A key role for the peroxisomal ABCD2 transporter in fatty acid homeostasis

Stéphane Fourcade, Montserrat Ruiz, Carme Camps, Agatha Schlüter, Sander M. Houten, Petra A. W. Mooyer, Teresa Pamps, Georges Dacremont, Ronald J. A. Wanders, Marisa Giròs, and Aurora Pujol. A key role for the peroxisomal ABCD2 transporter in fatty acid homeostasis. Am J Physiol Endocrinol Metab 296: E211–E221, 2009. First published October 14, 2008; doi:10.1152/ajpendo.90736.2008.—Peroxisomes are essential organelles exerting key functions in fatty acid metabolism such as the degradation of very long-chain fatty acids (VLCFAs). VLCFAs accumulate in X-adrenoleukodystrophy (X-ALD), a disease caused by deficiency of the Abcd1 peroxisomal transporter. Its closest homologue, Abcd2, exhibits a high degree of functional redundancy on the catalabolism of VLCFA, being able to prevent X-ALD-related neurodegeneration in the mouse. In the search for specific roles of Abcd2, we screened fatty acid profiles in organs and primary neurons of mutant knockout mice lacking Abcd2, we screened fatty acid profiles in organs and primary neurons of mutant knockout mice lacking Abcd2, demonstrating a defective VLCFA degradation of long-chain saturated and monounsaturated fatty acids in brain (51). A growing body of evidence based on epidemiological and interventional studies shows that low levels of DHA may play a role in age-related neurodegenerative diseases and depression (30, 39, 53). The precursors of DHA and EPA, C24:6 and C24:5, respectively, are synthesized in the endoplasmic reticulum and transported to peroxisomes, where they must be imported across the membrane to undergo shortening via β-oxidation in the peroxisomal matrix (59). Four ATP-binding cassette transporters (ABC transporters) have been identified at the peroxisomal membrane in mammals (ABCD1 to ABCD4 genes, encoding homologous proteins). Rather than being full transporters such as the cystic fibrosis transmembrane regulator (CFTR, ABC7) or the multidrug resistance gene (MDR1, ABC1), the peroxisomal ABCDs are half-size transporters and need to homo or heterodimerize to become functional. ABCDs are thought to be involved in the transport of fatty acids, based on three principal findings: 1) peroxisomal membranes are not freely permeable, the entrance and exit of metabolites requires the presence of peroxisomal transporter proteins; 2) the two peroxisomal ABC transporters of Saccharomyces cerevisiae, Pxa1 and Pxa2, form functional heterodimers that transports long-chain fatty acyl-CoAs into the peroxisome (20, 63); and 3) inactivation of ABCD1 causes the human disease X-linked adrenoleukodystrophy (X-ALD), which is biochemically characterized by the pathognomonic accumulation of saturated VLCFAs, mainly hexacosanoic acid (C26:0), in plasma and tissues. Oxidation of the VLCFA C26:0 and C24:0 is impaired in X-ALD fibroblasts, suggesting that ABCD1 is involved in the import of VLCFAs into the peroxisome for degradation (45). Thus the observed β-oxidation defect must be secondary to the impaired transport of the substrate across the peroxisomal membrane in

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the absence of a functional transporter in these cells (20, 58, 64). In analogy with the work in yeast, it has been postulated that ABCD1 transports the CoA esters of VLCFAs across the peroxisomal membrane in mammals (63).

