruptly stops at the junction to the ependymal layer (arrows in insets d and e). In the cerebellar cortex of 5-month-old *MFP-2* knockout mice, Oil Red O-positive inclusions (black arrows in F, compare to aged-matched WT mice in E) are only present in the molecular zone (MoZ), but remain notably absent from the white matter (WM) and the (neuronal) internal granule cell layer (IGL). The lipid droplets line up in straight pearl chain patterns extending to the surface (small arrows in G) and also concentrate on cell bodies (large arrows) between Purkinje cells (PC), both features indicative of Bergmann glial cells. This is further confirmed by a co-stain of Oil Red O and GFAP (H). Although no lipid droplets are detectable even with the more sensitive dark field optics in adult WT cerebella (I), at 4 weeks postnatally lipid droplets accumulate between (but not in) Purkinje cell bodies, but only at low levels in the molecular zone, and sometimes meningeal cells (Men) (J). The characteristic pearl chain pattern is established at 8 weeks (K), but intensifies further later on (compare to F). Scale bars: 200 μm (A–C); 5 μm (D); 100 μm (E, F); 20 μm (G, H); 50 μm (I–K).

Table 1. Lipid Analyses in Cerebellum of *MFP-2* Knockout and Control Mice

	Control	<i>MFP-2</i> knockout	
Phospholipids	50.83 ± 0.75	42.08 ± 1.05	nmol/mg cerebellum
Neutral glycerolipids	1.07 ± 0.44	1.23 ± 0.15	nmol/mg cerebellum
Free cholesterol	33.97 ± 1.90	25.90 ± 1.81*	nmol/mg cerebellum
Cholesteryl esters	0.72 ± 0.34	3.43 ± 0.60†	nmol/mg cerebellum
DHA in PL	12.70 ± 0.31	12.61 ± 0.40	nmol/mg cerebellum
C _{26:0} in PL	16.39 ± 1.15	337.13 ± 22.50†	pmol/mg cerebellum
C _{26:0} in NL	57.12 ± 2.79	68.40 ± 4.11	pmol/mg cerebellum

Values represent means ± SEM of three or four independent samples. **P* < 0.05, †*P* < 0.005.
 PL, phospholipids; NL, neutral lipids.

where they were predominantly present in radial Bergmann glial fibers (Figure 2, E–H). Besides their highly characteristic distribution pattern, this was confirmed by co-staining with Oil Red O and anti-GFAP (Figure 2H). Smaller inclusions, albeit rarely, occurred in Purkinje cell dendrites (data not shown).

Lipid inclusions in both ependyma and cerebellar molecular layer develop between the fourth and eighth post-natal week, ie, clearly after the period during which the first cohort of *MFP-2*-deficient mice dies (shown for Bergmann glia in Figure 2, I–K) and further increase thereafter (Figure 2, compare K with F). Within Bergmann glia, accumulation of lipid droplets follows a striking gradient, affecting cell body and (to a low degree) distal fiber segments first, while the pearl chain pattern of droplet-filled fibers becomes fully visible only from ~8 weeks onward. Lipid accumulations were only rarely found in the cerebral cortex in fibrous astrocytes close to blood vessels (data not shown). Furthermore, lipid droplets were found in the hypothalamus and in the inner segment of the pallidum, where they were always present in the gray matter and no accumulating lipids were seen in the white matter.

To clarify the biochemical nature of the lipid droplets, lipid analysis of the cerebellum, the brain region with the highest amount of lipid droplets, was performed. Cholesterylester levels were found to be fivefold increased but levels of triglycerides and phospholipids were unaltered (Table 1). Analysis of the fatty acid composition revealed that the C_{26:0} levels were at least 10-fold increased in the phospholipid fraction but not in the neutral lipids (including cholesterylesters) (Table 1). Surprisingly, the concentration of DHA, a PUFA that depends on peroxisomal β -oxidation for its synthesis, was the same in phospholipids of *MFP-2* knockout and control mice. Because this was an unexpected finding, food pellets were analyzed for the presence of DHA. This PUFA could indeed be detected (0.09 μ mol/g

pelleted chow), but was substantially lower than the main fatty acids C18:2, C16:0, C18:0, C18:1, and C18:3, respectively 28.80, 9.84, 4.98, 3.47, and 3.04 μ mol/g chow (mean of two determinations).

