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Gérard Lizard *Editor*

Peroxisome Biology: Experimental Models, Peroxisomal Disorders and Neurological Diseases

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Editor

Peroxisome Biology: Experimental Models, Peroxisomal Disorders and Neurological Diseases

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This book is dedicated to my family (Sarab, Diane, Denis and Garance) and to my collaborator Amira Z.: you are my "lighthouses".

I also thank my parents for having instilled in me the sense of work, effort and perseverance without forgetting curiosity and resourcefulness which are indispensable qualities in research.

Louis Pasteur's quote: "Le hasard ne favorise que les esprits préparés"

Preface

The discovery of the peroxisome as well as that of the lysosome is due to Prof. Christian de Duve (1917–2013). This work earned him to share the Nobel Prize for Medicine and Physiology in 1974 with Albert Claude and George E. Palade. At present, the peroxisome is still a poorly known organelle, and it is well established that peroxisomal alterations (absence of biogenesis or deficiency in enzymes or peroxisomal transporters) due to genetic abnormalities are capable of inducing diseases called peroxysomopathies, some of which can be fatal. These peroxysomopathies are often associated with developmental disorders and anomalies of the central or peripheral nervous system. Currently, there are no drugs to treat these diseases, and for some of them, such as X-linked adrenoleukodystrophy (X-ALD), only allogeneic bone marrow transplantation can be effective and great hopes are currently pinned on gene therapy. A better understanding of the peroxisome, its biogenesis, its activities and its functions is therefore an important medical issue. Recently, several studies have suggested that the peroxisome may also be involved in more common neurodegenerative diseases such as Alzheimer's disease and multiple sclerosis. These results, which deserve confirmation, are in favour of the involvement of peroxisomal dysfunction in neurodegeneration. As neurodegeneration is often associated with oxidative stress which can generate lipid peroxidation products, it has been shown that some of these products, called oxysterols (formed by the auto-oxidation of cholesterol, by enzymatic reactions or by both), induce cytotoxicity in nerve cells and lead to morphological and functional peroxisomal modifications. Today, as it is known that peroxisomal dysfunction can favour oxidative stress and inflammation as well as mitochondrial dysfunctions which are hallmarks of neurodegenerative diseases, and that mitochondria and peroxisome are tightly connected organelles, these arguments have reinforced the interest to study the peroxisome in neurodegenerative diseases other than peroxisomopathies. This book by internationally renowned European, Canadian and Japanese researchers on the biology and biochemistry of the peroxisome and its associated diseases is divided into five parts: I—Biology and biochemistry of peroxisomes; II—Peroxisomal diseases: biological characteristics and diagnosis; III—Potential roles of peroxisomes in major neurodegenerative diseases; IV—Cell and animal model systems; and V—Treatments of peroxisomal diseases.

This book provides information intended for a wide biomedical public as well as for researchers wishing to acquire broad and recent knowledge on peroxisome, the pathologies associated with it, their diagnoses, possible treatments and treatment perspectives.

Dijon, France Gérard Lizard

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Part I

Biology and Biochemistry of Peroxisomes

Peroxisome: Metabolic Functions and Biogenesis

1

Kanji Okumoto, Shigehiko Tamura, Masanori Honsho, and Yukio Fujiki

Abstract

Peroxisome is an organelle conserved in almost all eukaryotic cells with a variety of functions in cellular metabolism, including fatty acid β-oxidation, synthesis of ether glycerolipid plasmalogens, and redox homeostasis. Such metabolic functions and the exclusive importance of peroxisomes have been highlighted in fatal human genetic disease called peroxisomal biogenesis disorders (PBDs). Recent advances in this field have identified over 30 *PEX* genes encoding peroxins as essential factors for peroxisome biogenesis in various species from yeast to humans. Functional delineation of the peroxins has revealed that peroxisome biogenesis comprises the processes, involving peroxisomal membrane assembly, matrix protein import, division, and proliferation. Catalase, the most abundant peroxisomal enzyme, catalyzes decomposition of hydrogen peroxide. Peroxisome plays pivotal roles in the cellular redox homeostasis and the response to oxidative stresses, depending on intracellular localization of catalase.

Keywords

Lipid metabolism · Cellular redox metabolism · Catalase · Bak · Peroxisome biogenesis · Peroxins

Abbreviations

AGPS	alkylglyceronephosphate synthase
AGT	alanine glyoxylate aminotransferase
CHO	Chinese hamster ovary
DHA	docosahexaenoic acid
DHAPAT	dihydroxyacetone phosphate acyltransferase
DLP1	dynamin-like protein 1
DYNAMO1	dynamin-based ring motive-force organizer 1
ER	endoplasmic reticulum

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Far1	fatty acyl-CoA reductase 1
H_2O_2	hydrogen peroxide
IRD	infantile Refsum disease
MD	mitochondrial division
Mff	mitochondrial fission factor
NALD	neonatal adrenoleukodystrophy
NDP kinase	nucleoside diphosphate kinase
PBDs	peroxisome biogenesis disorders
PlsEtn	ethanolamine plasmalogen
PMP	peroxisomal membrane protein
POD	peroxisome-dividing
PTS	peroxisomal targeting signal
RCDP	rhizomelic chondrodysplasia punctata
ROS	reactive oxygen species
TPR	tetratricopeptide repeat
VDAC2	voltage-dependent anion channel 2
ZSDs	Zellweger spectrum disorders

1.1 Introduction

Peroxisome is a single membrane-bounded organelle in almost all eukaryotic cells. It was morphologically discovered in mouse kidney in 1954 and named peroxisomes in 1965 by the definition that peroxisomes contain one or more oxidases which produce hydrogen peroxide (H_2O_2) using molecular oxygen and catalase which degrades the H_2O_2 [1]. Peroxisomes are variable in the size, number, and functions, depending on the cell type, physiological status, and environment conditions. Peroxisomes contain a variety of enzymes participating in important metabolic pathways, including α- and β-oxidation of fatty acids, biosynthesis of ether phospholipids plasmalogens, bile acids, and docosahexaenoic acids (DHA), as well as the oxidization of D-amino acids and polyamines [2]. Of these, fatty acid β-oxidation is a notable function of peroxisomes in mammals, which is essential for the chain shortening of very long-chain fatty acids that cannot be oxidized in mitochondria.

Metabolic functions and the vital importance of peroxisomes have been also uncovered by investigation of human genetic peroxisomal biogenesis disorders (PBDs). PBDs are fatal human inherited diseases caused by defects in peroxisome biogenesis, which are classified to two groups, Zellweger spectrum disorders (ZSDs) and rhizomelic chondrodysplasia punctata (RCDP) (see details in the Chap. 4 "Peroxisome Biogenesis Disorders"). ZSDs include Zellweger syndrome (or cerebro-hepato-renal syndrome [3]) that is the prototype and most severe form, and the less severe form is neonatal adrenoleukodystrophy (NALD), and the milder forms are infantile Refsum disease (IRD) and Heimer syndrome, respectively [4] (Chap. 4 "Peroxisome Biogenesis Disorders"). Patients of Zellweger syndrome manifest multiple symptoms, such as severe hypotonia, seizures, facial dysmorphisms, brain atrophy, hepatomegaly, retinal degeneration, and hearing loss, usually resulting in death within a year after birth [2, 5]. PBDs have been categorized into 14 complementation groups by analysis using cell fusion and recent *PEX* gene transfection to fibroblasts derived from PBD patients [4, 6, 7]. So far, over 30 *PEX* genes encoding peroxins have been identified as essential factors for peroxisome biogenesis in various species ranging from unicellular organisms, such as budding yeast to higher ones, including plant *Arabidopsis thaliana* and humans. Fourteen *PEX* genes are shown to be causal genes for known human PBDs [7] (see Chap. 4 "Peroxisome Biogenesis Disorders"). In this chapter, we summarize functions and biogenesis of peroxisomes in mammals with recent advances.

1.2 Biochemistry and Functions of Peroxisomes

Peroxisome contains a number of enzymes required for both catabolic and anabolic metabolisms [2]. Peroxisomal catabolic pathways include the α- and β-oxidation of fatty acids, detoxification of glyoxylate, and degradation of purine and polyamine. Peroxisomal anabolic functions include biosynthesis of ether phospholipids (plasmalogens), bile acids, and DHA. Moreover, peroxisomes are involved in cellular redox metabolism by generating and degrading H_2O_2 in the matrix (Table 1.1).

Table 1.1 Functions of peroxisome[a]

1.	Hydrogen peroxide metabolism
2.	Fatty acid β-oxidation
3.	Ether glycerolipid (plasmalogen) biosynthesis
4.	Transaminations and oxidation (gluconeogenesis)
5.	Purine catabolism
6.	Polyamine catabolism
7.	Bile acid biosynthesis
8.	Pipecolic acid catabolism
9.	Phytanic acid catabolism

[a]Representative functions in mammalian peroxisomes are listed

1.2.1 α- and β-Oxidation of Fatty Acids in Peroxisome

Fatty acid β-oxidation is an essential function of peroxisomes in organisms from yeast to higher eukaryotes, including humans. In mammals, both peroxisomes and mitochondria are responsible for fatty acid β-oxidation catalyzed by similar reactions but by different enzymes with distinct substrate specificities, while fatty acid β-oxidation occurs exclusively in peroxisomes in yeast and plants [2]. The first step of β-oxidation, acyl-CoA dehydrogenation, is catalyzed in peroxisome by FAD-dependent acyl-CoA oxidase that directly transfers electron from FAD to O_2, generating H_2O_2, while in mitochondria re-oxidation of $FADH_2$ is coupled to the electron transport chain to produce ATP. In mammals, peroxisomal β-oxidation is essential for the chain shortening of very long-chain fatty acids (C22 and higher) that cannot be degraded in mitochondria. Shortened long-chain fatty acids are further degraded by mitochondrial β-oxidation pathway to produce energy [8, 9]. Peroxisomal β-oxidation also accepts long-chain dicarboxylic acids, branched-chain fatty acids, such as phytanic acid and pristanic acid, and bile acid intermediates (dihydroxycholestanoic acid and trihydroxycholestanoic acid). In the cases of phytanic acid and pristanic acid harboring a methyl group at the 3 position, they are first subjected to α-oxidation to produce a 2-methyl fatty acid, which are subsequently degraded by peroxisomal β-oxidation [9, 10].

1.2.2 Peroxisomal Glyoxylate Detoxification

In mammals, peroxisome is essential for detoxification of glyoxylate, a toxic metabolite mainly produced in the liver. The reaction is mediated by the peroxisomal matrix enzyme, alanine glyoxylate aminotransferase (AGT) [11], or serine–pyruvate aminotransferase (SPT) [12], which catalyzes the transamination of glyoxylate to glycine with alanine. Deficiency of AGT in either the enzyme activity or mislocalization to mitochondria causes hyperoxaluria type 1, indicating the importance of AGT in human health [13].

1.2.3 Plasmalogens and Lipid Metabolism

Plasmalogens are a major subclass of glycerophospholipids and mostly present as ethanolamine-containing ethanolamine plasmalogen (PlsEtn) with vinyl-ether bond in the *sn*-1 position. In mammals, PlsEtn is present in several organs, such as the brain, kidney, and heart, in which PlsEtn constitutes approximately 50% of ethanolamine-containing phospholipids [14–16]. Peroxisome is essential for plasmalogen synthesis, as the anabolic pathway starts in peroxisomes and completed in the endoplasmic reticulum (ER) via seven-step reactions [15]. The initial two steps of PlsEtn synthesis are catalyzed by peroxisomal matrix enzymes,

dihydroxyacetone phosphate acyltransferase (DHAPAT), and alkylglyceronephosphate synthase (AGPS). AGPS generates alkyl-DHAP by replacing the acyl chain of acyl-DHAP, the product of DHAPAT, where a long-chain fatty alcohol is supplied by fatty acyl-CoA reductase 1 (Far1), a peroxisomal C-tail anchored protein [17–19]. Alkyl-DHAP is further reduced, and PlsEtn synthesis proceeds via the four steps in the ER (reviewed in [14, 15, 20]). In humans, inherited mutations in DHAPAT, AGPS, and FAR1 cause RCDP types 2 [21], 3 [15], and 4 [22], respectively, which are the subtypes of a fatal genetic disease, RCDP [5, 23].

Cellular homeostasis of PlsEtn is largely maintained by the de novo synthesis as assessed by less than 10% of PlsEtn level detectable in plasmalogen-deficient mutant cells, as compared with wild-type cells [15]. Far1 is a rate-limiting enzyme in plasmalogen synthesis. The plasmalogen synthesis is regulated by modulating the stability of Far1 on peroxisomal membranes by means of sensing plasmalogens in the inner leaflet of plasma membranes [18–20, 24]. In addition to various symptoms in RCDP, plasmalogen deficiency also exhibits the abnormality in cholesterol metabolisms, including an impaired high-density lipoprotein-mediated cholesterol efflux [25], delayed transport of internalized cholesterol to the ER [26], and reduced cholesterol synthesis [27]. Recent findings showed that plasmalogen homeostasis tightly links to cholesterol synthesis via regulating the stability of squalene monooxygenase [27], the proposed second rate-limiting enzyme in cholesterol synthesis [28].

1.2.4 Synthesis of Bile Acids and Docosahexaenoic Acid

Peroxisomal β-oxidation plays an important role in the synthesis of bile acids and DHA (C22:6ω-3). Synthesis of bile acids is started from cholesterol in the ER. The intermediates, C27 bile acids, dihydroxycholestanoic acid, and trihydroxycholestanoic acid are racemized and subjected to one cycle of β-oxidation in peroxisomes to produce the CoA-conjugated C24 bile acids, chenodeoxycholic acid, and cholic acid, respectively. The CoA moieties are subsequently replaced with the corresponding taurine or glycine conjugates by peroxisomal enzyme bile acid-CoA:amino acid N-acyltransferase and are excreted into bile [29]. DHA is an omega-3 fatty acid constituting the most abundant class in polyunsaturated fatty acids in the brain and retina. Peroxisomal β-oxidation is required for the final step of DHA synthesis, converting the precursor tetracosahexaenoic acid (C24:6ω-3) to DHA [9, 30].