The four human ABCD transporters share a high sequence homology, suggesting similarity of function to a certain degree. ABCD2 shares 80%, ABCD3 shares 60%, and ABCD4 shares 46% of homology with ABCD1 (24, 33, 57). The function of ABCD4 has not been studied yet; mice deficient for ABCD3 have been shown to accumulate bile acid precursors [3α,7α,12β-trihydroxy-5β-cholestan-26-oic acid (THCA) and 3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA)] and pristanic acid (Wanders, R. J. A., personal communication). ABCD2 and ABCD3 can compensate for β-oxidation defects in X-ALD fibroblasts when overexpressed (42). Recently, we (12, 47) reported a functional overlap between ABCD1 and ABCD2 transporters on the metabolism of VLCFA in vivo, as concluded from observations in mouse models in which either Abcd1 or Abcd2 was disrupted or overexpressed. However, the differences in sequence, expression patterns (62), and phenotype of these mice suggest specific roles for ABCD2 in fatty acid homeostasis. Indeed, Abcd2−/− mice exhibit a late-onset cerebellar and sensory ataxia, areflexia, loss of cerebellar Purkinje cells, and dorsal root ganglia cell degeneration (12), together with oxidative damage in adrenal gland (35). These pathological features, not shared with Abcd1 null mice, might indicate that substrate specificity occurs in spite of a certain degree of overlap on C26:0 and/or that expression patterns at the cellular level are different between both and so are the cell types most sensitive to demise upon loss of function (62). In the present study, we take advantage of the available Abcd1 and Abcd2 null mice and set out to gain insight into ABCD2 biochemical roles related to peroxisomal metabolism. After comparative genomics analysis, we determined the composition of fatty acids of tissues with good expression of Abcd2, and subsequently, designed functional tests aimed to give strength to our observations, such as dietary manipulations and fasting. Of particular relevance, we set up an ex vivo peroxisomal β-oxidation assay, which allowed us to directly assess the role of ABCD2 and ABCD1 in fatty acid oxidation in the mouse brain.

MATERIALS AND METHODS

Comparative genomic analyses. BLAST (2) searches obtained all recognizable ABCD paralogues from 39 eukaryotic genome projects by using ENSEMBL genomes, National Center for Biotechnology Information genomes, IntegR8 portal maintained by the European Bioinformatics Institute, Sanger Institute, Center for the Study of Biological Complexity, The Institute for Genomic Research, BROAD Institute, The Josephine Bay Paul Center in Comparative Molecular Biology and Evolution, Joint Genome Institute, and Cyanobioschyzon merolae Genome Project.

Multiple and phylogenetic analyses. ABCD proteins were analyzed by ClustalX (61) and refined manually with MEGA version 3.0 (27) and GeneDOC (43). Insertions and sequence characters not alignable with confidence and incomplete sequences were removed. The final phylogenetic alignment included 87 taxa. The phylogenetic tree was created using ProtTest (1). ProtTest selects the most appropriate model of protein evolution among 64 different ones based on the smallest Akaike Information Criterion or Bayesian Information Criterion score. This software takes advantage of the PAL library (10) and of the Phyml program (16). The best-fit model of protein evolution for ABCD protein family according to ProtTest corresponds to a RtREV model with variable site parameter (+I), different evolution rate among sites (+G), and site frequencies (+F). The ABCD1 and ABCD2 incomplete sequences from draft genomes (for instance, Pan troglodytes, Takifugu rubripes, Tetraodon nigroviridis, and Bos taurus) have not been included for the sake of reliability of the multialignement and phylogenetic tree.

Transmembrane predictors. Secondary structure and transmembrane predictions of ABCD1 and ABCD2 were performed using PredictProtein (www.predictprotein.org; Ref. 30) and PHDhtm, a program to predict the location and topology of transmembrane helices from multiple sequence alignments (49).

Mouse breeding. The genotyping of Abcd1−/− and Abcd2−/− mice has been previously described (12, 36). To obtain double heterozygous mutants, we crossed Abcd1 null females (mutation is on the X chromosome) with Abcd2−/− males. Double heterozygous in the F1 generation were intercrossed to obtain double knockout mice and wild-type littermate controls; the offspring obeyed Mendelian ratios. Mice used for quantitative-PCR experiments and fatty acid analysis in serum were on a pure C57BL/6J background (backcrossed at least 12 generations into C57BL/6J), whereas for the rest of experiments they were on a mixed C57BL/6J/129Sv background (~87% C57BL/6J and ~13% 129Sv). Only male littermates were used for all the experiments. All animals were housed under the same controlled conditions between 22 and 25 °C on a 12-h light-dark cycle with free access to food and water. Animals were killed, and tissues including the brain, spinal cord, sciatic nerve, adrenal gland, and liver were snap frozen in liquid nitrogen and conserved at −80°C. All methods employed in this work are in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85–23, revised 1996). The project was authorized by the IDIBELL IACUC, Barcelona.