Increased Levels of Branched Chain Fatty Acids Have No Impact on Disease Progression in MFP-2-Deficient Mice

It is well established that phytanic acid, a 3-methyl branched chain fatty acid is α -oxidized in peroxisomes to pristanic acid, a 2-methyl branched chain fatty acid. The latter is in turn degraded by peroxisomal β -oxidation and *MFP-2* plays an essential role in this process. It was therefore not surprising to find that these branched chain fatty acids were elevated six- to eightfold in plasma (Table 2) and in brain phospholipids (Table 2) of *MFP-2* knockout mice. A deleterious impact of these fatty acids on the central and peripheral nervous system was observed in Refsum disease and in α -methylacyl-CoA racemase deficiency.^{15,17} Typically, plasma levels can reach concentrations of 2 mmol/L in these patients with normal values <30 μ mol/L. The low levels of branched chain fatty acids in *MFP-2* knockout mice (<5 μ mol/L) are probably attributable to very low amounts of 2-methyl branched chain fatty acids or their precursors present in standard mouse chow. We therefore fed 7-week-old *MFP-2* knockout and WT mice a diet supplemented with phytol (5 mg/g food pellets), the precursor of phytanic acid with the purpose to further increase branched chain fatty acid levels in brain (Table 2). An additional group was fed with phytanic acid-enriched food (1 mg/g food pellets).

Both the treated and the untreated knockouts developed a comparable dyskinesia of the hind limbs during the 3-week period whereas no signs of motor impairment were observed in the heterozygous mice. Analysis of

Table 2. Branched Chain Fatty Acid Levels in Plasma and in Whole Brain Homogenates of *MFP-2* Knockout and Control Mice

	Control	<i>MFP-2</i> knockout	
Phytanic acid in plasma	0.21 ± 0.03	1.84 ± 0.40*	μ mol/L
Pristanic acid in plasma	0.73 ± 0.22	4.40 ± 1.45†	μ mol/L
Phytanic acid in PL (standard chow)	ND (<0.5)	1.16 ± 0.78	pmol/mg brain
Phytanic acid in PL (0.5% phytol diet)	21.85 ± 4.20	65.37 ± 10.29	pmol/mg brain
Pristanic acid in PL (standard chow)	ND (<0.5)	0.78 ± 0.17	pmol/mg brain
Pristanic acid in PL (0.5% phytol diet)	ND (<0.5)	69.47 ± 7.10	pmol/mg brain

Values represent means ± SEM of four or five independent samples. **P* < 0.05, †*P* < 0.005.
 ND, not detectable.

brain phospholipids revealed that phytanic acid accumulated massively both in WT and knockout mice treated with phytol (Table 2). On the contrary, severely elevated levels of pristanic acid were only seen in *MFP-2* knockouts treated with phytol (Table 2) or phytanic acid (not shown) but not in WT mice. No differences in the histology of the nervous system were found between WT and *MFP-2* knockout mice treated with 0.5% phytol or with 0.1% phytanic acid. No overt pathological changes were observed in other tissues, all knockout and heterozygous mice survived the phytol treatment.

MFP-2 Deficiency in the Mouse Results in Up-Regulation of Catalase and Severe Gray Matter Gliosis but No Demyelination in the CNS

We further traced the expression of catalase, GFAP, MBP, and synaptophysin to monitor reactions of peroxisomes, astroglia, oligodendroglia/white matter, and neurons, respectively, in different brain regions of *MFP-2*-deficient and WT mice by Western blotting of brain homogenates (Figure 3). Because both the neuromotor deficits and lipid accumulations could only be observed in young adult mice, these analyses were performed from the early postweaning period (3 weeks postnatally) to the prefinal stage (5 months). Both GFAP and catalase were clearly elevated in all brain regions investigated and in spinal cords of 4-month-old *MFP-2* knockout mice (Figure 3A). In contrast, no changes in either MBP or synaptophysin were recorded even at late stages (Figure 3B), indicating that no major degeneration took place in either white matter or synapses. It is striking thereby that although catalase up-regulation was seen early on, GFAP as a brain damage marker was not up-regulated before 8 weeks of age (Figure 3C).