1.2.5 Cellular Redox Metabolism

Peroxisomes contain H_2O_2-generating enzymes and H_2O_2-degrading catalase, being suggested to play a role in cellular reactive oxygen species (ROS) metabolism. A number of peroxisomal enzymes produce H_2O_2 as a byproduct in normal metabolic processes, including acyl-CoA oxidases, urate oxidase, D-amino acid oxidase, D-aspartate oxidase, L-pipecolate oxidase, 2-hydroxy acid oxidases, polyamine oxidase, and xanthine oxidase [2, 31]. The generated H_2O_2 is detoxified by catalase, one of the most abundant peroxisomal proteins in mammalian cells. Proteome of mammalian peroxisomes revealed other ROS-eliminating enzymes in this organelle, such as superoxide dismutase 1, peroxiredoxin 5, and glutathione S-transferase kappa 1 [32]; therefore peroxisomal H_2O_2 metabolism is most likely maintained by the balance between production and degradation of H_2O_2. Indeed, a recent report showed that succinylation of lysine residue, a posttranslational modification enhancing the acyl-CoA oxidase 1 activity, modulated the H_2O_2 generation in peroxisomes [33]. In contrary, the enzyme activity of acyl-CoA oxidase 1 was downregulated by desuccinylation that was catalyzed by peroxisome-localized sirtuin 5 (SIRT5), a NAD-dependent desuccinylase. Knockdown of SIRT5 indeed elevated the acyl-CoA oxidase 1 activity, resulting in a higher level of peroxisomal H_2O_2 and cellular oxidative stresses [33].

Table 1.2 Peroxins and functional roles

Function	Molecular function	Human	Yeast	Characteristics
Matrix protein import				
Receptor of matrix proteins	PTS1 receptor	Pex5S/L	Pex5, Pex9	TPR motif
	PTS2 receptor	Pex7	Pex7	WD domain
	PTS2 co-receptor	Pex5L	Pex20, Pex18, Pex21	TPR motif (human)
Protein translocation machinery	Pex5-docking complex	Pex14	Pex14	PMP
		Pex13	Pex13	PMP, SH3
			Pex17	PMP
			Pex8	PMP
	E3 ligase with RING finger	Pex2	Pex2	PMP, RING
		Pex10	Pex10	PMP, RING
		Pex12	Pex12	PMP, RING
	E2	UbcH5a/b/c	Pex4	E2
	Recruiter of Pex4		Pex22	PMP
Export complex for PTS receptor	Export of PTS receptor	Pex1	Pex1	AAA-ATPase
		Pex6	Pex6	AAA-ATPase
	Recruiter of Pex1-Pex6 complex	Pex26	Pex15	PMP
Membrane protein import				
	Chaperone and receptor of PMP	Pex19	Pex19	CaaX motif
	Pex19-docking receptor	Pex3	Pex3	PMP
	Pex3 receptor	Pex16	Pex16*, Pex36*	PMP
Division of peroxisome				
	Pex11 family	Pex11α/β/γ	Pex11, 25, 27, 34	PMP
			Pex23, 28, 29, 30, 31, 32	PMP

*, *PEX16* and *PEX36* are identified in limited yeast species, *Y. lipolytica* and *P. pastris*, respectively
TPR tetratricopeptide repeat, *SH3* Src homology-3, *RING* really interesting new gene, *AAA* ATPases associated with diverse cellular activities, *CaaX* farnesylation motif (C, cysteine; a, aliphatic residue; X, any amino acid), *PMP* peroxisomal membrane protein, *WD* Trp-Asp motif

1.3 Biogenesis of Peroxisomes

PEX genes have been isolated by genetic phenotype complementation of peroxisome biogenesis-deficient mutants of mammalian somatic cells, such as Chinese hamster ovary (CHO) cells and several yeast species, including *Saccharomyces cerevisiae*, *Pichia pastoris*, *Yarrowia lipolytica*, and *Hansenula polymorpha* [7, 34–36] (Table 1.2; see Chap. 4 "Peroxisome Biogenesis Disorders"). Identification of *PEX* genes and functional analysis of the peroxins have revealed that peroxisome biogenesis comprises the processes, including membrane assembly, matrix protein import, division, and proliferation [7] (Fig. 1.1, Table 1.2).

1.3.1 Membrane Biogenesis

Three peroxins, Pex3, Pex16, and Pex19, are essential factors for the biogenesis of peroxisomal membranes in several species, including humans [37–46]. Their critical roles in the assembly of peroxisomal membrane proteins (PMPs) were first recognized by the lack of any peroxisomal membrane structures in either *PEX3*-, *PEX16*-, or *PEX19*-defective fibroblasts derived from ZSD patients and CHO cell mutants [37–42]. Pex19

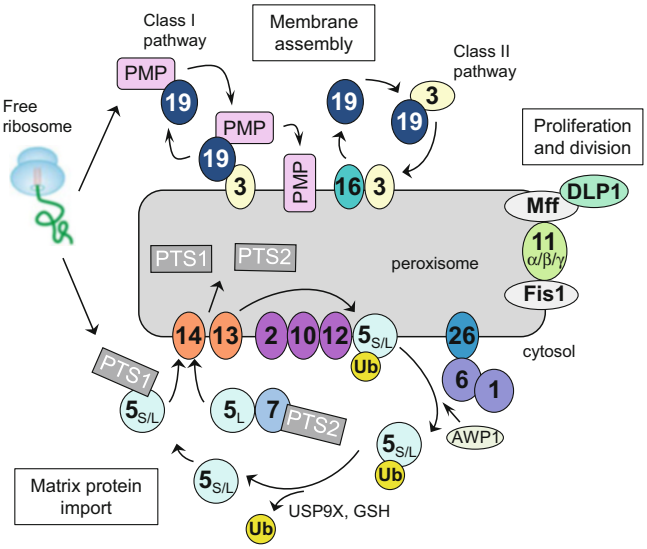

Fig. 1.1 The subcellular localization and molecular characteristics of peroxins are shown. Peroxins are divided into three groups by the functions: membrane assembly, matrix protein import, and proliferation and division. Both peroxisomal membrane proteins (PMPs) and matrix proteins are newly synthesized in free ribosomes in the cytosol and posttranslationally imported to peroxisomes. Pex3, Pex16, and Pex19 are responsible for peroxisome membrane assembly by mediating PMP import via classes I and II pathways. Matrix proteins with PTS1 in majority and rest of those with PTS2 are recognized in the cytosol by PTS1 receptor Pex5 and PTS2 receptor Pex7, respectively. In mammals, two isoforms of Pex5, Pex5S, and Pex5L interact with PTS1 proteins. Pex5L specifically binds Pex7, functioning in targeting the PTS2-Pex7 complex to peroxisomes. Pex5-cargo complexes target peroxisomes by binding to Pex14, the initial docking site on peroxisome membrane. Pex5 releases the cargo proteins into the peroxisome matrix during sequential association with the components of the putative import machinery, including a docking complex consisting of Pex14 and Pex13 and a translocation complex comprising RING peroxins, Pex2, Pex10, and Pex12. Pex1 and Pex6 of the AAA family mediate Pex5 export to the cytosol, where Cys-ubiquitination of Pex5 is essential for Pex5 exit. An ubiquitin-binding protein AWP1 is involved in Pex5 export. USP9X and/or glutathione (GSH) catalyze deubiquitination of released Pex5 for next round import. Pex11β, one of three Pex11 family members, plays a pivotal role in peroxisome proliferation where DLP1, Mff, and Fis1 coordinately function

is a mainly cytosolic protein that has a chaperone-like activity to stabilize PMPs in the cytosol, functioning as an import receptor of newly synthesized PMPs [47, 48]. Pex19 forms stable complexes with PMPs in the cytosol, which are targeted to peroxisomal membrane by docking to an integral membrane protein Pex3, termed Class I pathway [49–51]. Recent study showed that the Pex19-Pex3-mediated Class I pathway received broad types of PMPs with distinct topology, such as multi-membrane-spanning PMPs and an N-terminally signal-anchored protein in mammalian PMP import [51]. In mammalian cells, a peroxisomal membrane peroxin Pex16 functions as the membrane receptor for Pex19 complexes with newly synthesized Pex3, named Class II pathway [50]. The function of Pex16 is not conserved between different species, as *PEX16* is absent in most yeast species [40, 52]. Although *PEX16* has not been identified in *S. cerevisiae*, a novel *PEX* gene, *PEX36* was recently identified in *P. pastoris* [53]. *P. pastoris* Pex36 shows a high similarity to human Pex16 in the deduced secondary structures and membrane topology despite only a weak homology in the amino acid sequence. The finding that the growth defect in Pex36-defective mutant of *P. pastoris* was restored by the expression of human Pex16 suggested that Pex36 is a functional orthologue of human Pex16 [53], plausibly allowing

comparison of Pex16 functions across the species. In Class I pathway, Pex19 unloads the cargo PMP via docking with Pex3 on peroxisomal membrane and shuttles back to the cytosol for a next round of PMP transport. Integration of the released PMP into the peroxisomal membrane proceeds in the absence of ATP [54–57]. Pex19 and Pex3 coordinately facilitate the insertion of hydrophobic transmembrane segments [58, 59]. Molecular mechanisms underlying the membrane integration of the cargo PMPs remain to be better refined.

1.3.2 Matrix Protein Import

Two topogenic signals are identified in peroxisomal matrix proteins: peroxisome-targeting signal type-1 (PTS1) is a C-terminal tripeptide sequence SKL and its derivatives found in the majority of matrix proteins [60, 61] and PTS2 are present in a few proteins as an N-terminal cleavable nonapeptide presequence [62, 63]. In mammals, 10 peroxins, including Pex1, Pex2, Pex5, Pex6, Pex7, Pex10, Pex12, Pex13, Pex14, and Pex26, are essential for protein import into peroxisomal matrix [7, 64] (Fig. 1.1). Pex5 and Pex7 are the cytoplasmic receptors for PTS1 and PTS2, respectively [65–69]. Mammalian *PEX5* codes for two isoforms of Pex5 by alternative splicing are the shorter (Pex5S) and longer one (Pex5L) with a 37-amino acid insertion at the N-terminal part, both of which interact with PTS1 via the highly conserved tetratricopeptide repeat (TPR) motifs in the C-terminal region [65, 66].

Newly synthesized PTS1 proteins are recognized by Pex5 in the cytosol and transported to peroxisomal membrane, where Pex14 forms an 800-kDa complex as the initial Pex5-docking site [70, 71]. After releasing the cargoes, Pex5 translocates to a 500-kDa complex comprising of the RING peroxins, Pex2, Pex10, and Pex12 [71]. Then Pex5 shuttles back to the cytosol for another round of the protein import by ATP-dependent Pex5 export mediated by AAA peroxins, Pex1 and Pex6, and their membrane recruiter Pex26 [71–73]. Pex5L specifically binds Pex7 [74, 75] and thereby targets the PTS2-Pex7 complex to peroxisomes in a Pex14-dependent manner [70, 76, 77]. In yeast *S. cerevisiae*, *P. pastoris*, *Y. lipolytica*, and *H. polymorpha*, additional peroxins, are involved in matrix protein import, which include Pex7 co-receptors, Pex18 and Pex21, import machinery components Pex8 and Pex17, and an E2 Pex4 and its membrane-anchoring protein Pex22 [78, 79].

At the export step from peroxisomes, Pex5 becomes mono-ubiquitinated at the conserved cysteine residue of position 11 in the N-terminal region via a thioester bond (Cys-mono-ubiquitinated Pex5). This unique modification of Pex5 is catalyzed by RING peroxins in peroxisomal membrane, which is also required for the peroxisomal matrix protein import [80–83] as in yeast [84, 85]. A cytosolic factor, AWP1/ZFAND6, is essential for the Pex5 export in mammals, interacting with the Cys-mono-ubiquitinated Pex5 and Pex6 [83]. The conserved cysteine residue of Pex5 is shown to be redox-sensitive and suggested to be involved in a redox regulation of PTS1 protein import [86, 87] (See Sect. 1.3.4.). USP9X and Ubp15 are suggested as a potential deubiquitinase in mammals [88] and yeast [89], respectively, while glutathione non-enzymatically disrupts ubiquitin-Pex5 thioester conjugate [81]. Moreover, distinct ubiquitination of Pex5 via lysine residues is reported: RING peroxin-mediated, multiple mono-ubiquitination required for efficient export of Pex5 [90], mono-ubiquitination of Lys520 in the TPR motifs of Chinese hamster Pex5 by cytosolic unknown E3 involved in binding to PTS1 cargo [90], and an E3 TRIM37-catalyzed mono-ubiquitination of human Pex5L at Lys464 likely in stabilizing Pex5 and controlling the Pex5 abundance [91]. Moreover, ubiquitination at Lys209 of Pex5 is found upon autophagic degradation of peroxisomes (pexophagy) induced by ROS [92]. Amino acid starvation-induced pexophagy is also shown to lead to a Lys-ubiquitination of Pex5 at unknown residue(s) [93]. Therefore, the ubiquitin modification of Pex5 is a pivotal process for regulating peroxisome biogenesis in response to a variety of cellular conditions.

1.3.3 Division of Peroxisomes

1.3.3.1 Mechanism of Peroxisome Fission

Following the membrane assembly and matrix protein import, peroxisomes proliferate by division accompanied with multiple processes, including elongation, constriction, and fission [94–96]. Pex11β is a membrane peroxin playing a pivotal role in peroxisomal division and morphogenesis. Peroxisomal division also requires other factors, including dynamin-like protein 1 (DLP1) [97, 98], mitochondrial fission factor (Mff) [99, 100], and fission 1 (Fis1) [101, 102] in mammals. These proteins except for Pex11β are originally identified as fission factors of mitochondria, showing that peroxisomes share a common division machinery with mitochondria [95, 101, 103]. Ectopic expression of Pex11β induces proliferation of peroxisomes [104], whereas the knocking out of *PEX11β* in mouse [105] and genetic defect of human *PEX11β* decrease peroxisome abundance [106, 107]. The morphogenic activity of Pex11β is suggested to depend on the homo-oligomerization via its N-terminal region [102]. Furthermore, amphipathic helixes located in the N-terminal region of Pex11β are essential for the formation of Pex11β homo-oligomer and for interaction with membrane phospholipids, leading to deformation of peroxisomal membrane [108–110]. Remarkably, DHA, a polyunsaturated fatty acid of peroxisomal β-oxidation metabolites, induces elongation of peroxisomes, hyper-oligomerization of Pex11β on the elongated regions, and extension of Pex11β-enriched membrane [100]. These findings demonstrate a critical role of Pex11β in the elongation step of peroxisomes, requiring the regulated oligomerization of Pex11β via coordination of its N-terminal amphipathic region and DHA-containing phospholipids [96, 111].

DLP1, a member of the dynamin GTPase family, is essential for membrane fission of peroxisomes and mitochondria by the translocation to the membrane constriction sites [98, 112, 113]. DLP1 is thought to mediate the fission step by forming large multimeric spirals [114, 115]. Accumulating evidence reveals that Pex11β forms a ternary fission machinery complex with Mff and DLP1 at the constricted membrane region of elongated peroxisomes, promoting fission during peroxisome division (see reviews: [96, 111]). Pex11β enhances GTPase activity of DLP1 [116], indicating the multiple roles throughout processes of peroxisome division. Patients with deficiency in *PEX11β* gene have been reported, manifesting a defect of peroxisome division and a mild ZSD phenotype, albeit normal peroxisomal metabolism [106, 107, 117, 118]. This suggests the physiological significance of Pex11β-mediated homeostasis of peroxisome abundance and/or peroxisomal morphology. Related to this notion, Pex11β-defective epidermal cells are reported to cause abnormal spindle alignment and mitotic delay, leading to the failure in terminal differentiation [119]. Noteworthily, an adaptive regulation of peroxisome abundance is shown to contribute to cell viability against noise-induced oxidative stress in hair cells in mouse cochlea [120].