Mouse diets. Animals were fed with either a standard chow ad libitum consisting of 5.5% fat, 23% protein, and 50% digestible carbohydrate (D03, SAFE, Scientific Animal Food and Engineering, Villemoisson sur Orge, France), or a diet based on the standard chow enriched in saturated VLCFAs (UAR, ref: UAR9124CH, SAFE, Villemoisson sur Orge, France). For fasting experiments, animals were fed with the standard chow ad libitum or fasted for 48 h. Only males and pure background animals were used for these studies.

Fatty acid determination. Tissues from three to eight mice of each genotype were dissected and stored frozen at −80°C. After chloroform/methanol extraction (2.1, v/v), the upper phase was removed and the lower phase was dried under nitrogen and weighted for total lipid weight. A volume of 0.1 ml of lower phase fatty acids were subjected to one-step transmethylation after the Lepage and Roy method (31), quantified only. We measured C16:0, C18:0, C20:0, C22:0, C24:0, C18:1, C20:1, C22:1, C24:1, C18:2, C20:3, C20:4, C22:4, C22:5n6, C18:3, C20:5, C22:5, and C22:6n3. Unless otherwise stated, sig-
significant differences have been determined by ANOVA followed by post hoc Tukey’s honestly significantly different test (P ≤ 0.05, P = 0.01, and P ≤ 0.001) or by Wilcoxon test (P ≤ 0.05).

Hippocampal-cortical primary neurons culture. Hippocampi and cerebral cortices were dissected from E16 mice embryos of pure C57BL/6J background, and the blood vessels and meninges were removed under microscope and placed in ice-cold HBSS. Tissue was harvested and fatty acid levels were quantified.

Cortical slices preparation and peroxisomal fatty acid β-oxidation. The brain from 7-wk-old male mice on C57BL/6J was immediately removed; the cortex was dissected and chopped into 350-μm slices in the paraxial plane using a McIlwain tissue chopper. The resulting m slices in -oxidation.

Synthesis of [1-14C]6,9,12,-36 was purified on a small silica gel column with hexane-ether-acetic acid (60:40:1; v/v/v) as eluent. Thin-layer chromatography of the purified acid showed only one labeled band, and GC-MS analysis of the methyl ester showed one single peak with a molecular ion at m/z 367 ([C14]M+ 4.5%) and 365 ([C12]M+ 0.5%). The synthesized [1-14C]24:6n-3 had a specific activity of 50 mCi/ mmol (11).

RNA extraction and quantitative real-time PCR. Total RNA was extracted using RNeasy Kit (Qiagen), and quantitative-PCR experiments were performed according to manufacturer’s instructions (LightCycler, Roche Diagnostics). PCR were carried out with 36b4 (also called Rpl0) used as a standard gene. The nucleotide sequences of primers are available (Supplemental Figs. 1 and 2 and Supplemental Table 2). Data are means ± SE.

RESULTS

Origin of ABCD1 and ABCD2. Phylogenetic analysis of ABCD family members of 39 eukaryotic full sequenced genomes indicated that ABCD1 and ABCD2 are close paralogues that diverged rather late in evolution at the vertebrate lineage (Fig. 1). Indeed, the ascidian Ciona intestinalis, a sea squirt and the only chordate invertebrate whose genome is available, contains only one bona fide ABCD, similar to both ABCD1 and ABCD2. The same is true for worms and insectae; only

![Phylogenetic tree of ABCDs](http://ajpendo.physiology.org/)