At the immunohistochemical level, up-regulation of catalase and GFAP (Figure 4) was outspoken in gray matter regions, whereas the immunostaining in white matter tracts appears mainly unaltered as compared to controls (note that corpus callosum and fimbria retain a comparatively low staining intensity for both markers, similar to the situation in WT controls). A similar distribution to gray matter and sparing white matter was observed for the microglia marker F4/80 (Figure 5) and the murine microglial activation marker RCA-1 (Figure 5). In line with these results, no obvious change was found with myelin stains such as Luxol fast blue (not shown) or anti-MBP (Figure 5).

Although both astrogliosis and microglia activation are sensitive but relatively unspecific markers of an ongoing brain pathology, their co-distribution with catalase up-regulation confirms that white matter is not the prime target of murine *MFP-2* deficiency. It is striking that neither the increased catalase expression nor astrogliosis and microglia activation coincide in their distribution with the extent of lipid storage. The only partial exception is the cerebellar cortex, where lipid inclusions are strictly limited to the molecular zone (Figure 2), thus coinciding with increased GFAP and catalase immunoreactivity, whereas activated microglia cells were in comparable density present in the Purkinje cell layer and internal granular layer.

Figure 3. Time course of expression of catalase as well as neuronal and glial markers in *MFP-2*-deficient mice. **A:** A significant up-regulation of catalase protein could be seen by Western blotting in brain stem (BS), cerebellum (cere), diencephalon (Di), hippocampus (hippo), cortex, olfactory bulb (OB), and spinal cord (SC), with a concomitant increase of GFAP expression. In whole brain homogenates (shown for 5 months in **B**) the expression of oligodendroglial MBP and neuronal synaptophysin (SY) remained unchanged, indicating the absence of major white matter and neuronal defects. **C:** It is striking thereby that catalase up-regulation is a very early event, clearly being present at ~3 weeks postnatally, a time at which GFAP expression levels were still fully normal. At 8 weeks a slight increase in GFAP expression but clear-cut increase of catalase were noticed (**C**). Incubation times and concentration of the primary and secondary antibodies were the same for all blots but developing time with the alkaline-phosphatase substrate was different.

The up-regulation of catalase (visible from 3 weeks onward) preceded the overexpression of GFAP and F4/80 (visible at 5 weeks), confirming the Western blot data (Figure 6, A–F, and data not shown). The primary involvement of the gray versus the white matter is further confirmed by immunostaining for the lysosome-associated membrane protein-1 (LAMP-1), which revealed an intense staining for lysosomes especially in the gray matter (astrocytes) of 5-month-old *MFP-2* knockout mice (data not shown).

MFP-2-Deficient Astroglial Cells, but Not Neurons, Stain for a Neural Degeneration Marker

To test for cell degeneration, we screened brains of adult *MFP-2*-deficient mice by a Fluoro-Jade assay that stains the membranes of degenerating neurons as well as reactive and degenerating glial cells. By this approach, no degenerating neurons could be identified in any of the

Figure 4. MFP-2 deficiency causes up-regulation of catalase and gliosis in the gray matter. Five-month-old MFP-2-deficient brains are characterized by a significant up-regulation of catalase in all brain regions, whereby only in white matter tracts like fimbria or corpus callosum immunoreactivity is not visibly increased beyond WT level. This increase is paralleled by reactive astrogliosis (GFAP staining) in a similar distribution, again sparing white matter tracts. Co, cortex; Cc, corpus callosum; Fi, fimbria hippocampi; Hi, hippocampus; Cb, cerebellum. Scale bars, 2 mm.

brain regions investigated (shown for isocortex and cerebellum in Figure 6, G, H, and L). Strikingly, astroglial cells and cerebellar Bergmann glia, ie, the same cells that stand out by their early catalase up-regulation, are regularly stained by this technique, whereas their counterparts in WT brains are consistently negative. Only a subpopulation of the Fluoro-Jade-positive cells were stained in a consecutive analysis for apoptotic cell death by antibodies against cleaved caspase 3 (data not shown).