1.3.3.2 Fission Machinery of Peroxisome and Mitochondrion: A Role of the GTP Supplier for DLP1, DYNAMO1

As described above, the fission of peroxisomes and mitochondria is mediated by a dynamin-like GTPase, DLP1 or Dnm1 [96, 98, 111]. During the division of peroxisomes, DLP1 polymerizes and forms a ring or spiral structure, peroxisome-dividing (POD) machinery, to constrict and pinch off the peroxisomal membrane [121]. Although DLP1 requires a large amount of GTP for the conformational change [122], it remained unknown how GTP is supplied to the division site. A unicellular red alga, *Cyanidioschyzon merolae*, contains a single of each mitochondrion, peroxisome, and plastid. Taking advantage of the feature that division of these organelle can be highly synchronized by light and dark cycles [121] and by proteomic analysis of highly purified mitochondrial division

(MD) and POD machinery, Imoto et al. [123] recently identified 17-kDa nucleoside diphosphate (NDP) kinase-like protein, named dynamin-based ring motive-force organizer 1 (DYNAMO1). DYNAMO1 colocalizes with DLP1 as an essential component of MD and POD machineries, where it locally generates GTP from ATP and GDP for DLP1 [124]. This enzyme activity is essential for the DLP1-mediated fission of peroxisomes and mitochondria [123]. More recently identified *C. merolae* DYNAMO2, an isoform of DYNAMO1 NDP kinase localizes in the cytoplasm, maybe functioning as a regulator of cellular GTP levels during the cell cycle [125]. In eight mammalian NDP kinases so far identified, nucleoside diphosphate kinase 3 (NME3) may be a mammalian orthologue of DYNAMO1, based on the similarity of amino acid sequences. A patient with NME3 deficiency shows a phenotype that has a reduced level of mitochondrial fusion, resulting in a fatal neurodegenerative disorder [126]. It is of interest whether NME3 is localized in peroxisomes and participates in peroxisome morphogenesis.

1.3.4 Regulation and Homeostasis of Peroxisomal Functions

Isolation of *PEX* genes and delineation of peroxin functions have provided highly outstanding advances in understanding peroxisome biogenesis. However, it remains largely enigmatic how multiple peroxisomal functions are regulated in response to the changes in cellular and environmental conditions. In this context, several lines of evidence have been accumulating in revealing pivotal roles of catalase in peroxisomal functions involved in oxidative stress and antioxidant defense. In mammals, catalase is encoded by a single gene and imported into peroxisomes by the PTS1 receptor Pex5 in a manner dependent on a non-canonical PTS1, KANL [127, 128]. Catalase is mainly localized in peroxisomes in normal condition. However, an increased level of catalase is observed in the cytosol in aged human skin fibroblasts with higher intracellular ROS [129] and under the condition of oxidative stress [130]. This is consistent with the finding that the conserved cysteine residue of Pex5, the acceptor for mono-ubiquitination, is redox-sensitive, thereby Pex5-mediated PTS1 protein import especially for catalase is reduced under oxidative stress, with concomitant increase of cytosolic catalase [86, 87]. In contrast, catalase chronically residing in the cytosol impairs redox homeostasis in peroxisomes [131, 132], leading to mitochondrial dysfunction or cell senescence [87, 133, 134]. These findings evidently suggest physiological importance of cytosolic catalase.

Furthermore, another distinct way to regulate the subcellular localization of catalase was serendipitously discovered by genetic phenotype-complementation assay with a peroxisome-defective CHO cell mutant, ZP114 [135, 136]. ZP114 was shown to be deficient in voltage-dependent anion channel 2 (VDAC2) encoding mitochondrial outer membrane channel [136]. Remarkably, in the VDAC2-defective ZP114, a pro-apoptotic Bcl-2 protein BAK shifts its localization from mitochondria to peroxisomes, where it elevates peroxisomal membrane permeability, giving rise to the release of catalase from peroxisomes into the cytosol. Further compelling evidence strongly suggested that BAK partially localizes to peroxisomes in normal cells as well and regulates peroxisome membrane permeability involving catalase export from peroxisome matrix [136, 137]. Cytosol-localized catalase confers higher cell viability against exogenous H_2O_2 [136], thus supporting the protective role of peroxisomes in response to oxidative stress [124].

Such antioxidant function of peroxisomes was most recently corroborated by a comprehensive survey of oxidative stress regulators in mammalian cells [138]. Genome-wide screens by means of CRISPR/Cas9 knockout and short hairpin RNA knockdown identified multiple genes showing protective or sensitizing effects against exogenous H_2O_2, revealing a broad set of regulators and diverse genetic pathways for modulating oxidative stress sensitivity. Of these, disruption of any of nine *PEX* genes essential for peroxisomal matrix protein import, except for

PEX7, was protective against oxidative stress, which was evidently dependent on the cytosol-residing catalase. This demonstrates that perturbation of the peroxisomal matrix protein import pathway protects cells from oxidative stress by increasing catalase in the cytosol, further highlighting the consequence of intracellular localization of catalase as a key controller in the cellular oxidative stress response.

1.4 Conclusions and Perspectives

Many lines of evidence have accumulated in understanding the functions and biogenesis of peroxisomes. In the biochemical functions, peroxisomes cooperate with other organelles, including metabolic pathways, such as fatty acid β-oxidation and synthesis of plasmalogens with mitochondria and the ER, respectively, by a direct or indirect interaction and communication. Membrane contact sites more likely mediate the inter-organelle interactions [139–141]. However, it remains enigmatic how cells establish such coordination of peroxisomal metabolisms with other organelles. Peroxisome homeostasis is tightly linked to the biogenesis and breakdown of peroxisomes. Functional analysis of peroxins have better elucidated the molecular mechanism of peroxisomal matrix protein import, including its regulation mediated by ubiquitination of Pex5 in response to a variety of cellular conditions. Furthermore, intracellular localization of catalase is fine-tuned by several regulations, including one under the cellular oxidative stress. Several further tackling issues to be addressed include delineation of the molecular mechanisms underlying how cells sense cellular status and environmental changes, such as oxidative stress, and how the signal is conveyed to peroxisomes to initiate the response. Posttranslational modifications may well be also involved in these cascades. In addition to the issues addressed here mostly concerning the various peroxisomal functions at cellular level, higher-ordered functions of peroxisomes have been unveiled at a tissue level and/or an animal level using *Pex*-knockout mice [142] (see Chap. 10, Abe et al. A mouse model system to study peroxisomal roles in neurodegeneration of peroxisome biogenesis disorders). Further investigations towards understanding the functions and biogenesis of peroxisome will uncover the long-standing issues in this field, including molecular mechanisms underlying pathogenesis of neurodegeneration in PBDs.

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2 Peroxisomal Dysfunction and Oxidative Stress in Neurodegenerative Disease: A Bidirectional Crosstalk

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Abstract

Peroxisomes are multifunctional organelles best known for their role in cellular lipid and hydrogen peroxide metabolism. In this chapter, we review and discuss the diverse functions of this organelle in brain physiology and neurodegeneration, with a particular focus on oxidative stress. We first briefly summarize what is known about the various nexuses among peroxisomes, the central nervous system, oxidative stress, and neurodegenerative disease. Next, we provide a comprehensive overview of the complex interplay among peroxisomes, oxidative stress, and neurodegeneration in patients suffering from primary peroxisomal disorders. Particular examples that are discussed include the prototypic Zellweger spectrum disorders and X-linked adrenoleukodystrophy, the most prevalent peroxisomal disorder. Thereafter, we elaborate on secondary peroxisome dysfunction in more common neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and multiple sclerosis. Finally, we highlight some issues and challenges that need to be addressed to progress towards therapies and prevention strategies preserving, normalizing, or improving peroxisome activity in patients suffering from neurodegenerative conditions.

Keywords

Peroxisome · Redox balance · Peroxisomal disorder · Mitochondria · Central nervous system · Neurological disease

Abbreviations

ABCD	ATP-binding cassette subfamily D
AD	Alzheimer's disease
ALDP	Adrenoleukodystrophy protein
ALS	Amyotrophic lateral sclerosis
CNS	Central nervous system
DAO	D-amino acid oxidase
DHA	Docosahexaenoic acid
EAE	Experimental allergic encephalomyelitis
MS	Multiple sclerosis
PD	Parkinson's disease
PEX	Peroxin
PPA	Peroxisome-proliferating agent
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid

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ROS	Reactive oxygen species
VLCFA	Very-long-chain fatty acid
X-ALD	X-linked adrenoleukodystrophy
ZSD	Zellweger spectrum disorder

2.1 Peroxisomes and the Central Nervous System

Peroxisomes are morphologically and functionally remarkably plastic organelles that play a prominent role in diverse metabolic and signaling pathways [1]. In humans, they are pivotal for the oxidative degradation of a broad range of fatty acids and D-amino acids as well as the biosynthesis of plasmalogens, bile acids, and all-*cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA; 22:6 n = 3) [1, 2]. Peroxisomes can also serve as a source or sink for hydrogen peroxide (H_2O_2) [3]. Given that (i) this non-radical reactive oxygen species (ROS) acts as a physiologically important redox signaling messenger, (ii) plasmalogens and DHA function as potential reservoirs for lipid second messengers (e.g., prostaglandins, leukotrienes, thromboxanes, resolvins, and neuroprotectins), and (iii) some of these bioactive lipids can be degraded through peroxisomal β-oxidation, peroxisomes have the intrinsic capability to mediate and modulate redox- and lipid-driven signaling cascades [4]. Besides this, they have emerged as indispensable signaling platforms in innate immunity and inflammation [5]. To perform their functions effectively, peroxisomes must communicate and cooperate with other subcellular compartments, including mitochondria [6], and a defect in one or more of these functions often result in a range of neurological symptoms and brain pathology (see Sect. 2.4).

Peroxisomes are present in all major cell types of the central nervous system (CNS), including neurons, astrocytes, oligodendrocytes, microglia, and endothelial cells [7]; their abundance and intracellular distribution can differ between cell types. For example, peroxisomes in white matter tracts are highly abundant in myelin-forming oligodendrocytes but only sparsely present in axonal projections [8]. In comparison with other tissues, brain peroxisomes are smaller in size [9]. In addition, their abundance varies between different brain areas and significantly decreases during postnatal development [7]. From the functional point of view, peroxisomes play diverse roles in brain physiology: they (i) provide key metabolites (e.g., DHA and plasmalogens) for the formation and maintenance of myelin membranes and neuronal signaling; (ii) degrade important mediators of lipotoxicity (e.g., hydroperoxy fatty acids, very-long-chain fatty acids (VLCFAs), and the dietary fatty acid phytanic acid and its metabolite pristanic acid); (iii) metabolize D-serine and D-aspartate, two endogenous molecules involved in brain neurotransmission, synaptic plasticity, and neurodegeneration; and (iv) play a role in the maintenance of redox balance ([3]; and references therein) (Fig. 2.1). Finally, it is interesting to note that, despite the fact that oligodendrocyte-, astrocyte-, and neuron-selective inactivation of peroxisomes in mice adversely affects the animals' postnatal thriving, only the oligodendrocyte-specific inactivation results in a reduced lifespan [10].

2.2 Neurodegenerative Disease and Oxidative Stress

The CNS, which comprises the brain and spinal cord, is made up of complex networks of functionally diverse cell types that control virtually all activities of the human body and mind. The brain is intrinsically vulnerable to oxidative stress, a phenomenon that can be explained by—among others—its high oxygen consumption, rich content of peroxidation-sensitive polyunsaturated fatty acids (PUFAs), regionally high abundance of redox-active transition metals, and relatively modest antioxidant defenses [11]. Increased oxidative stress is one of the key hallmarks of neurodegenerative disorders, the most common being Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS). Other distinctive features include mitochondrial dysfunction, protein misfolding and aberrant localization,

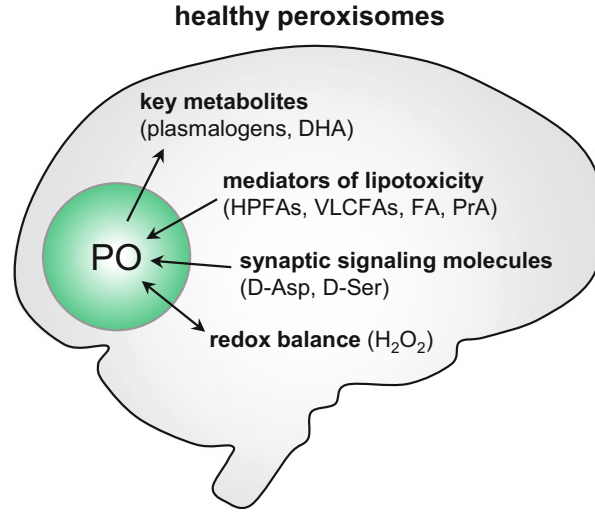

Fig. 2.1 Emerging roles of peroxisomes in brain physiology. Schematic overview depicting the role of healthy peroxisomes (indicated in green) in the maintenance of normal brain physiology. *DHA* docosahexaenoic acid, *HPFAs* hydroperoxy fatty acids, *VLCFAs* very-long-chain fatty acids, *PA* phytanic acid, *PO* peroxisome, *PrA* pristanic acid

inflammation, and neuronal cell death [12]. Importantly, despite the fact that oxidative stress has been associated with the onset and progression of neuropathological alterations, it is not yet clear whether disturbances in the cellular redox balance represent a secondary response to brain damage or play a causative role in disease development.

2.3 Peroxisomes and Cellular Redox Balance

Mammalian peroxisomes contain about 100 different proteins, of which approximately a dozen generate H_2O_2 as a metabolic by-product [13]. In addition, one of the most abundant peroxisomal proteins is catalase, an enzyme that efficiently decomposes H_2O_2 to H_2O and O_2 without consuming reducing equivalents. Currently, there is compelling empirical evidence that peroxisomes serve a key role in cellular redox balance and stress responses ([3]; and references therein). In the context of this chapter, it needs to be emphasized that disturbances in peroxisomal (redox) metabolism can rapidly perturb mitochondrial redox state and function, a key feature of neurodegenerative diseases. Unfortunately, little is known about how disturbances in peroxisomal redox metabolism affect other subcellular compartments. Given that the main focus of this thematic review lies on the interplay between peroxisome (dys)function and oxidative stress in neurodegenerative disease (see Sects. 2.4 and 2.5), we refer the reader to other recent reviews for detailed discussions on how peroxisomes may act as redox signaling hubs [3] and why these organelles need to maintain a healthy relationship with mitochondria [6].