Fig. 1. Phylogenetic tree of ABCDs. Scale length indicates 0.5 substitutions per site. Incomplete sequences are not represented for the sake of reliability of the phylogenetic distribution. Human: Homo sapiens; Chimp: Pan troglodytes; Dog: Canis familiaris; Cow: Bos Taurus; Mouse: Mus musculus; Rat: Rattus norvegicus; Chicken: Gallus gallus; Tetra: Tetraodon nigroviridis; Fugu: Takifugu rubripes; Zebraf: Danio rerio; Frog: Xenopus tropicalis; Ciona: Ciona intestinalis; Cele: Caenorhabditis elegans; Brig: Caenorhabditis briggsae; Honeybee: Apis mellifera; Mosquito: Anopheles gambiae; Ylipo: Yarriowia lipolytica; Afum: Aspergillus fumigatus; Ncras: Neurospora crassa; Pchry: Phanerochaete chrysor- Phanerochaete chrysosporium; Cne: Cryptococcus neoformans; Dhans: Debaryomyces hansenii; Klac: Kluyveromyces lactis; Egos: Eremothecium; gossypii; Scer: Saccharomyces cerevisiae; Cgla: Candida glabrata; Lmajor: Leishmana- nia; major; Ddis: Dictyostelium discoid- um; Cner: Cyanidioschyzon merolae; Rice: Oryza; sativa; Atha: Arabidopsis thali- ana; and Tpsu: Thalassiosira pseudonana.)
one ABCD halfway between ABCD1 and ABCD2 can be found in their genomes. Plants, here represented by Oryza sativa and Arabidopsis thaliana, do not have any ABCD1-ABCD2 homologue; their closest ABCD, the comatosome protein, is more similar to ABCD3 than to ABCD1 in contrast to previous reports (13). Fungi have two ABCD related proteins: Pxa2p and Pxa1p, which appear closer to ABCD1-2 than to ABCD3 or ABCD4, although they are more similar to one another than to any metazoan ABCD (Fig. 1 and Mr Bayes phylogenetic tree in www.peroxisomeDB.org; Ref. 54). Compared with model organisms such as yeasts, drosophila, or Caenorhabditis elegans, the mouse thus appears as a suitable model to resolve the biological function of ABCD1 and ABCD2.

Nonconserved domains between ABCD1 and ABCD2. The human ABCD1 and ABCD2 proteins share an overall 80% homology. The most conserved part encompasses the ABC domain (92% of homology), whereas the N-terminal part (1-496 in ABCD2), exhibits only 74% of homology (Supplemental Fig. 1). To get a better view on the nonconserved domains between ABCD1 and ABCD2, we performed a multialignment by CLUSTALW, excluding the ABC domain. This revealed three main nonconserved regions: 1) the largest region of divergence is located in the first 100 N-terminal residues; 2) the second region is encompassed between amino acids 380 and 395 with an extra motif for ABCD1; and 3) the third region is located between amino acids 460-480. Based on transmembrane predictors (see MATERIALS AND METHODS), these three regions should be located outside the intramembrane domains, suggesting involvement in the conformation of the ligand binding domain, and thus might account for substrate specificities between ABCD1 and ABCD2 (Supplemental Fig. 1).

Changes in the fatty acid profile of the sciatic nerve, adrenal gland, and spinal cord from Abcd2<sup>–/–</sup> and Abcd1<sup>–/–</sup> Abcd2<sup>–/–</sup> mice. Fatty acids are delivered to the either complexed into lipoproteins or as nonesterified fatty acids, which enter the cells via fatty acid transporters (17). Once in cells, the nonesterified fatty acids are rapidly converted to fatty acyl-CoA thioesters by acyl-CoA synthetases, specific for carbon length. Four types of fatty acids are most commonly found in tissues: saturated, ω9 monounsaturated, and ω6 fatty acids as acyl-CoA esters, and the free fatty acids, the latter in little amounts. We have shown the altered profiles in Fig. 2.

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Fig. 2. C20:0 levels in adrenal gland, sciatic nerve, and spinal cord from 8-mo-old wild-type (Wt), Abcd1−/−, Abcd2−/−, and Abcd1−/−/Abcd2−/− mice (A). ω9-monounsaturated fatty acids levels in adrenal gland (B), in sciatic nerve (C), and in spinal cord (D) from 8-mo-old mice. C22:6ω3 levels (E), C22:5ω6 levels (F), and C22:5ω6-to-C22:4ω6 ratio (G) in adrenal gland, in sciatic nerve, and in spinal cord from 8-mo-old mice. Concentration of fatty acid in total tissue lipids is expressed as percentage of total fatty acids relative to Wt of 3–8 samples. For adrenal gland and sciatic nerve, a given value results from pooling the organs of 2–4 animals, respectively. A minimum of 3 independent pools were used for lipid analysis. Significant differences have been determined by ANOVA followed by Tukey’s honestly significantly different (HSD) post hoc (*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001).
diet, reaching statistical significance at 12 mo (Fig. 3A). C24:0 and C26:0 levels were raised as well, although not enough to be statistically different from controls. No effect of the diet was noticed on the ω3-, ω6-, or ω9-fatty acid series; also, no additional differences were observed in the other organs analyzed.