Fluoro-Jade stainability did not fully coincide with lipid storage, as it was, eg, absent in ependymal cells, further confirming that lipid storage is not predetermin-

ing further reactive or degenerative changes and that the main pathology of MFP-2 deficiency is storage-independent. The only possible involvement of neurons is indicated by the presence of scattered Fluoro-Jade-positive axons in the cerebellar white matter (Figure 6, O and P), arguing for an incipient neuronal degeneration that within the lifespan of the mice remains however confined to the axonal compartment. Because in several cases of rhizomelic chondrodysplasia punctata type I, a partial peroxisome biogenesis disorder, selective degeneration and loss of cerebellar Purkinje cells had been observed,^{30,31} their distribution was assessed by staining with calbindin antibodies. Normal

Figure 5. MFP-2 deficiency causes microglia activation in the gray matter, but no apparent white matter defects. The up-regulation of catalase and the astrogliosis seen in the gray matter of MFP-2-deficient mice is paralleled by an intense microglial reaction, both evidenced by a drastically increased stainability for F4/80 and binding of RCA-1, both again sparing white matter tracts like fimbria (Fi) or corpus callosum (Cc). Note that no change is visible in the expression of the myelin marker MBP. Co, cortex; Cc, corpus callosum; Fi, fimbria hippocampi; Hi, hippocampus; Cb, cerebellum. Scale bars, 2 mm.

arborization and density of Purkinje cells were observed (Figure 6, M and N).

Axonal Degeneration in the Spinal Cord of MFP-2-Deficient Mice

A small number of degenerating axons were regularly found in the dorsal tracts. Relatively few axons were affected, and degenerating fibers were not more abundant in the thoracic or cervical spinal cord than in the

lumbar spinal cord. This suggested that these fibers were central processes of lumbar dorsal root ganglion cells. The ventral and lateral white matter tracts were normal in knockout mice. A small number of anterior horn cells in the lumbar spinal cord of knockout mice were vacuolated and contained lipid deposits. Rarely small groups of microglial cells were seen in the anterior horn, possibly marking sites of lost neurons. These changes were, however, rare and were only detectable when a large number of spaced serial sections was

assessed. Comparable changes were never seen in age-matched WT mice.

Lesions Were Absent from Peripheral Nervous System and Skeletal Muscles

In light micrographs of 3- to 5- μ m epoxy sections stained with *p*-phenylenediamine, phrenic, sciatic, tibial, and peroneal nerves of *MFP-2* knockout mice did not show signs of axonal degeneration or of demyelination. No difference as compared to WT control mice could be detected. The number of myelinated fibers in the phrenic nerve of knockout mice was normal. No degenerating fibers, no remnants of axonal degeneration, and no signs of demyelination were found in lumbar ventral roots L4 to L6. Soleus and extensor digitorum longus muscles and the diaphragm of knockout and WT mice did not differ; no atrophic fibers and no fibers with internal nuclei were present in knockout mice.

No demyelination and no signs of axonal regeneration were found in the mixed nerves investigated, in the sural nerve, or in intramuscular nerve branches close to muscle spindles. Some neurons of lumbar dorsal root ganglia in knockout mice contained more lipofuscin than others, but similar differences were found in age-matched WT mice. It was therefore not possible to ascertain neuronal degeneration in knockout mice. Nageotte granules were never found in knockout or in WT mice. Nevertheless, few unequivocally degenerating myelinated fibers were present in some lumbar dorsal roots of knockout mice but not in controls.

The sensory conduction velocity recorded in tail nerves of seven WT and seven knockout mice aged 13 weeks was 32 ± 1.4 m/s in WT mice (age 13 weeks, weight 31.0 ± 0.4 g) and 25 ± 0.7 m/s in knockout mice (age 13 weeks, weight 23 ± 1.4 g), which is not different taking into account the reduced weight of the knockouts. The investigation was repeated both after 6 weeks as well as in severely symptomatic preterminal mice. Again, no change in conduction velocity was noted.