2.4 Peroxisomal Dysfunction, Oxidative Stress, and Neurodegeneration

The observation that virtually all patients with peroxisomal dysfunction present with neurological symptoms clearly highlights that maintenance of a healthy peroxisomal population is critical for neuronal health [9]. Depending on the metabolic pathways and cell types involved, the CNS pathology may be more or less severe [14]. Symptoms can range from abnormalities in neuronal migration or differentiation to defects in the formation or maintenance of central white matter and even post-developmental neuronal degeneration [9]. The exact immuno- and inflammatory dysregulations causing these injuries remain unclear, but potential factors may include reduced plasmalogen content [15], toxic

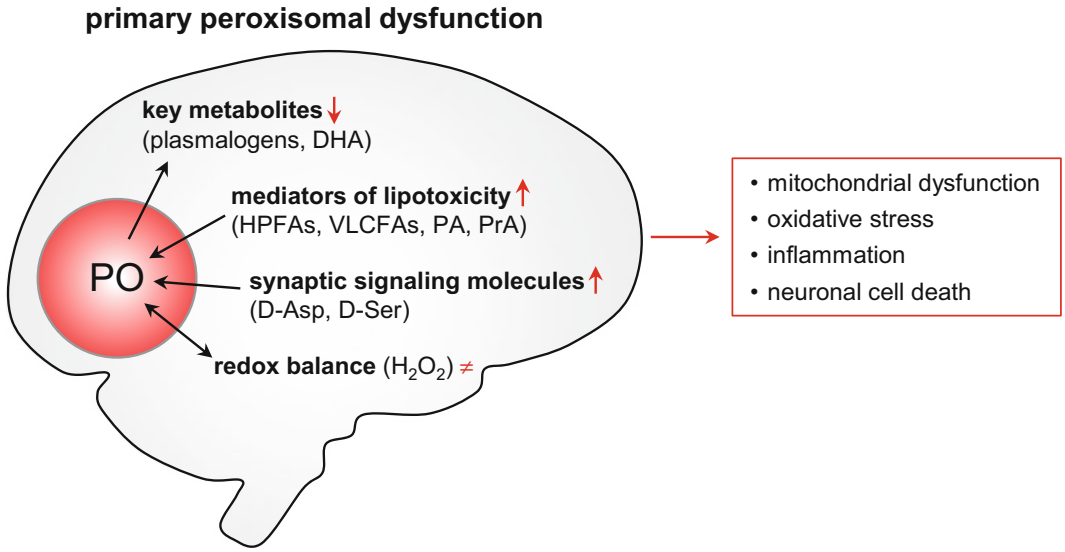

Fig. 2.2 Emerging roles of primary peroxisome dysfunction in brain pathology. Schematic overview depicting how primary peroxisomal dysfunction (indicated in red) may contribute to brain pathology. *DHA* docosahexaenoic acid, *HPFAs* hydroperoxy fatty acids, *VLCFAs* very-long-chain fatty acids, *PA* phytanic acid, *PO* peroxisome, *PrA* pristanic acid

accumulation of peroxisomal α- (e.g., phytanic acid) and β-oxidation (e.g., pristanic acid and VLCFAs) substrates, and changes in peroxisomal H_2O_2 metabolism and signaling (Fig. 2.2).

Plasmalogens have been shown to protect myelin from oxidative damage [15], and a general shortage of this class of glycerophospholipids in mitochondrial membranes has been reported to reduce respiratory chain activity and ATP production, a condition negatively affecting cerebral energy metabolism [16]. Also phytanic and pristanic acid as well as the VLCFAs, docosanoic acid (C22:0), tetracosanoic acid (C24:0), and hexacosanoic acid (C26:0), have been reported to exert harmful effects on neural cells and brain tissues through mitochondrial dysfunction and oxidative stress, at least in vitro. Indeed, in vitro studies with neural cells have shown that low micromolar concentrations of these lipids can distort mitochondrial respiration, Ca^{2+} retention, and ROS production, thereby impairing cell physiology and viability ([17, 18]; and references therein); and ex vivo studies on brain cortex and cerebellum of young rats have shown that micromolar concentrations of both phytanic and pristanic acid elicit lipid and protein oxidative damage [19, 20]. However, in vivo injection of phytanic acid (estimated final concentration: 450–500 μM) into the cerebellum of adolescent rats did only provoke oxidative damage to lipids [21]. Interestingly, treatment of primary mixed neural cultures of rat hippocampus revealed that the pathological consequences of VLCFA accumulation are more severe for myelin-producing oligodendrocytes than for astrocytes and neurons [17]. In addition, the disease pathology associated with the accumulation of VLCFAs in the CNS appears to be more severe in humans than in mice ([22]; and references therein).

A major concern regarding the studies referred to in the previous paragraph is that the administered concentrations of fatty acids are probably way outside the physiological range. In this context, it is mandatory to point out that the concentrations of phytanic acid, pristanic acid, C22:0, C24:0, and C26:0 in cerebrospinal fluid of patients with peroxisomal disorders are below the detection limit or do not exceed the values of 16, 34, 100, 350, and 300 nM, respectively (for comparison, with exception of the C26:0 levels in

patients with an acyl-CoA oxidase deficiency, these concentrations are less than 1% of those found in the serum of these patients) [23, 24].

In the following sections, we focus on what is currently known about the peroxisome-oxidative stress-neurodegeneration nexus in patients suffering from a Zellweger spectrum disorder (see Sect. 4.1) or X-linked adrenoleukodystrophy (X-ALD) (see Sect. 4.2). When relevant, animal studies are also discussed.

2.4.1 Zellweger Spectrum Disorders

The Zellweger spectrum disorders (ZSDs) are a group of rare (combined incidence: ~1:50,000 live births), clinically and genetically heterogeneous disorders caused by mutations in *PEX* genes. The encoded proteins, referred to as peroxins, are involved in peroxisome biogenesis and maintenance. Mutations leading to a loss of peroxin function result in the absence of functional peroxisomes and, consequently, the accumulation of peroxisomal substrates (e.g., VLCFAs, phytanic and pristanic acid, and C27-bile acid intermediates) and loss of peroxisomal products (e.g., plasmalogens and DHA) [14]. ZSD patients present with a continuum of symptoms ranging from sensorineural hearing loss, vision impairment, and low muscle tone to profound liver, kidney, and brain dysfunction [14].

The molecular mechanisms underlying the neuropathology of ZSD are not yet well-understood. Solid evidence that the absence of functional peroxisomes in ZSD patients causes lipid-induced mitochondrial dysfunction and oxidative damage in the CNS is lacking. However, by employing mice with a brain-specific deletion of PEX13, Crane and co-workers could show that the neuronal cell death (and subsequent impairment of cerebellar development) caused by absence of functional peroxisomes is associated with aberrant mitochondrial ROS production and dynamics [25]. These findings are in line with the observations that deletion of even a single allele of PEX11β is sufficient to cause oxidative stress and neuronal death in mouse brain [26]. In contrast, no specific relationship could be observed among peroxisome dysfunction, mitochondrial failure, generalized oxidative stress, and CNS lesions in mice with a brain-restricted deletion of PEX5; mitochondria in cortical plate neurons did not exhibit obvious abnormalities; oxidative stress markers were only upregulated in Purkinje cells; and treatment of the animals with an antioxidant cocktail did neither improve their motor performance nor have an effect on their health status [27]. As such, the present data are not sufficiently robust to accept or reject the hypothesis that a general defect in peroxisome biogenesis in ZSD patients causes neuronal cell death and brain dysfunction through mitochondria-mediated oxidative stress.

2.4.2 X-Linked Adrenoleukodystrophy

X-ALD, the most prevalent peroxisomal disorder (~1:17,000 live male births), is a clinically heterogeneous and progressive neurometabolic disease caused by mutations in the *ABCD1* gene [28]. This gene encodes a peroxisomal ATP-binding cassette-containing transmembrane protein (ALDP) that mediates the import of CoA-esters of VLCFAs into the organelle. Mutations in ALDP may result in the accumulation of VLCFAs in body tissues, including the brain and spinal cord (e.g., as cerebrosides, phosphatidylcholine, sphingomyelin, sulfatides, and cholesteryl esters) [29], due to impaired peroxisomal β-oxidation. Depending on yet-to-be-identified environmental factors and modifier genes, a reduction or absence of ALDP activity may induce—among others—adrenocortical insufficiency, progressive inflammatory demyelination, axonal damage, and neurodegeneration [28].

The molecular mechanisms of how the VLCFA-containing lipids can drive these symptoms are currently under intense investigation. Besides the accumulation of VLCFAs, other early hallmarks of X-ALD are oxidative damage and mitochondrial dysfunction, both in patients as well as in experimental cell and mouse models

[30]. Approximately a decade ago, Singh and Pujol proposed a "three-hit hypothesis" to explain the physiopathogenesis of cerebral ALD, the most severe X-ALD phenotype. This hypothesis postulates that, as a direct consequence of peroxisomal metabolic impairment, oxidative stress functions as the first hit, neuroinflammation acts as the second insult, and a generalized loss of peroxisome function serves as the third hit [31]. The observations that (i) the levels of 7-ketocholesterol (0.5–4.0 μM), a reliable biomarker of oxidative damage, are on average 10- to 20-fold higher in the plasma of X-ALD patients than in the plasma of healthy control subjects [32]; (ii) this bioactive product of lipid oxidation, which can trigger inflammation and an oxiapoptophagic (OXIdation, APOPTOsis, and autoPHAGY) mode of cell death, also induces general peroxisome dysfunction [32]; and (iii) upregulation of endogenous antioxidant responses can rescue mitochondrial function, prevent inflammatory imbalance, and halt axonal degeneration and locomotor deficits in an X-ALD mouse model are in line with this hypothesis [30]. However, it remains to be investigated whether or not the inflammatory and cell death responses elicited by 12.5–50 μM of 7-ketocholesterol in murine microglial BV-2 cells [32] also occur under conditions more closely mimicking the in vivo microenvironment and at 7-ketocholesterol concentrations (0.5–4.0 μM) found in the plasma of X-ALD patients.

2.5 Neurodegenerative Disease and Peroxisomal Redox Dysfunction

Increasing evidence indicates that, among other processes, peroxisome function is declined with aging ([4]; and references therein). In addition, a number of studies have suggested that this phenomenon may contribute to the onset and progression of more widespread age-related neurological disorders ([9, 33]; and references therein). Common brain abnormalities in individuals suffering from such diseases include lipid changes, mitochondrial dysfunction, oxidative stress, inflammation, deposits of misfolded protein aggregates, cell death, and demyelination (Fig. 2.3). Given that many of these manifestations can also be linked to peroxisome dysfunction (see Sect. 4), this organelle too may play a role in the etiology and pathogenesis of age-related neurodegenerative diseases. Importantly, many of the abnormalities listed are not independent but promote each other. This is perhaps best illustrated by the discoveries that oxidative stress can be either causative or consecutive, to protein aggregation, and that both phenomena can be part of a vicious cycle ([34]; and references therein). In the following sections, we further elaborate on what is currently known about the peroxisome-oxidative stress-neurodegeneration nexus in AD (see Sect. 5.1), PD (see Sect. 5.2), and MS (see Sect. 5.3). Importantly, given the unavailability of sufficient empirical studies on peroxisome dysfunction in amyotrophic lateral sclerosis (ALS), this progressive motor neuron disorder will not be discussed. However, for completeness, it is necessary to mention that (i) a deficiency in D-amino acid oxidase (DAO), a peroxisomal H_2O_2-producing enzyme, triggers motor neuron degeneration in both mice and familial ALS patients and (ii) the spinal cord levels of D-serine are ~five-fold increased in $DAO^{-/-}$ mice relative to control strains [35].

2.5.1 Alzheimer's Disease

AD, the most common form of dementia (prevalence: ~10% of all people aged 65 and over), is a progressive neurodegenerative disorder whose pathology is related to the deposition of intracellular hyperphosphorylated tau aggregates and extracellular amyloid-β plaques [36]. Despite the fact that the molecular pathogenesis underlying this devastating disease is not yet fully understood, there is evidence that neuronal damage and death are associated with mitochondrial dysfunction, increased oxidative stress, and alterations in lipid metabolism [36, 37].

Currently, there is compelling evidence to support the notion that both peroxisome function and dysfunction can be linked to AD. For example, lipid analyses of human cortical brain samples

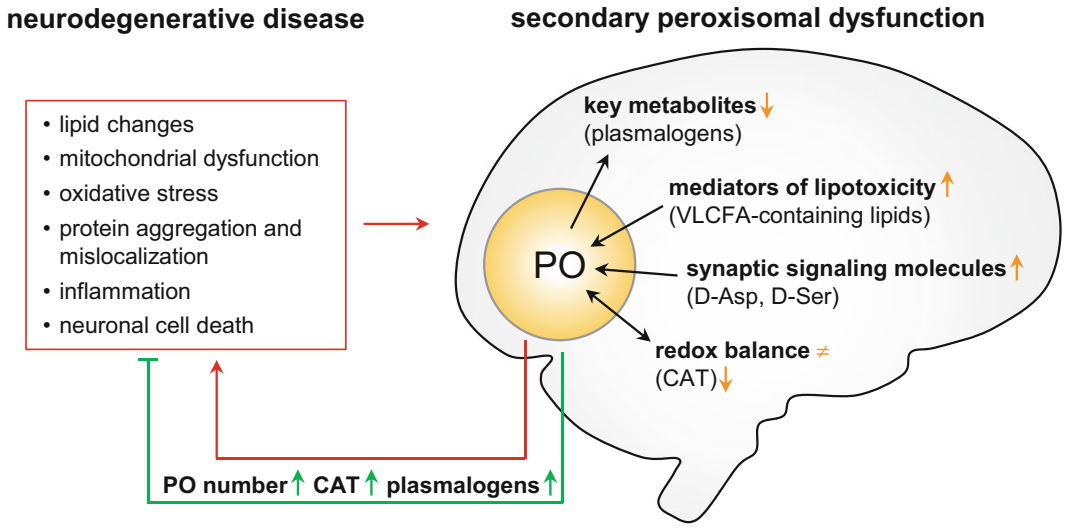

Fig. 2.3 Neurodegenerative disease and secondary peroxisomal dysfunction. Schematic overview depicting the potential relationships between hallmarks of neurodegenerative diseases and secondary peroxisome dysfunction (indicated in orange). The red arrows show the potential vicious cycle between neurodegeneration and peroxisome impairment. The green T-bar provides literature-based suggestions of how this cycle may be broken. *CAT* catalase, *PO* peroxisome, *VLCFA* very-long-chain fatty acid

have documented that the levels of ethanolamine plasmalogens with a PUFA at the sn-2 position and the concentrations of C24:0 plus C26:0 are reduced (~7.7%) and elevated (~36.6%), respectively, in late-stage (V and VI) compared with early-stage (I and II) AD patients [38], and another study reported that, in the prefrontal cortex of AD patients, choline plasmalogens (decrease: ~73%) are more affected than ethanolamine plasmalogens (decrease: ~20%) [39]. In addition, the first study demonstrated that the transport of peroxisomes to neurites is compromised in AD-affected neurons. Numerical and functional changes of peroxisomes were also found in the neocortex of the widely-used Tg2576 mouse model of AD ([37]; and references therein), and in rat, a decline in peroxisomal β-oxidation (as suggested by elevation of cortical C26:0 levels) positively correlated with significant elevations of the lipid peroxidation marker malondialdehyde, the accumulation of amyloid-β peptides, and spatial memory deficits [40]. In line with these observations, others found that an increase in peroxisome number with concomitant increased catalase activity (and plasmalogen levels) mitigates amyloid-β-induced toxicity in primary rat hippocampal neuron cultures and in brains of transgenic APPswe/PS1dE9 mice, another commonly used mouse model of AD ([41]; and references therein). This phenomenon appears to be at least partially mediated through enhanced redox buffering capacity and mitochondrial fitness. Note that the oxidative stress link is further reinforced by the observation that intraperitoneal injections of CAT-SKL, a catalase variant with enhanced peroxisomal targeting capacity, attenuate the loss of cholinergic neurons and long-term reference memory deficits in rats that received intracerebroventricular injections of amyloid-β [42].