ABCD2 plays a role in fatty acid catabolism during fasting. Fasting is a physiological stress condition that leads to lipolysis in adipose tissue and the release of free fatty acids into the circulation, which are subsequently taken up and degraded as an energy source in most organs. We investigated whether ABCD2 could have a functional role in fasting by first checking its expression levels. Interestingly, Abcd2 mRNA levels were increased 2.8-fold times in livers from mice fasted for 48h (Fig. 3B). Knowing this, we hypothesized that the fatty acids transported via Abcd2 under normal conditions, would most likely accumulate in Abcd2 knockouts in a given situation that would require extra-work to be performed by Abcd2. We then fasted wild-type and Abcd2−/− mice for 48h and quantified fatty acid levels in serum (Fig. 3C). We noticed that C22:1ω9 content was higher in Abcd2−/− mice under basal and fasting conditions. Moreover, while levels of circulating C20:0 decreased in wild-type mice on fasting, most likely because of degradation in liver or adipose tissue obeying energetic requirements, in Abcd2 knockouts this was not the case. These results reinforce the notion that ABCD2 could be involved in the degradation of saturated VLCFAs and ω9-MUFAs.

Changes in the fatty acid profile of primary hippocampal-cortical culture from Abcd2−/− and Abcd1−/−/Abcd2−/− mice. Because Abcd2 is highly expressed in mouse hippocampal and cortical neurons, particularly during differentiation in embryonic development (12, 62), we investigated the fatty acid profile in hippocampal-cortical primary neurons from E16 mouse embryos (Abcd1−/−, Abcd2−/−, Abcd1−/Abcd2−/−, and wild-type controls). After 4 days of culture, levels of saturated and monounsaturated fatty acids and PUFAs were measured. As observed in several other tissues, C20:0 and C20:1ω9 levels were increased in Abcd2−/− and Abcd1−/Abcd2−/− neurons. The PUFAs C22:6ω3 and C22:5ω6 were lowered in Abcd1−, Abcd2−/−, and Abcd1−/Abcd2−/− neurons, again suggesting a role for Abcd2 (and also Abcd1) in C22:6ω3 and C22:5ω6 biosynthesis (Fig. 4A).

Decreased C26:0 and C24:6ω3 β-oxidation rates in cortical slices from Abcd1−/−, Abcd2−/−, and Abcd1−/Abcd2−/− mice. To demonstrate directly a role of ABCD2 in the oxidation of VLCFAs and PUFAs, we performed β-oxidation assays with radiolabeled fatty acids as a measure of fatty acid transport across the peroxisomal membrane. We chose as a target tissue the brain cortex, which provides a sufficient amount of material and highly expressed Abcd2. To create conditions as close as possible to the in vivo situation, we used freshly chopped cortical brain slices from the different mouse models as starting material, and prepared cortical brain slices from 6-wk-old Abcd1−/−, Abcd2−/−, and Abcd1−/Abcd2−/− mice. As we needed an internal control to validate the experiment, we sought to test the capacity of Abcd1− intact tissue to degrade C26:0, which we expected to be defective as is the case in human X-ALD fibroblasts (65). We incubated cortical slices with 1,14C-radiolabeled C26:0 during 3 h to demonstrate for the first time a marked impairment up to 50% of C26:0 β-oxidation in tissues from Abcd1− mice. The same result holds true for Abcd2−/− and Abcd1−/Abcd2−/− mice, as a further proof of functional redundancy between both transporters (Fig. 4B). This functional assay confirms that the detected accumulation of C26:0 in the brain (Abcd1− and Abcd1−/
**Abcd2 in Fatty Acid Metabolism**