Nerve Regeneration

To test whether degradation of fatty waste may be impeded in *MFP-2*-deficient mice, the breakdown of myelin was investigated which is a crucial step in Wallerian degeneration without which nerves do not regenerate. The right sciatic nerve was crushed in seven *MFP-2* knockout and in seven WT mice aged 13 weeks. The compound motor action potentials of the gastrocnemius

muscles were recorded before the operation and after 6 weeks when the toe-spreading reflex had recovered in all mice. The regenerated tibial nerves were investigated histologically.

The compound motor action potentials before denervation was 53 ± 5 mV in knockout as compared to 54 ± 6 mV in WT mice; after regeneration it had recovered to 53 ± 3 mV in knockout and to 54 ± 4 mV in WT mice. The motor latencies increased from 1 ± 0.04 ms to 1.5 ± 0.06 ms in knockout and from 0.9 ± 0.04 ms to 1.4 ± 0.06 ms in WT mice. The regenerated tibial nerves of both knockout and WT mice contained numerous new myelinated nerve fibers, removal of debris was almost completed, and no difference between the two groups of mice was detectable.

Discussion

Murine MFP-2 Deficiency Shows Major Differences to Human Inactivating MFP-2 Mutations

In man, inactivating mutations of the peroxisomal β -oxidation enzyme *MFP-2* cause a severe pathology, resembling the peroxisome biogenesis disorder, Zellweger syndrome. It is characterized by neonatal hypotonia and seizures, neuronal migration defects, and early postnatal death.¹³ In contrast, in the mouse the neurodevelopmental abnormalities, hypotonia, and seizures are absent,²⁰ but a significant fraction of the mice dies postnatally with a general failure to thrive related to metabolic problems.³² The surviving knockouts develop during early adulthood abnormal extension reflexes of the limbs, biochemically and histopathologically paralleled by an overexpression of catalase in glial cells, astrogliosis and microglia activation, and possible signs of glial cell degeneration in the CNS gray matter, ie, features not known from human patients.

The Clinical and Biochemical Phenotype of Murine MFP-2 Deficiency Resembles that of Peroxisomal Defects with Accumulating Branched Chain Fatty Acids

In humans, a somewhat related clinical course, histopathologically characterized by a slowly developing, progressive peripheral neuropathy and by severe motor weakness, retinopathy, and ataxia is known from two

Figure 6. Increased reactivity for catalase and glial activation and degeneration. Increased catalase immunoreactivity in Bergmann glial fibers is visible as early as 3 weeks postnatally (shown at 5 weeks in **A** and **B**) and becomes more evident thereafter. **C** and **D** show a stain with GFAP and catalase on adjacent sections at 8 weeks. **E** and **F** a comparison of WT and *MFP-2*^{-/-} mice at 22 weeks (see **insets** in **F** for a double stain of catalase and GFAP in Bergmann glia at 22 weeks). A similar localization of catalase overexpression to astroglial cells is seen in the neocortex (shown at 22 weeks in **I–K**). Intriguingly, similar cells in *MFP-2*-deficient, but not WT brains stain positive for Fluoro-Jade B, a marker for neuronal and possibly astroglial degeneration and glial activation (**G**, **H**, and **L**). Whereas the localization of Fluoro-Jade reactivity to Bergmann glial fibers (**G**, **H**) is evident from their unique morphology, the direct vascular and surface relationship (processes with endfeet formation) serves as an additional criterion for neocortical astrocytes next to their morphology (**arrow** in **L**). Purkinje cells, as stained by anti-calbindin, are present in normal numbers (**M**, **N**) and do not stain for Fluoro-Jade (**G**, **H**). However, in the cerebellar, but not cerebral white matter a moderate number of Fluoro-Jade-positive axons is invariably present (**O**, **P**), in knockout, but not WT mice. Scale bars: 40 μ m (**A–H**); 60 μ m (**I**, **J**); 40 μ m (**K**, **L**); 120 μ m (**M–P**).