2.5.2 Parkinson's Disease

PD, the second-most widespread neurodegenerative disorder after AD (prevalence: ~1–2% of all persons over 60 years of age), is characterized by a progressive loss of dopamine-producing neurons in the substantia nigra pars compacta, a process causing motor deficits, such as resting

tremor, rigidity, and bradykinesia ([43]; and references therein). A typical pathological hallmark of PD is the presence of intraneural α-synuclein aggregates. The exact molecular mechanisms in the pathology of PD remain to be clarified. Once again, growing evidence supports the view that mitochondrial dysfunction and oxidative stress play a pivotal role in PD pathogenesis [43].

To the best of our knowledge, there are only a handful of studies examining the potential links between α-synuclein-related brain pathology and peroxisome dysfunction. On one hand, it has been reported that in PD patients frontal cortex lipid rafts contain less plasmalogens and DHA than those in the control group [44]. On the other hand, it has been observed that α-synuclein accumulates in neurons and glial cells from X-ALD patients [45] and that generalized defects in peroxisome biogenesis promote the oligomerization and phosphorylation of α-synuclein in mouse brain, a process that appears to be positively correlated with an increase in VLCFAs and long-chain n-6-PUFAs, and a drop in most n-3-PUFAs [46]. However, in a follow-up study, the same authors concluded that high or prolonged dietary levels of DHA enhance the α-synuclein pathology in A53T α-synuclein transgenic mice [47]. In addition, they demonstrated that, in the brain of these mice, peroxisomal α- and β-oxidation are normal, plasmalogen levels are slightly increased, and catalase activity is decreased by 25–30% [48].

Importantly, some studies have hinted that compounds known as peroxisome-proliferating agents (PPAs) may provide neuroprotection, at least in PD animal models ([49]; and references therein). The precise underlying mechanisms are unknown but are most likely multifactorial. Indeed, the effects of PPAs, such as fibrates and thiazolidinediones, are mediated by distinct peroxisome proliferator-activated receptors (PPARs), which tune the gene expression of proteins involved in a wide range of cellular processes, including—among others—peroxisome biogenesis, mitochondrial biogenesis, lipid metabolism, and antioxidant and inflammatory responses. In this context, it is interesting to note that, in peroxisome-defective fibroblasts overexpressing α-synuclein, the pathogenic accumulation of intracellular α-synuclein inclusions did not correlate with oxidative stress or mitochondrial dysfunction [46]. However, whether or not this is also true for nigral dopaminergic neurons remains to be determined. Finally, it has been demonstrated that PPI-1011, a DHA-containing ethanolamine plasmalogen precursor, protects against striatal dopamine loss in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-intoxicated mouse model of PD [50].

2.5.3 Multiple Sclerosis

MS, whose prevalence can vary considerably (e.g., from 2 to >200 per 100,000 residents) between different regions and populations, is a chronic inflammatory condition of the CNS with a varying clinical picture ([51]; and references therein). Pathological hallmarks of the disease include—among others—impaired homeostasis of glial cells with production of ROS, mitochondrial damage, demyelination, and axonal degeneration [51].

Once again, the precise molecular mechanisms leading to the disease pathology of MS are not yet well-understood. However, as for AD and PD, MS has been associated with impaired peroxisomal function, both in patient-derived tissues [52] and in the widely-accepted experimental allergic encephalomyelitis (EAE) animal model for MS [53]. Indeed, in the brain of EAE rats, C26:0 levels are increased (by ~40%), and plasmalogen levels and catalase activity are decreased (by 16–30% and 37%, respectively) [53], and in grey matter from brain autopsies of MS patients, C26:0 levels are ~two-fold elevated, and peroxisomal ABCD3 membrane protein-positive neurons are negatively correlated with the disease duration [52]. In addition, more recently, it has been demonstrated that a combined administration of lovastatin and the AMP-activated protein kinase activator AICAR to EAE mice (i) increases peroxisomal and mitochondrial biogenesis and function through PPARα/γ- and PPARγ coactivator-1 alpha-

mediated pathways, (ii) alleviates the inflammation-induced mitochondrial and peroxisomal defects, and (iii) provides protection against myelin and axonal pathology in EAE [54]. In the cerebrospinal fluid of MS patients, only PPARγ appears to be upregulated [55]. Based on these findings, it is tempting to speculate that alterations in peroxisome function may contribute to neuronal dysfunction and degeneration in MS and that restoring peroxisome function may be beneficial for MS disease prognosis [54]. However, given that targeted lipidomic profiling studies of cerebrospinal fluid samples from MS patients do not provide any evidence for peroxisomal dysfunction [56], this view should be considered with caution.

2.6 Conclusions and Outlook

The studies reviewed in this chapter clearly demonstrate that peroxisomes play an important role in normal brain function (Fig. 2.1). In addition, they strengthen the view that peroxisome function is compromised in the brain of patients suffering from common age-related neurodegenerative diseases (Fig. 2.3). The specific mechanisms underlying peroxisome dysfunction-related brain impairment remain obscure, but potential elements include the shortage of peroxisomal products (e.g., plasmalogens), the toxic accumulation of peroxisomal substrates (e.g., VLCFA-containing lipids), and perturbations in peroxisomal H_2O_2 metabolism and signaling (Fig. 2.2). Importantly, imbalances in any of these factors can be firmly linked to inflammation [31], mitochondrial dysfunction [17], and protein aggregation [34], all hallmarks of neurodegeneration [12]. Given that virtually all these processes are tightly interconnected, it is difficult to pinpoint the primary triggering event (s).

A major challenge is to decipher which shortage or excess of peroxisome-related metabolites and signaling molecules drives CNS pathology. In this context, it is mandatory to note that most of the in vitro lipotoxicity studies have been performed with fatty acid concentrations in the low micromolar range, which closely reflect the situation in plasma [23, 24]. As already pointed out above (see Sect. 2.4), these concentrations are often 100–1000-fold higher than those found in the brain [23, 24], thereby raising doubts regarding the physiological relevance of these studies. In addition, given that the concentrations of phytanic and pristanic acid in cerebrospinal fluid from patients with a peroxisomal disorder only seldom exceed the control range, it is unlikely that these substances play a direct pathogenic role in the neurological manifestations of such patients [23].

With respect to the age-related neurodegenerative disorders, it is unclear to which extent the observed decline in peroxisome function actively contributes to the disease phenotype or merely represents a secondary change associated with the pathologic process. However, in this context, it is relevant to mention that various inflammatory stimuli have been reported to reduce plasmalogen levels in glial cell lines and microglia in mouse cortex through NF-κB activation and subsequent c-myc-mediated transcriptional repression of *GNPAT*, a gene coding for a peroxisomal membrane protein essential for ether-phospholipid biosynthesis [57]. In addition, it has been demonstrated that peroxisome number and function are also altered at sites of secondary neurodegeneration (e.g., post-stroke or post-doxorubicin chemotherapy). Indeed, induction of transient focal cerebral ischemia in mice significantly increases peroxisome number as well as catalase activity in peri-infarct neurons [58], and treatment of mice with the antineoplastic agent doxorubicin increases the number of peroxisomes in neurons [59]. Interestingly, in the first case, the observed changes were considered to be an adaptive response, protecting neurons against ischemia/reperfusion-induced oxidative and metabolic stress [58]. In contrast, in the second case, the expansion of the peroxisomal compartment was attributed to a downregulation of pexophagy, a phenomenon that coincided with enhanced peroxisome-derived oxidative stress and was considered to contribute to the deterioration of

cognitive function and accelerated brain aging in cancer patients and survivors [59].

A final key message is that peroxisomal fitness is pivotal to protect the CNS from inflammation, mitochondrial dysfunction, oxidative stress, and neurodegeneration. This implies that diet strategies [60] and therapeutic approaches [41, 49, 54] aimed at preserving, normalizing, or improving peroxisome abundance and activity may have beneficial effects for patients suffering from neurodegenerative conditions.

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3

7-Ketocholesterol- and 7β-Hydroxycholesterol-Induced Peroxisomal Disorders in Glial, Microglial and Neuronal Cells: Potential Role in Neurodegeneration

7-ketocholesterol and 7β-hydroxycholesterol-Induced Peroxisomal Disorders and Neurodegeneration

Thomas Nury, Aline Yammine, Franck Menetrier, Amira Zarrouk, Anne Vejux, and Gérard Lizard

Abstract

Peroxisomopathies are qualitative or quantitative deficiencies in peroxisomes which lead to increases in the level of very-long-chain fatty acids (VLCFA) and can be associated with more or less pronounced dysfunction of central nervous system cells: glial and microglial cells. Currently, in frequent neurodegenerative diseases, Alzheimer's disease (AD) and multiple sclerosis (MS), peroxisomal dysfunction is also suspected due to an increase in VLCFA, which can be associated with a decrease of plasmalogens, in these patients. Moreover, in patients suffering from peroxisomopathies, such as X-linked adrenoleukodystrophy (X-ALD), AD, or MS, the increase in oxidative stress observed leads to the formation of cytotoxic oxysterols: 7-ketocholesterol (7KC) and 7β-hydroxycholesterol (7β-OHC). These observations led to the demonstration that 7KC and 7β-OHC alter the biogenesis and activity of peroxisomes in glial and microglial cells. In X-ALD, AD, and MS, it is suggested that 7KC and 7β-OHC affecting the peroxisome, and

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which also induce mitochondrial dysfunctions, oxidative stress, and inflammation, could promote neurodegeneration. Consequently, the study of oxisome in peroxisomopathies, AD and MS, could help to better understand the pathophysiology of these diseases to identify therapeutic targets for effective treatments.

Keywords

Oxysterol · 7-ketocholesterol · 7β-hydroxycholesterol · Peroxisome · Very-long-chain fatty acids · Alzheimer's disease · Multiple sclerosis · X-linked adrenoleukodystrophy

Abbreviations

ABC	ATP-binding cassette
ACALD	adolescent cerebral adrenoleukodystrophy
ACOX1	Acyl-CoA oxidase 1
AD	Alzheimer's disease
ALDP	adrenoleukodystrophy protein
AMN	adrenomyeloneuropathy
CCALD	childhood cerebral adrenoleukodystrophy
CNS	central nervous system
DHA	docosahexaenoic acid
DHAPAT	dihydroxyacetone-phosphate acyltransferase
DMF	dimethyl fumarate
DNA	deoxyribonucleic acid
LC3	protein light chain 3
LXR	Liver X receptor
MFP2	multifunctional protein 2
MMF	monomethyl fumarate
MS	multiple sclerosis
PARP	poly-ADP-ribose polymerase
PD	Parkinson's disease
P-MS	progressive MS
PPAR	peroxisome proliferator-activated receptor
RNS	reactive nitrogen species
RR-MS	remittent recurrent-MS
ROS	reactive oxygen species
TEM	transmission electron microscopy
VLCFA	very-long-chain fatty acid
X-ALD	X-linked adrenoleukodystrophy
7β-OHC	7β-hydroxycholesterol
7KC	7-ketocholesterol

3.1 Neurodegeneration and Peroxisomal Disorders

3.1.1 Peroxisome and Peroxisomopathies

Peroxisomes, which are devoid of DNA, are single cell membrane organelles present in nearly all eukaryotic cells [1]. Depending on the cell type, elongated tubular (>2 μm in length) or spherical (0.1–1 μm) peroxisomes can be observed by transmission electron microscopy (TEM) [2] (Fig. 3.1). The identification of peroxisomes by immunofluorescence with the use of appropriated antibodies directed against specific peroxisomal antigens, such as the Abcd3, gives information on the peroxisomal topography (conventional immunofluorescence, confocal microscopy) and on the peroxisomal mass per cell (flow cytometry) (Fig. 3.2). Peroxisomes are essential to maintain Redox homeostasis and have important roles in lipid metabolism: β-oxidation of very-long-chain fatty acids (VLCFAs) and branched fatty acids, synthesis of docosahexaenoic acid (DHA, C22:6 n-3) and of plasmalogens which are major components of the myelin sheath [1]. There are also several evidences that peroxisome and mitochondria are tightly connected organelles playing key roles in cellular ageing [3]. It is now well-established that peroxisomal changes can directly or indirectly affect mitochondrial activity, and reciprocally [3].