A C20:0, C20:1ω9, C22:6ω3, and C22:5ω6 in primary neurons

![Graph A](image)

B C26:0 and C24:6ω3 β-oxidation in cortical slices

![Graph B](image)

**Abcd2**/− mice; Refs. 14, 36, 46) is indeed due to an impaired oxidation of this fatty acid. Next, we incubated the slices of the different mutants with 1-14C-radiolabeled C24:6ω3. We evidenced a 25% decrease in the capacity to β-oxidize C24:6ω3 in Abcd2**/−** and Abcd1**/−**/Abcd2**/−** mice. Interestingly, we found no deficiency of C24:6ω3 oxidation in Abcd1**/−** mice (Fig. 4B). These results indicate a selective function of Abcd2 in the chain shortening of C24:6ω3 or synthesis of DHA in the cortex, as the underlying reason for the lowered DHA levels found in primary neurons.

**Lack of transcriptional effects on sterol regulatory element binding protein, peroxisome proliferator-activated receptor, liver X receptor-α, or hepatocyte nuclear factor 4α target genes.** Fatty acyl-CoAs are metabolized in many different metabolic pathways (β-oxidation, elongation, desaturation, triglyceride or cholesterol synthesis, and prostanoid or leukotriene synthesis), where each intermediate metabolite or end product can be responsible for the transcriptional effect of LCFAs. For instance, it was shown that nonesterified fatty acids, long chain acyl-CoAs, lipoxygenase-derived metabolite leukotriene B4, prostacyclins, and 15-deoxy-12,14-prostaglandin J2 are potent regulators of transcription depending upon the gene considered and the cell-specific context; for general review see Refs. 34, 41. In particular, the manipulation of ω3-PUFA and/or the ratio of ω6-PUFA to ω3-PUFA may influence the expression of many genes in the brain (52). Specific PUFAs might act as ligands of several nuclear receptors, such as peroxisome proliferator-activated receptors-α, -β, and -γ (PPARs), liver X receptor-α (LXRα), hepatocyte nuclear factor 4α (HNF4α), or as modulators of nuclear transcription factors such as sterol regulatory element binding protein (SREBP; Refs. 9, 23). To investigate whether disturbances in metabolic pathways controlled by these transcription factors might be affected in our mouse models, we analyzed the expression levels of selected target genes. We studied acyl-CoA oxidase (Acox1), fatty acid synthase (Fasn; Ref. 32), Aca1, Aca1g (56), stearoyl-CoA desaturase 1 (Sdes1; Ref. 32), δ5-desaturase (Fads1), and δ6-desaturase (Fads2; Ref. 48) mRNA expression levels in the spinal cord, sciatic nerve, and adrenal gland from 12-mo-old Abcd1**/−**, Abcd2**/−**, and Abcd1**/−**/Abcd2**/−** mice. We found no differences in the expression levels of the above-mentioned genes in any of the mutants (data not shown), which suggests that despite significant changes in the fatty acid profiles, transcriptional dysregulation of key genes in pathways controlled by SREBP, PPARs, LXRα, and HNF4α does not occur.