peroxisomal diseases, Refsum disease and α -methylacyl-CoA racemase deficiency.^{15,17} Because both diseases have in common the accumulation of branched chain fatty acids (phytanic and/or pristanic acid) in blood and tissues it was hypothesized that they are progressively deleterious agents and are responsible for the adult-onset neurological defects. As a striking parallel on first glance, MFP-2-deficient mice exhibit a similar pattern, not only clinically developing late-onset motoric dysfunction, but also retinopathy (Huyghe et al, unpublished observations) and accumulation of both phytanic and pristanic acid in plasma.

Our extensive screen did not reveal any signs of a peripheral sensorimotor neuropathy because both skeletal muscles and peripheral nerves even at late stages were consistently free from signs of cell degeneration or any evidence of pathological changes in their physiological characteristics. Only the scattered profiles of degenerating axons within the spinal cord dorsal funiculus could possibly be interpreted as related to a low-level damage to or loss of spinal ganglion cells. However, the absence of any substantial damage within the ganglia themselves indicates that such a mechanism is unlikely to contribute to a major degree to the observed neurological deficits. Finally, the normal regeneration capacity even after severe experimental trauma to a peripheral nerve including the timely removal of lipid debris basically rules out major problems of the turnover of peripheral nerve constituents including myelin sheaths as an underlying cause. These findings are in agreement with data from human MFP-2 patients, in which biopsies failed to reveal any muscular lesion.³³ The dissimilarity with Refsum disease and α -methylacyl-CoA racemase deficiency was further illustrated by the fact that elevating the levels of branched chain fatty acids in MFP-2 knockout mice did not aggravate the phenotype.

Murine MFP-2 Deficiency Causes Overexpression of Catalase in Astroglial Cells and Lipid Storage in Ependyma and Bergmann Glia

The earliest and most sensitive marker changing postnatally with MFP-2 deficiency is the overexpression of catalase, both evidenced by Western blotting and immunohistochemical stainings in glial cells and some brain endothelia from as early as 3 weeks post partum onwards. The cause and significance of this up-regulation is unclear at this point, but it was also seen in liver tissue of MFP-2 knockout mice and was shown not to be accompanied by increased catalase transcripts in either tissue (S. Huyghe, unpublished observations).

At a later stage, we observed a conspicuous accumulation of Oil Red O- and Sudan Black-positive lipid inclusions within cerebellar Bergmann glia fibers and the ependymal cells of the entire ventricular system of the CNS, ranging from the lateral ventricles down to the spinal canal, which became evident after the fourth postnatal week. Strikingly, choroid plexus epithelium was un-

affected, whereby it remains unclear as yet whether the eg, absent vascular blood brain barrier or their active secretory and transport function during CSF formation could be linked to this difference. Histological examinations in brains of MFP-2-deficient patients are scarce but in a few pioneering reports on patients with peroxisomal β -oxidation defects lipid droplet accumulations were reported^{34,35} besides demyelination, gliosis, and Purkinje cell heterotopias. These patients were recently reinvestigated and identified as MFP-2-deficient patients.^{36,37}

As yet, the nature of the stored lipids in the ependymal cells of the CNS and in the molecular layer of the cerebellum remains obscure but the absence of any major degeneration in lipid-storing cells indicates that the accumulated lipids are not toxic. Biochemical analysis documented increased levels of cholesterylesters in cerebellum, but no marked accumulation of fatty acids that are substrates of the peroxisomal β -oxidation pathway were found in this lipid fraction. In contrast, in the phospholipids of MFP-2 knockout mice extensive accumulation of the very-long-chain fatty acid C_{26:0} was found. It is striking that the lipid droplet accumulation is confined to the astroglial lineage and almost selectively avoids neurons and white matter. The reason for this selectivity of lipid accumulation remains enigmatic so far.