Peroxisomopathies, which are rare diseases of genetic origin affecting the central and/or peripheral nervous system, include peroxisome biogenesis disorders (Zellweger syndrome), multiple peroxisomal enzyme deficiency (rhizomelic chondrodysplasia punctata), and single peroxisomal enzyme deficiency, such as X-linked adrenoleukodystrophy (X-ALD, MIM 300100), Acyl-CoA oxidase deficiency, and

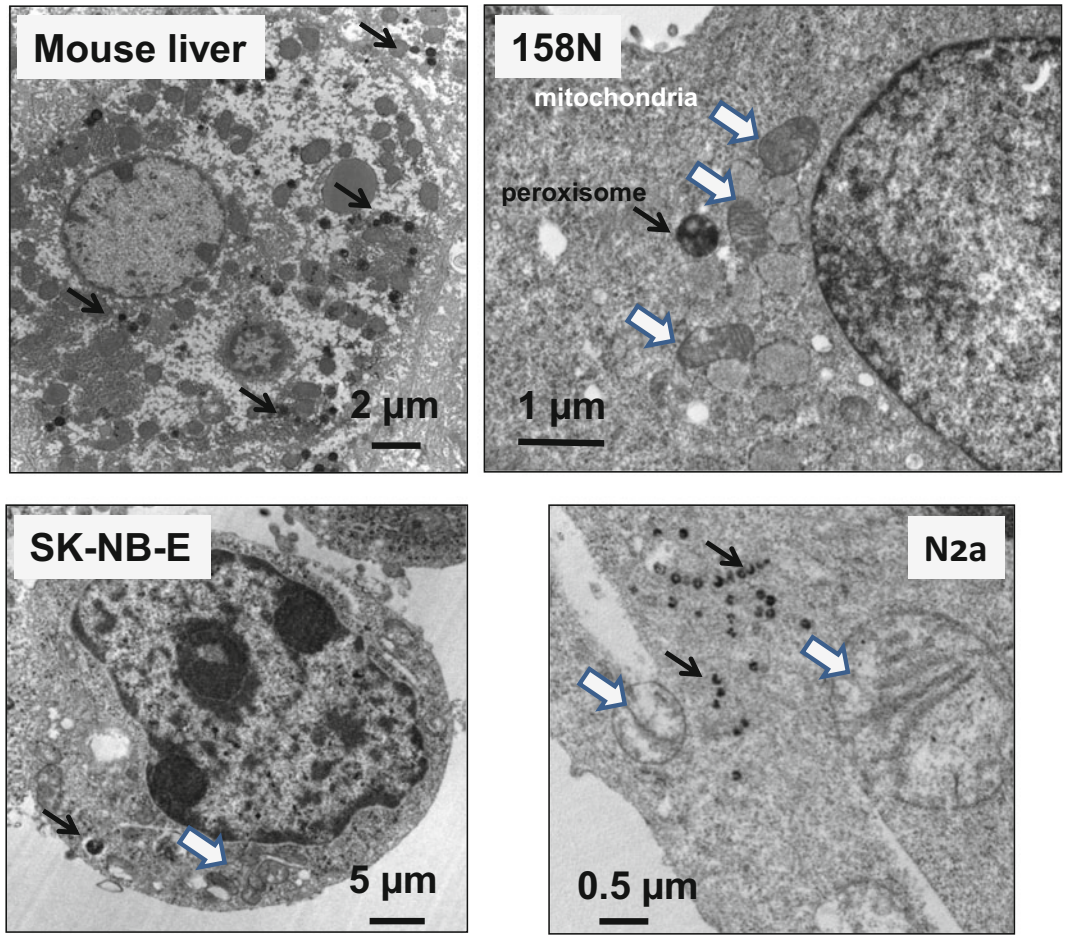

Fig. 3.1 Identification of peroxisomes in neural cells by transmission electron microscopy. Peroxisomes can be identified by various techniques, including transmission electron microscopy (TEM). Dark arrows point towards peroxisomes and white arrows towards mitochondria. Round peroxisomes were observed. The diameters were in the range of 0.5 µm in mouse liver, murine oligodendrocytes 158 N, and human neuronal SK-NB-E cells and of 0.1 µm in murine neuronal N2a cells

D-Bifunctional protein deficiency [4]. X-ALD, which is the most frequent peroxisomal leukodystrophy of childhood, is characterized by progressive central nervous system (CNS) demyelination [5]. X-ALD is caused by mutations of the *ABCD1* gene which encodes for a peroxisomal ABC half-transporter (ATP-binding cassette member 1 (ABCD1) also named adrenoleukodystrophy protein (ALDP)) involved in the import of very-long-chain fatty acids (VLCFAs) into the peroxisome [6]. These different forms of peroxisomopathies are all characterized by increased plasma levels of VLCFAs due to an impaired β-oxidation in the peroxisome and/or increased elongation [7]. The initial diagnosis of peroxisomopathies relies on the clinical presentation, brain-imaging, and biochemical analyses of VLCFAs, especially C24:0 and C26:0, which are elevated in the plasma and tissues of patients [7]. Newborn screening is based on the measurement of C26:0 lysophosphatidylcholine (26:0-lyso-PC) in dried blood spots [7]. At the moment, several studies have shown cytotoxic effects of VLCFA in vitro and in vivo [8, 9]. In vitro, on different types of nerve cells (neurons, glial, and microglial cells), C24:0 and C26:0 often induce a

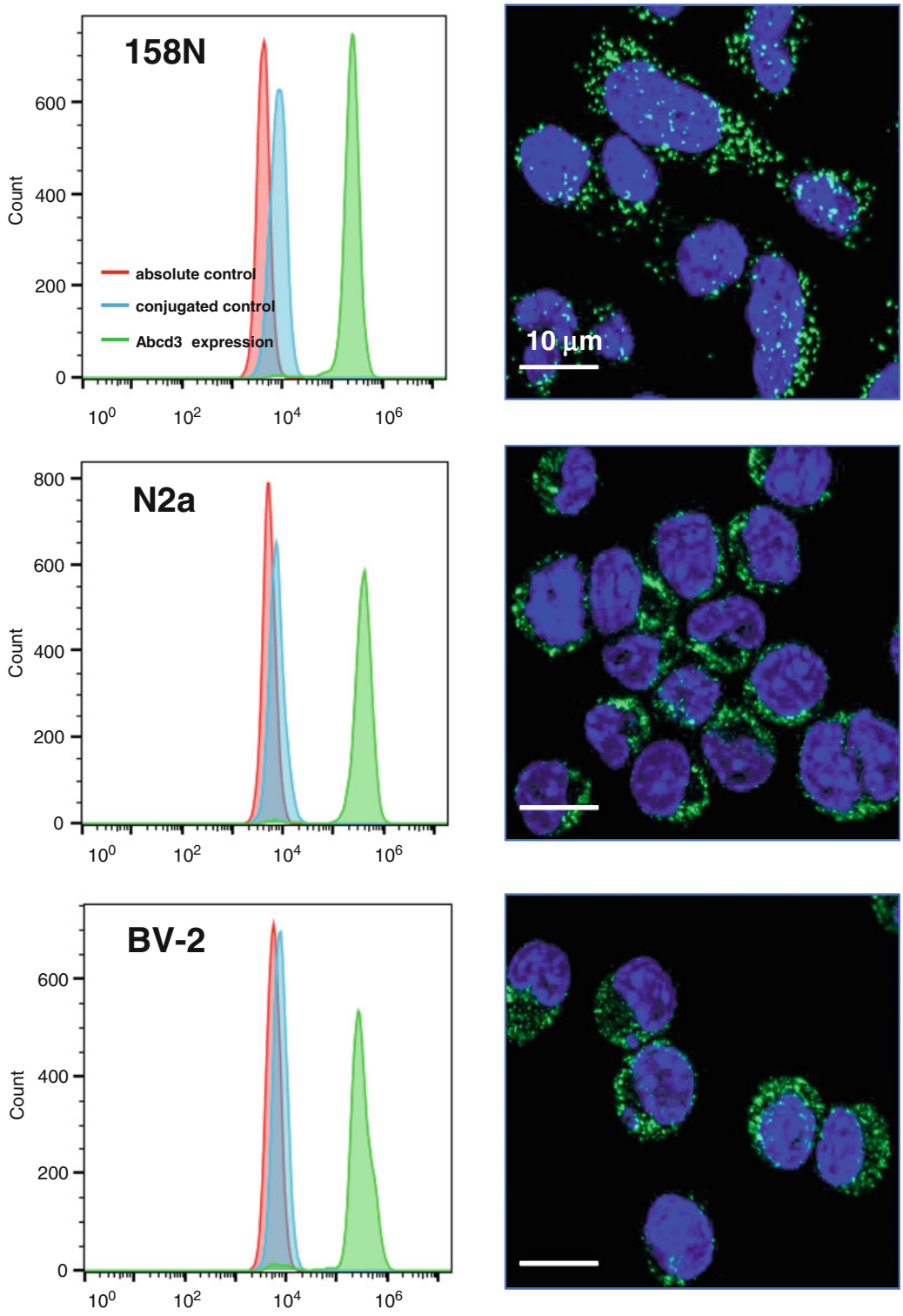

Fig. 3.2 Analysis of peroxisomes in neural cells by flow cytometry and fluorescence microscopy. The peroxisomes were revealed by indirect immunofluorescence with a rabbit polyclonal primary antibody directed against Abcd3 (ref # 11523651, Pierce / Thermo Fisher Scientific, Montigny le Bretonneux, France) and with a secondary

non-apoptotic mode of cell death associated with Ca^{2+} raise [10], K^+ homeostasis disruption [11], reactive oxygen species (ROS) and reactive nitrogen species (RNS) overproduction [8, 12], and autophagic criteria [13]. In vivo, lysophosphatidylcholine (C24:0) injection into the parietal cortex of mice led to widespread microglial activation and apoptosis [14]. However, in human, there is no apparent correlation between the VLCFA level and the phenotype in X-ALD patients [7]. Therefore, other factors than VLCFA, including lipid and non-lipid compounds, are suspected to trigger neurodegeneration in peroxisomopathies. At the moment, some signs of oxidative stress, considered as a hallmark of neurodegeneration, have been detected in the plasma and brain of X-ALD patients [15] and in patients with frequent neurodegenerative diseases: Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) [16]. As VLCFA (C24:0; C26:0) are potent inducers of oxidative stress [8, 12], they can favor the production of protein and lipid oxidation products capable to trigger cytotoxic effects. The lipid peroxidation products generated include several aldehydes [8], which are known to induce protein carbonylation, as well as some cholesterol oxidation products (oxysterols), including 7-ketocholesterol (7KC; also named 7-oxocholesterol) and 7β-hydroxycholesterol (7β-OHC) [8]. 7KC and 7β-OHC, which are mainly formed by cholesterol auto-oxidation, are strongly cytotoxic [17]. These oxysterols induce a mode of cell death by oxiapoptophagy involving oxidative stress, apoptosis, and autophagy [18], which are hallmarks of neurodegeneration. Currently, increased levels of 7KC and 7β-OHC have been described in the plasma of patients with different forms of X-ALD [19]: (i) cerebral demyelination, inflammatory childhood phenotypes (CCALD: childhood cerebral ALD), which is associated with a poor prognosis and rapidly progresses to dementia and death; (ii) cerebral juvenile and adult forms with demyelination in the CNS (ACALD: adolescent cerebral ALD); (iii) adulthood forms without CNS demyelination (AMN: adrenomyeloneuropathy) defined as a spinal cord and peripheral nerve disease; and (iv) Addison's disease, which is characterized by adrenal insufficiency only. It is therefore hypothesized that 7KC and 7β-OHC could contribute to neurodegeneration in peroxisomopathies, especially in X-ALD, and also in other major neurodegenerative diseases [20].

3.1.2 Potential Involvement of Peroxisomal Changes in Major Neurodegenerative Diseases: Alzheimer's Disease and Multiple Sclerosis

Peroxisomal abnormalities associated with peroxisomopathies led to the clarification of the role of this organelle in neurodegeneration occurring in frequent neurodegenerative diseases, such as AD and MS. Currently, potential roles of peroxisomes are suspected in AD and in dementia of the Alzheimer's type. Indeed, an accumulation of C22:0 and VLCFAs (C24:0; C26:0), all substrates for peroxisomal β-oxidation, was observed in the cortical regions of AD patients with stages V–VI of the disease compared with those modestly affected (stages I–II); conversely, the level of plasmalogens, which need intact peroxisomes for their biosynthesis, was decreased [21]. In addition, in demented patients, including AD patients, the variations of fatty acid levels and the accumulation of C26:0 in the plasma and red blood cells highlight an alteration of fatty acid

Fig. 3.2 (continued) goat anti-rabbit antibody coupled with 488-Alexa (Thermo Fisher Scientific). The peroxisomal mass was quantified by flow cytometry. A fluorescent microscope coupled to an Apotome-structured illumination system (Imager M2, Zeiss) was used to visualized the peroxisomes; the fluorescent signals of the samples were collected with the ZEN software (Zeiss). For microscopical observations, the nuclei were stained with Hoechst 33342 (2 μg/mL)

metabolism and point towards possible peroxisomal dysfunction [22]. In MS patients, a reduction in neuronal peroxisomes in MS grey matter has been reported [23], and alteration in the metabolism of VLCFAs, which could be a consequence of defective peroxisomes, has been described [24]. In the brain of patients with AD, increased levels of 7KC and 7β-OHC have also been shown [25]. It is important to underline that these oxysterols can be formed under the action of VLCFA-induced oxidative stress [8] and under stress conditions induced by amyloid-β (Aβ) proteins, mainly Aβ42, which accumulates in brain lesions of patients with AD. High amount of 7KC have been reported in the cerebrospinal fluid of MS patients [26] as well as low levels [27]. 7KC values might depend on the type of MS considered (Progressive-MS (P-MS) versus remittent recurrent – MS (RR-MS)) [28]. It is however widely accepted that oxysterols are considered as bonafide lipid mediators. To be qualified as a bonafide lipid mediator, a lipid compound should meet three conditions: (i) to be endogenous, (ii) to have its levels altered depending on the physiological or pathological situation, and (iii) to induce a signaling response when its levels are altered. As a result, abnormal levels of 7KC (low or high) can alter cellular behavior. It is therefore hypothesized that 7KC, 7β-OHC could favor peroxisomal and mitochondrial dysfunction leading to neurodegeneration in patients with AD and MS [20] (Fig. 3.3). This led us to specify the impact of different concentrations of 7KC and/or 7β-OHC on murine glial cells (murine oligodendrocyte 158 N [29], murine microglial BV-2 cells [19], murine neuronal N2a cells, human neuroblastoma SK-N-BE cells, and on rat C6 astrocytoma cells [30, 31], taking into account the effects on organelles (peroxisomes, mitochondria and lysosomes), oxidative stress, and the ability to induce cell death (apoptosis/necrosis) and to activate autophagy.