**DISCUSSION**

We have previously shown a functional overlap between Abcd1 and Abcd2 peroxisomal transporters. Overexpression of Abcd2 is able to correct the defective catabolism of VLCFA in Abcd1**/−** mice and even to prevent the development of a late onset neurodegenerative phenotype caused by loss of function of Abcd1 (12, 46). Phylogenetic analysis suggests that the divergence of ABCD1 and ABCD2 originated as the vertebrate lineage appeared. Both transporters are well conserved among all sequenced vertebrate genomes, strongly suggesting that
they have kept specific substrates and functions. It is tantalizing to speculate that this divergence could represent a “gain of function,” belonging to an integrated evolutionary program and necessary to allow the development of higher complexity systems, i.e., the nervous system of vertebrates, for which compounds such as DHA are of paramount importance. In search for ABCD2-specific roles, we set out to explore fatty acid profiles in the tissues of the three different mouse mutants and used dietary manipulation and fasting to give strength to our observations. We readily detected an accumulation of saturated C20:0 and \( \omega \)-monounsaturated LCFA:s in an ABCD2-dependent manner, indicating that this transporter is involved in the homeostasis of these fatty acids. Since the different ABCDs have been suggested to act as metabolite transporters, one simple explanation would be that Abcd2 either as homo- or heterodimer would catalyze the uptake of these fatty acids, as CoA-ester or otherwise, across the peroxisomal membrane. A diet enriched in saturated VLCFA:s strengthened this defect as a function of time. Fasting is a metabolic stress condition that results in upregulation of Abcd2 expression; analysis of serum from \( \text{Abcd2}^{+/+} \) fasted mice therefore provided further evidence pointing to the impaired degradation of C20:0, most likely as a mirror of the situation in liver or white adipose tissue. Concerning the \( \omega \)9 series, our results are in agreement with other studies in humans, suggesting that erucic acid (22:1\( \omega \)9) degradation is preferentially peroxisomal (5, 7, 44). Moreover, accumulation of C20:0, C20:1\( \omega \)9, C22:1\( \omega \)9, and C24:1\( \omega \)9 has been noticed in Zellweger fibroblasts (3) but not in X-ALD fibroblasts. This indicates that \( \omega \)-monounsaturated VLCFA:catabolism requires functional peroxisomes but is independent of Abcd1, at least in fibroblasts (3, 6). In conclusion, our observations underline the role of peroxisomes in the degradation of saturated and \( \omega \)-monounsaturated LCFA:s and VLCFA:s and uncover specific functions for Abcd2, distinct from its overlapping function with Abcd1. It is worth noting that in organs, such as adrenals and the sciatic nerve, Abcd1 seems to function in cooperation with Abcd2, resulting in significant increases of C22:1\( \omega \)9 when both transporters are missing. This is even clearer in the spinal cord, where only the inactivation of both transporters results in a significant increase of C22:1\( \omega \)9 levels, providing a strong indication of functional redundancy regarding this specific metabolite. This is also seen in neuronal cultures, where significantly increased levels of C20:1\( \omega \)9 are accumulated in double mutants only. This indicates that Abcd1 and Abcd2 can be functionally redundant for C22:1\( \omega \)9 and C20:1\( \omega \)9 in addition to C26:0 as formerly reported. The fact of having found specific accumulation of saturated and \( \omega \)-monounsaturated long- and VLCFA:s in the adrenal gland, spinal cord, or sciatic nerve of \( \text{Abcd2}^{-/-} \) mice, but not in other organs, might indicate that the physiological role of Abcd2 in these tissues is more important than, for instance, in the liver or brain or, alternatively, that loss of ABCD2 function in these particular organs or cell types is not compensated by any of the other members of the ABCD subfamily or by means of an alternative metabolic route.

We have also investigated another major function of peroxisomes: the biosynthesis of the PUFA DPA (C22:5\( \omega \)6) and DHA (C22:6\( \omega \)3; Ref. 68). In cultures derived from cortical neurons, where \( \text{Abcd2} \) is highly expressed, we observed a 30% decrease of DHA and DPA levels, comparable with the decrease found in the brain of \( \text{Pex5}^{-/-} \) mice (22) and the brain of Zellweger patients, which are devoid of functional peroxisomes (37). DHA is one of the major building structures of membrane phospholipids; its deficiency can prevent proper renewal of membranes and accelerate aging. DHA has been shown to exert positive effects on neuronal differentiation, function, and neurotransmission (4, 25, 26). Given the low

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**Fig. 5.** Proposed model for the roles of ABCD1 and ABCD2 transporters in fatty acid homeostasis. Activated saturated, monounsaturated fatty acid (MUFA), or polyunsaturated fatty acids (PUFA) are translocated into peroxisomes via ABCD1, ABCD2, or both for degradative or biosynthetic (retroconversion) \( \beta \)-oxidation.
levels of DHA detected in our primary neuronal cultures, in-depth behavioral analysis directed at exploring higher cognitive functions of ABCD2 knockouts seems appropriate. Moreover, conclusive evidence for a role of Abcd2 in PUFA biosynthesis is provided by the reduced β-oxidation capability for C24:6ω3, as seen in Abcd2−/− and double Abcd2/Abcd1 knockout tissue. These results are in favor of a central role of Abcd2 in fatty acid homeostasis, most likely by acting in the uptake of the precursors C24:6ω3 and C24:5ω6 into the peroxisome.