Glial Cells of the Gray Matter Show a Pathological Activation and Stainability for a Degeneration Marker

In parallel with the lipid storage and catalase overexpression within the CNS, MFP-2-deficient mice are characterized by a combination of astrogliosis and microglia activation during late juvenile and young adult life predominantly in the gray, but not in white matter regions of the entire CNS. Interestingly, lipid storage and glial activation only partially coincide in spatial distribution. They co-localize perfectly in Bergmann glial fibers, which featured both lipid storage and increased immunoreactivity for GFAP. However, in the Purkinje and granule cell layer only microglia activation and increased density of stellate astrocytes were observed, which could contribute to the motor symptoms, whereas ependymal cells only display intense storage of lipids. A significantly more stringent correlation exists between catalase overexpression and the gray matter astrogliosis.

A screen for cell degeneration using the neural degeneration marker Fluoro-Jade did not, except for some scattered axons in the cerebellum, decorate neurons, but only and systematically astroglia and the related Bergmann glia, a pattern that has also been found in prion disease and caused speculations that Fluoro-Jade may also be used to identify degenerating glia before large-scale overt cell death.²³ The striking aspect of these results is again the strict confinement of cellular lesions to astroglia, ie, the absence of any marker for cell degeneration or death in neurons.

Could a Primarily Glial Lesion Cause Neuromotor Deficits?

The timetable of the glial lesions closely parallels the development of the finally fatal motor deficits and, given the absence of any discernible defect within the peripheral nervous system thus in all probability is a key factor in their pathogenesis. It remains unclear as yet, how precisely a loss of MFP-2 causes these severe glial lesions and how these are translated to neuronal dysfunction that stops short of overt neuronal degeneration. However, with respect to the metabolic functions of astroglial cells within the brain, some possibilities can be envisaged.

At present, one example of an astrocyte-based peroxisomal function to support neurons is known ie, the synthesis of the ω -3 PUFA DHA from ω -3 fatty acid precursors which requires a final single peroxisomal β -oxidation cycle after elongation and desaturation steps in the endoplasmic reticulum.^{38–40} Quite unexpectedly, no shortage of DHA was found in the MFP-2-deficient mouse brain, despite the fact that MFP-2 was shown to take part in the final oxidation step.^{41,42} This can possibly be ascribed to supplementation of DHA to the brain via the diet and the economic way by which the brain conserves DHA levels when it senses a decrease in DHA availability or concentration.⁴³ It is difficult to envisage alternative synthesis routes bypassing MFP-2, because studies with human fibroblasts⁴¹ and mouse fibroblasts and hepatocytes (P.P. Van Veldhoven, unpublished observations) demonstrated that β -oxidation of C_{24:6n-3} was impaired in MFP-2- but not in MFP-1-deficient cells. In fact, astroglia not only forms a metabolic factory for the neighboring neurons, but also constitutes a metabolic shield in between neurons and the blood-brain-barrier proper (ie, CNS capillary endothelia). Because any substance entering or leaving the neuronal compartment from and to the bloodstream has to pass these cells, the inability, because of MFP-2 deficiency, to degrade or modify certain lipids may indeed manifest first in these cells.

If astroglial cells are indeed predisposed as the prime victims of MFP-2 deficiency, it is intriguing that they are affected only in certain brain regions, ie, generally gray matter and cerebellar molecular zone, but not white matter. This argues against a generalized inability to handle lipids coming in from the blood stream and underscores a causative role of lipids secreted from the specific cellular environment. Accordingly, such lipids released within the gray matter (so most probably being derived from neurons) would then be more detrimental than those of the white matter.

As a second option next to a direct metabolic insufficiency of glial cells causing neuronal dysfunction, the mere pathological activation of astrocytes and microglia has repeatedly been shown to be a neurotoxic condition by itself. Giulian and co-workers^{44,45} have already shown during the 1980s that *in vitro* activated glia cells secrete neurotoxic factors, and the list of cytokines involved has grown ever since. Accordingly, in several neurodegenerative diseases such as, eg, Alzheimer's disease neurodegeneration is attributed to activated glial cells in the tissue

rather than a direct neuronal cell defect; and it is thus conceivable similar effects may play a role here as well.

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