3.2 In Vitro Evidence of 7-Ketocholesterol- and 7β-Hydroxycholesterol-Induced Peroxisomal Damages in Nerve Cells

The cytotoxic effects of 7KC and 7β-OHC have been studied on several nerve cell lines: murine oligodendrocyte 158 N cells, murine microglial BV-2 cells, murine neuronal N2a cells, and C6 rat glioma cells. On 158 N and/or BV-2 cells, 7KC and 7β-OHC induce a mode of cell death by oxiapoptophagy (OXIdative stress + APOPTOsis + autoPHAGY) characterized by ROS overproduction revealed by dihydroethidium staining, a decrease of oxidative phosphorylation associated with a loss of transmembrane mitochondrial potential (ΔΨm) measured with DiOC6(3), reduced expression of Bcl-2, caspase-3 activation, poly-ADP-ribose polymerase (PARP) degradation, and condensation and/or fragmentation of the nuclei which are typical criteria of oxidative stress and apoptosis [18, 19, 32, 33]. Moreover, 7KC and 7β-OHC also enhance cytoplasmic membrane permeability to propidium iodide and induce acidic vesicular organelle formation evaluated with acridine orange which evocate autophagic vesicles in agreement with observations realized by TEM [32, 33]. In addition, 7KC and 7β-OHC promote conversion of microtubule-associated protein light chain 3 (LC3-I) to LC3-II which is a characteristic of autophagy [18, 32], and 7KC increases the expression of p62 an autophagosome cargo protein involved in the sequestosome/aggresome formation preceding the autophagosome formation [19, 29]. On C6 rat glioma cells, it has been demonstrated that 7β-OHC induces survival autophagy, since rapamycin, an autophagic inducer, and 3-methyladenine, an autophagic inhibitor, reduce and increase 7β-OHC-induced cell death, respectively [31]. On 158 N cells, under treatment with 7KC used at sub-toxic (25 μM, 24 h) and toxic (50 μM, 24 h)

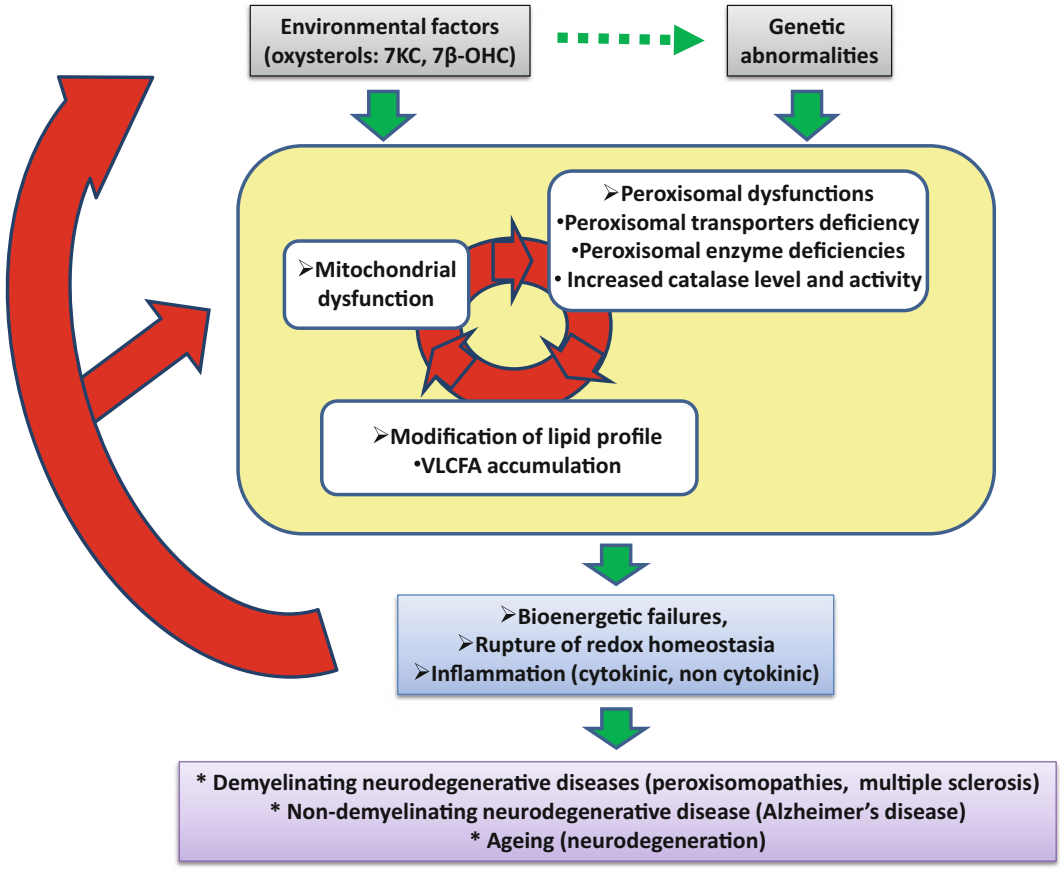

Fig. 3.3 Incidence of peroxisomal dysfunction in neurodegeneration: hypothetic model. Environmental factors (oxysterols: 7-ketocholesterol (7KC) and 7-β-hydroxycholesterol (7β-OHC)) resulting from rupture or Redox homeostasis, which is a hallmark of neurodegenerative diseases, are often enhanced in the biological fluids and in the brain of patients with neurodegenerative disease. These oxysterols could trigger peroxisomal and mitochondrial damages which could further favor bioenergetic failure, overproduction of ROS, and activation of inflammatory processes. In turn, these events could contribute to amplify brain damages via secondary mitochondrial and peroxisomal dysfunctions contributing to the overproduction of oxysterols, thereby creating an amplification loop [20]

concentrations, important peroxisomal changes were observed even in the absence of oxiapoptophagy which was only induced at 50 μM [31] (Fig. 3.4). In the presence of 7KC (25 μM), only slight mitochondrial dysfunction and oxidative stress were found as well as modifications of the cytoplasmic distribution of mitochondria: clusters of mitochondria were detected. Thus, the peroxisomal alterations observed were similar with 7KC used at 25 and 50 μM [29]: presence of peroxisomes with abnormal sizes and shapes observed by TEM; lower cellular level of ATP-binding cassette transporter member 3 (ABCD3) used as a marker of peroxisomal mass (measured by flow cytometry); lower mRNA and protein levels (measured by RT-qPCR and western blotting) of ABCD1 and ABCD3 (two ATP-dependent peroxisomal transporters), of two peroxisomal enzymes Acyl-CoA Oxidase 1 (ACOX1) and multifunctional protein 2 (MFP2) enzymes involved in peroxisomal β-oxidation, and lower mRNA level of dihydroxyacetone-phosphate acyltransferase (DHAPAT), involved in peroxisomal β-oxidation and plasmalogen synthesis; and increased levels of VLCFAs (C24:0, C24:1,

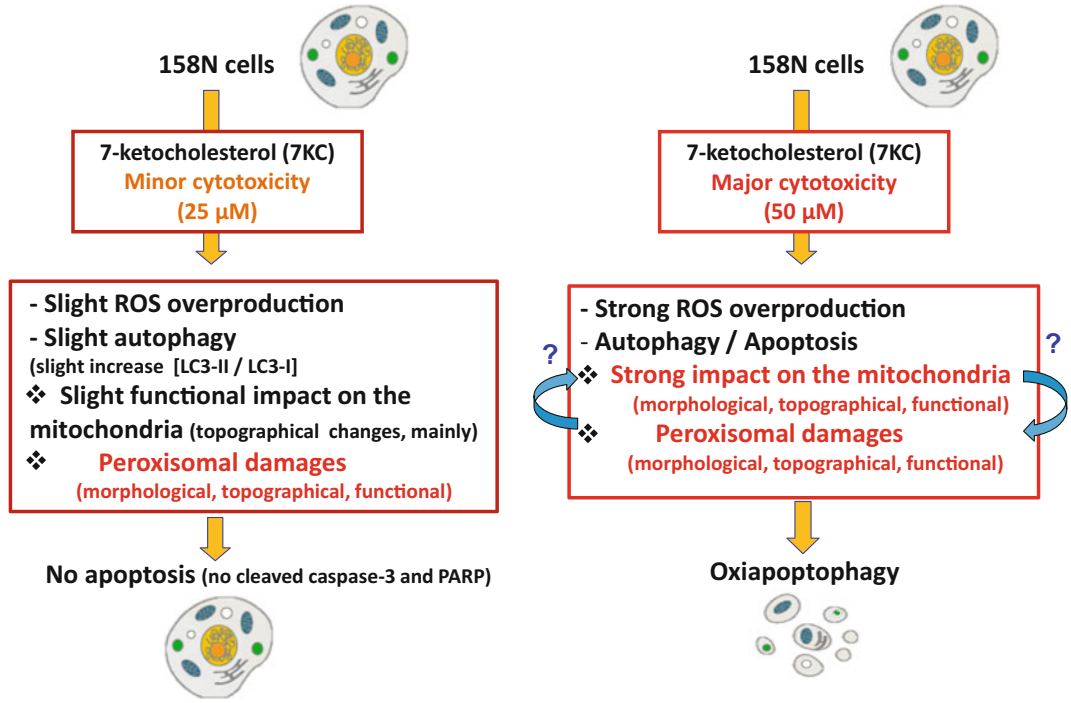

Fig. 3.4 Association of peroxisomal damages with mitochondrial dysfunctions in 7-ketocholesterol-treated 158 N murine oligodendrocytes. 7-ketocholesterol (7KC)-induced topographical, morphological, and functional peroxisomal changes are observed either with slight or strong mitochondrial dysfunctions, slight or strong ROS overproduction, and without or with cell death induction (oxiapoptophagy) on 158 N cells. The cellular model (158 N cells treated with 7KC) has been used to clarify the relationships between peroxisome and mitochondria (and vis versa) during 7KC-induced side effects leading either to slight cytotoxic effects (7KC: 25 µM) or oxiapoptophagy (7KC: 50 µM) The figure was reproduced with the written consent of Elsevier Editor (Nury T et al., Induction of peroxisomal changes in oligodendrocytes treated with 7-ketocholesterol: Attenuation by α-tocopherol [29])

C26:0, and C26:1) quantified by gas chromatography coupled with mass spectrometry metabolized by peroxisomal β-oxidation. On 158 N cells, under treatment with 7β-OHC (50 µM, 24 h), morphological alterations of mitochondria and peroxisomes were simultaneously observed [33]. These data obtained on 158 N cells support the following hypotheses: (i) the peroxisome would be more sensitive to 7KC than the mitochondria (7KC used at 25 µM induced important peroxisomal changes, whereas the mitochondria was slightly affected); (ii) peroxisomal changes can occur without signs of cytotoxicity (induction of oxiapoptophagy); and (iii) peroxisomal changes could precede mitochondrial dysfunctions required to induce oxiapoptophagy. These peroxisomal dysfunctions may be associated with oligodendrocyte degeneration and may contribute to demyelination in X-ALD and MS. On BV-2 cells, 7KC also induced several peroxisomal modifications: decreased Abcd1, Abcd2, Abcd3, Acox1, and/or Mfp2 mRNA and protein levels, increased catalase activity, and decreased Acox1-activity [19]. In microglial cells, 7-ketocholesterol- and 7β-hydroxycholesterol-induced peroxisomal alterations may favor the pro-inflammatory phenotype of these cells, thereby contributing to brain inflammation often seen in neurodegenerative diseases.

3.3 Pharmacological Consequences of the Involvement of 7-Ketocholesterol- and 7β-Hydroxycholesterol-Induced Peroxisomal Damages for the Treatment of Neurodegenerative Diseases

Under the effect of 7KC and 7β-OHC, the signaling pathways involved in oxidative stress, inflammation, and cell death, associated with apoptotic and autophagic criteria, have the advantage of being fairly well-known [17, 33]. This made it possible to identify natural and synthetic molecules as well as mixtures of molecules (argan, olive, and milk thistle oils) [34] having cytoprotective activities with respect to 7KC and 7β-OHC and capable to prevent peroxisomal and mitochondrial dysfunctions. Several molecules are able to attenuate the cytotoxic effects of 7KC on different cell types, but only few are very efficient. Currently, the only molecules identified strongly counteracting the toxicity of 7KC and 7β-OHC are α-tocopherol, docosahexaenoic acid (DHA, C22:6 n-3), and oleic acid (C18:1 n-9) [18]; biotin is only cytoprotective for 7β-OHC. Among the synthetic molecules, only dimethyl fumarate (DMF), marketed under the name Tecfidera for the treatment of MS, as well as its major metabolite, monomethyl fumarate (MMF), protect against oxiapoptophagy [33]. As more and more arguments are in favor of the relationships between neurodegeneration, peroxisomal, and mitochondrial dysfunction and the involvement of oxysterols, to oppose the cytotoxic activities of these molecules could pave the way for new therapeutic approaches in both peroxisomopathies and frequent neurodegenerative diseases, such as AD and MS.

3.4 Conclusion

Although the auto-oxidation of cholesterol essentially leads to the formation of oxidized cholesterol derivatives in the 7-position, the formation of oxidized cholesterol derivatives at the 4-, 5-, and 6-positions should be studied. An analysis of the oxisome by appropriate analytical biochemistry methods could also be informative. This approach would also take into account the metabolites of 7KC and 7β-OHC and also other enzymatically formed oxysterols (24S-hydroxycholesterol (also named cerebrosterol), 27-hydroxycholesterol, 25-hydroxycholesterol, and derivatives) known to be involved in neurodegeneration. The study of the impact of oxysterols on the organelles of nerve cells (glial and microglial cells, neurons) in relation to oxidative stress, inflammation, and cell death as well as the incidence of these molecules on certain nuclear receptors (peroxisome proliferator-activated receptors (PPARs), Liver X receptors (LXRs) in particular) offers many perspectives to better know and better treat neurodegenerative diseases.

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Part II

Peroxisomal Diseases: Biological Characteristics and Diagnosis

Peroxisome Biogenesis Disorders

4

Masanori Honsho, Kanji Okumoto, Shigehiko Tamura, and Yukio Fujiki

Abstract

Peroxisomes are presented in all eukaryotic cells and play essential roles in many of lipid metabolic pathways, including β-oxidation of fatty acids and synthesis of ether-linked glycerophospholipids, such as plasmalogens. Impaired peroxisome biogenesis, including defects of membrane assembly, import of peroxisomal matrix proteins, and division of peroxisome, causes peroxisome biogenesis disorders (PBDs). Fourteen complementation groups of PBDs are found, and their complementing genes termed *PEXs* are isolated. Several new mutations in peroxins from patients with mild PBD phenotype or patients with phenotypes unrelated to the commonly observed impairments of PBD patients are found by next-generation sequencing. Exploring a dysfunctional step(s) caused by the mutation is important for unveiling the pathogenesis of novel mutation by means of cellular and biochemical analyses.