Indeed, a major contribution of this work derives from the setup of the peroxisomal β-oxidation technique that allows ex vivo assessment of fatty acid oxidation in nervous tissue. Recent investigations (38) could not find alterations of the β-oxidation of C24:0 in any of the tissue homogenates from the X-ALD mouse model tested, which prompted the authors to argue against a role of Abcd1 in the degradation of VLCFA. It should be noted that the authors performed C24:0 β-oxidation assays using frozen postnuclear supernatants of tissue homogenates or purified peroxisomes from liver (19), which may well account for the different experimental results, especially since the peroxisomal membrane is notoriously fragile upon isolation. If membrane integrity is disrupted, substrates for the peroxisomal β-oxidation system can freely reach their site of oxidation in contrast to the situation in intact cells or tissue, as shown in this work. This is supported by the observation that yeast lacking one or both ABCD paralogues (Pxa1p and Pxa2p) show elevated VLCFA levels and defective β-oxidation in intact cells, whereas oxidation is normal in cell lysates. Thus we convincingly show that Abcd1 and Abcd2 are required for correct peroxisomal β-oxidation of C26:0 and C24:6ω3 in intact brain tissue.

Taking our findings together, we propose a model for the functioning of ABCD1 and ABCD2 in Fig. 5. As discussed above, these roles are based on the following: 1) the deficiency of C26:0 and C24:6ω3 β-oxidation, and 2) the changes in the fatty acid profile in tissues of mutant animals under basal or challenged conditions. Based on the overlapping functions of Abcd1 and Abcd2 and because deficiency of C26:0 and C24:6ω3 β-oxidation is only partial in the mutant mice studied, it seems plausible that the other members of the ABCD family (ABCD3 or ABCD4) might contribute to the remaining β-oxidation activity. To further clarify this issue, the same type of experiments should be repeated in single or double Abcd3−/−/Abcd4−/−/Abcd2−/− mice once they become available. Nonetheless, definitive establishment of the role of ABCDs awaits successful reconstitution of ABCDs, either as homo- or heterodimers, into liposomes followed by in vitro transport studies. Very recently, significant advances in the field (15) may eventually help to circumvent the technical complexity associated with the approach and complete our understanding of ABCD roles in fatty acid transport.

To date, no human disease has been linked to the ABCD2 gene locus. Mice lacking Abcd2 exhibit hyperactivity, late-onset peripheral neuropathy, and spinocerebellar ataxia (12), presumably as a consequence of the chronic disturbances of fatty acid metabolism as reported here. Our findings deliver specific profiles of altered fatty acids enhancing the previously described, rather broad phenotype, thus greatly facilitating associations of compatible phenotypes to the ABCD2 gene locus at 12q1.1–2. In analogy with X-ALD patients, in which we found a 4- to 10-fold accumulation of C26:0 in plasma, in spite of a milder accumulation of C26:0 in tissues and none in plasma in the Abcd1 knockout mice, we believe that patients with mutations in ABCD2 could present with lowered levels of C22:6ω3 and C22:5ω6 in plasma, mononuclear cells, or fibroblasts. The importance of our findings go well beyond the field of rare disease, as recent reports have demonstrated that lower levels of DHA in plasma or tissues are correlated with high risk for coronary heart disease (18) and Alzheimer’s disease (53). Moreover, low plasma levels of ω6 and DHA fatty acids are associated with the accelerated decline of peripheral nerve function with aging (29). This suggests that ABCD2 as peroxisome gate opener might well be a key player regulating DHA and EPA endogenous synthesis and, in view of the literature, a potential candidate modifier gene and/or modulating factor contributing to a growing list of common human disorders and aging.

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