Keywords

Peroxisomal biogenesis disorders (PBDs) · Zellweger spectrum disorders (ZSDs) · Peroxin gene *PEX* · Complementation groups (CGs) · Next-generation sequencing · CHO mutant cells

Abbreviations

CGs	complementation groups
CHO	Chinese hamster ovary
EST	expressed sequencing tag
IRD	infantile Refsum disease
NALD	neonatal adrenoleukodystrophy
PBDs	peroxisome biogenesis disorders
PTS	peroxisomal targeting signal
RCDP	rhizomelic chondrodysplasia punctata
VLCFA	very-long chain fatty acids
WGS	whole-genome sequencing
ZSDs	Zellweger spectrum disorders

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4.1 Introduction

Peroxisomes are single membrane-bounded organelles present in almost all eukaryotic cells. In mammals, peroxisomes participate in many metabolic pathways, including β-oxidation of fatty acids, oxidation of D-amino acids, and synthesis of ether-linked glycerophospholipids, such as plasmalogens [1]. These oxidation reactions in peroxisomes generate hydrogen peroxide, which is subsequently decomposed in situ by catalase, a typical marker enzyme of peroxisomal matrix [2]. The physiological significance of peroxisome is highlighted by fatal human genetic peroxisomal biogenesis disorders (PBDs), which is divided into two groups: Zellweger spectrum disorders (ZSDs) and rhizomelic chondrodysplasia punctata (RCDP) (Table 4.1). ZSDs comprise the most severe Zellweger syndrome (or cerebro-hepato-renal syndrome) [3], less severe neonatal adrenoleukodystrophy (NALD), and milder infantile Refsum disease (IRD) [4].

The patients with PBD caused by mutations of genes encoding proteins essential for the biogenesis of peroxisome manifest progressive metabolic disease as well as developmental abnormalities that produce distinct dysmorphic features, including abnormal morphology of the cerebellum, cerebrum, and inferior olivary nucleus [5]. The patients with PBD generally show biochemical abnormalities of peroxisomes, such as an elevation of very-long chain fatty acids (VLCFA) in plasma and a decreased level of plasmalogens in erythrocytes [6]. In contrast, patients with RCDP show the plasmalogen synthesis without accumulation of VLCFA [7–11]. In RCDP five genotypes are reported, in which RCDP1 and RCDP5 are classified as PBDs due to the inherited mutations in the genes encoding the cytosolic receptors essential for the transport of peroxisomal enzymes catalyzing the synthesis of plasmalogens [6, 12]. The remaining three RCDP disorders, RCDP2, 3, and 4, belong to the single peroxisomal enzyme deficiencies by the mutation in the genes encoding the peroxisomal enzymes essential for the synthesis of plasmalogens [6], including dihydroxyacetonephosphate acyltransferase [7], alkylglycerone phosphate synthase (AGPS) [13], and fatty acyl-CoA reductase 1 [14].

To date, genetic heterogeneity comprising 14 complementation groups (CGs) is found in PBDs, including ZSDs and RCDP1, and their complementing genes have been identified [15–19] (Table. 4.2). Moreover, exploring functions of individual peroxin led to understanding the impaired step of peroxisome biogenesis in the patients. Furthermore, combination of these earlier studies searching a causal gene responsible for the respective CG patient and the whole-exome sequencing and/or whole-genome sequencing (WGS) now allows to identify mutation in peroxin linking to novel disease phenotypes from patients with the adolescent-adult onset [20]. In this chapter, we summarize the approaches for identification of peroxins in mammalian cells and present our recent studies showing the pathogenesis of PBDs found in a patient with adolescent onset moderate to severe hearing loss.

4.2 Genetic Approaches for the Identification of Causal Genes of PBDs

Two mutually complementary approaches were applied for the identification of causal genes of PBDs, i.e., a forward genetics screening by means of phenotype complementation of peroxisome assembly-defective mutants of somatic cells, such as Chinese hamster ovary (CHO), and isolation of human *PEX* genes by homology search on the human expressed sequencing tag (EST) database using yeast *PEX* genes, followed by complementation analysis of phenotype in fibroblast cells derived from the patients with PBDs of 14 different genotypes.

Table 4.1 Peroxisome biogenesis disorders (PBDs)

Zellweger spectrum disorders (ZSDs)
Zellweger syndrome (ZS)
Neonatal adrenoleukodystrophy (NALD)
Infantile Refsum disease (IRD)
Heimler syndrome (HS)
Rhizomelic chondrodysplasia punctata (RCDP)

Table 4.2 Complementation groups (CGs) and *PEX* genes of peroxisome deficiencies

Gene	CG		PBD	CHO mutants	Biogenesis of peroxisomal membrane[a]	Characteristics
	US/EU	Japan				
PEX1	1	E	ZS, NALD*, IRD*	Z24, ZP107	+	AAA family
PEX2	10	F	ZS, IRD*	Z65	+	PMP, RING
PEX3	12	G	ZS	ZPG208	−	PMP, PMP-DP
PEX5	2		ZS, NALD, RCDP5[#]	ZP105*, ZP139, ZPG231[#], ZPEG241[#]	+	PTS1 receptor, TPR family
PEX6	4(6)	C	ZS, NALD*	ZP92	+	AAA family
PEX7	11	R	RCDP1	ZPG207	+	PTS2 receptor, WD repeat family
PEX10	7(5)	B	ZS, NALD		+	PMP, RING
PEX11β	16		ZS		+	PMP
PEX12	3		ZS, NALD, IRD	ZP109	+	PMP, RING
PEX13	13	H	ZS, NALD*	ZP128	+	PMP, PTS1-DP, SH3
PEX14	15	K	ZS	ZP110	+	PMP, PTS1-DP, PTS2-DP
PEX16	9	D	ZS		−	PMP, PMP-DP
PEX19	14	J	ZS	ZP119	−	CAAX motif, PMP receptor
PEX26	8	A	ZS, NALD*, IRD*	ZP124, ZP167	+	PMP, Pex1-Pex6 recruiter

*, temperature-sensitive phenotype
[a]Assembly of peroxisomal membrane is normal (+) or impaired (−)
[#], import of PTS2-protein is specifically impaired
PBD peroxisomal biogenesis disorders, *ZS* Zellweger syndrome, *IRD* infantile Refsum disease, *NALD* neonatal adrenoleukodystrophy, *RCDP* rhizomelic chondrodysplasia punctata, *DP* docking protein, *PMP* peroxisome membrane protein, *TPR* tetratricopeptide repeat

4.2.1 Mammalian Cell Mutants Deficient of Peroxisome

Genetic heterogeneity consisting of 14 CGs has been identified in PBDs by cell-fusion CG analysis using fibroblast cells derived from PBD patients [4, 22], whereas CG16 [16] and RCDP5 [23] are defined by the exome sequencing. Localization of peroxisomal matrix proteins are impaired in fibroblast cells derived from patients with ZS, a prototype of ZSDs, despite of the presence of peroxisomal remnant structures

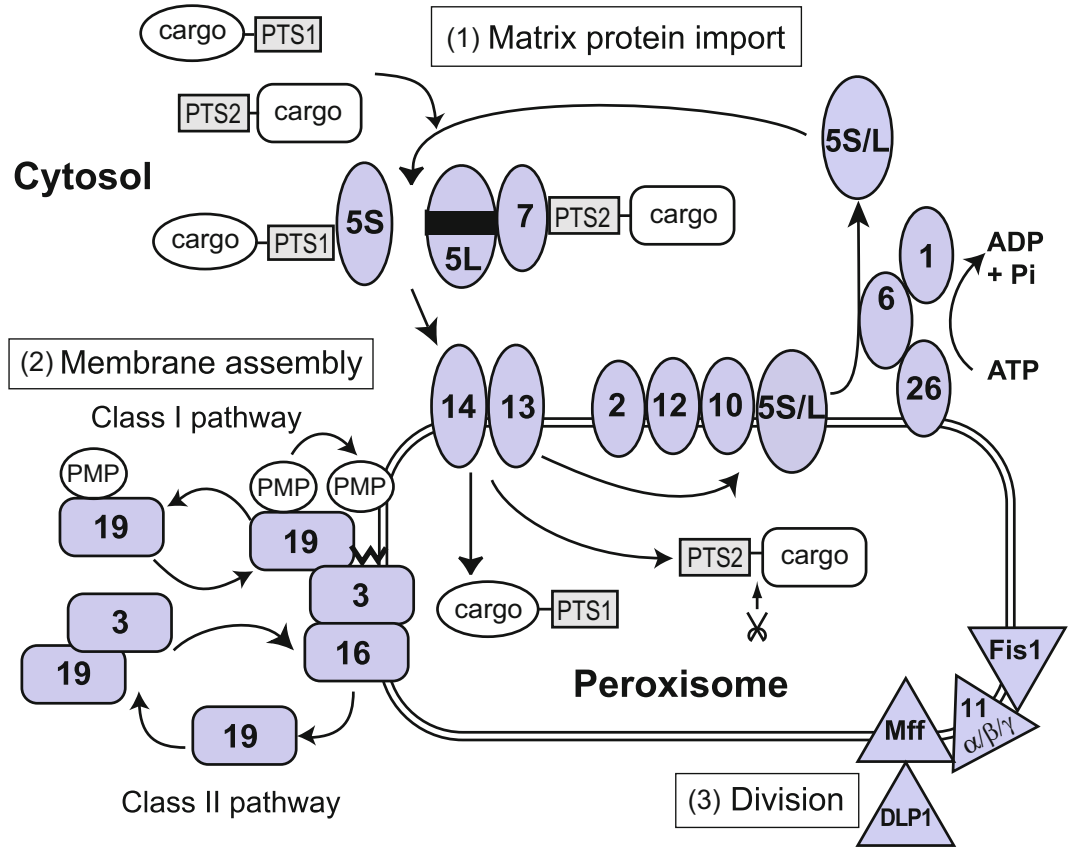

Fig. 4.1 A schematic model of peroxisome biogenesis in mammalian cells. Peroxins are classified into three groups: 1) 10 peroxins required for matrix protein import, 2) 3 peroxins essential for the membrane assembly, and 3) peroxins involved in the division of peroxisomes. PTS1- and PTS2-containing peroxisomal matrix proteins are recognized by Pex5 and Pex7, respectively. Note that both Pex5S, the shorter form of Pex5, and Pex5L, the longer form of Pex5, act as a cytosolic receptor for PTS1-protein. Pex5L contains an additional insert of 37 amino acids that is positioned after amino acid at 214 of Pex5S. This region is essential for binding to Pex7 carrying PTS2-protein [21]. Any impairment of Pex5L-Pex7-PTS2-protein complex formation abrogates transport of PTS2-protein. For peroxisome membrane assembly, three peroxins, Pex19, Pex3, and Pex16, participate in the targeting step of peroxisomal membrane proteins (PMPs). Pex19 binds to PMPs in the cytosol and recruits PMPs to the membrane receptor Pex3 (class I pathway), whereas newly synthesized Pex3 targets to Pex16 by the aid of Pex19 (class II pathway). Pex11β functions in the division step of peroxisomes in a manner dependent on DHA-containing phospholipids. DHA promotes the formation of Pex11β-enriched region on elongated peroxisomes [24]. Other factors, including dynamin-like protein 1 (DLP1), mitochondrial fission factor (Mff), and mitochondrial fission 1 (Fis1), are coordinately regulated peroxisome division. See the chapter of peroxisome biogenesis for the role of individual peroxins. The figure is adopted and modified from [19]

containing peroxisomal membrane proteins, while cells from patients classified in CG9, CG12, and CG14 of ZS are devoid of peroxisomal remnant structures (Table 4.2) [22]. These cellular phenotypes are valuable information for exploring the function of individual peroxins in peroxisome biogenesis, including membrane assembly, import of peroxisomal matrix proteins, and division of peroxisomes (Fig. 4.1).

As an alternative strategy, aiming for the identification of *PEX* genes, peroxisome-deficient CHO cell mutants were isolated by a combination of colony autoradiographic screening based on the activity of peroxisomal matrix protein, dihydroxyacetonephosphate acyltransferase [25, 26], or the combination method of colony autoradiographic screening and the 9-(10-pyrene) nonanol (P9OH)/ultraviolet (UV) selection

method enables selective killing of wild-type cells by efficient incorporation of P9OH into fatty alcohol of plasmalogens [27, 28]. Subsequent CG analyses using pairwise cell-fusion assay between CHO cell mutants and PBD patient fibroblasts revealed that 11 CGs of CHO mutants represent the human PBD CGs (Table 4.2). It is noteworthy that *PEX1* and *PEX6* are the most commonly affected and the second of the most commonly affected genes, respectively, in patients with ZSDs; the mutations in *PEX12* or *PEX26* are two of the more common ZSDs [29–32]. CHO cell mutants corresponding to the remaining three CGs, i.e., *PEX10*, *PEX16*, and *PEX11β*, have not been isolated despite of several rounds of experiments for isolation of peroxisome-deficient CHO cell mutants. However, unique peroxisome biogenesis-defective CHO cell mutant, ZP114, and dynamin-like protein 1 (DLP1) mutant, ZP121, were isolated by the same P9OH/UV selection method [33–35]. Interestingly, the impaired localization of catalase in ZP114 is caused by a localization shift of a mitochondrial protein BAK to peroxisomes due to a loss of expression of voltage-dependent anion channel protein 2 [34]. In ZP121, morphologically abnormal tubular peroxisomes and mitochondria are observed due to the dysfunction of DLP1, an essential fission regulator of both mitochondria and peroxisomes [35]. These two mutant cells showing a unique phenotype imply a crosstalk of peroxisomes with mitochondria. Therefore, an isolation of peroxisome biogenesis defective mutant cells has a potential to uncover the role of network between peroxisomes to other organelle, such as lysosome [36].

4.2.2 Isolation of *PEX* Genes

The advantage of a high transfection efficiency of CHO cells enables to isolate *PEX* gene encoding peroxin from cDNA library subcloned into mammalian expression vector by means of monitoring a restoration of peroxisomal matrix protein import and/or cell viability in a manner dependent on the synthesis of plasmalogens whose synthesis requires functional peroxisomes [22, 37].

The effectiveness of forward genetic approach for the isolation of *PEX* gene was demonstrated by the successful isolation of *PEX2* from rat liver cDNA library as a complementing gene of Z65, a CHO cell mutant belonging to CG10 in human PBDs [37, 38]. Expression of Pex2, a 35-kDa membrane peroxin, enhanced cell viability of Z65 from hypersensitive to resistant to the reactive oxygen species generated by irradiation of 12-(1′-pyrene)dodecanoic acid [37]. Moreover, impaired catalase localization in Z65 and fibroblasts from a CG10 patient were restored by the transfection of *PEX2*. Subsequent genome analysis of the patient identified that a homozygous nonsense point mutation at arginine 119 was responsible for CG10 PBD [38].

Development of a more practical approach by a transient transfection of cDNA library and direct monitoring of the localization of peroxisomal marker protein, such as peroxisomal targeting signal type-I-tagged green fluorescent protein (GFP-PTS1), in some cases facilitates identification of *PEX* cDNA, including seven others, *PEX1, PEX3, PEX6, PEX12, PEX13, PEX19,* and *PEX26* [22]. Other peroxins, including *PEX5* [39] and *PEX14* [40], were also successfully isolated by the CHO mutant-cell-based complementation assay, although these peroxins were earlier identified [41–43]. Of note, we isolated *PEX5*-defective CHO mutant ZPG231 showing an impaired import of PTS2-protein but not PTS1-protein, including catalase, from wild-type CHO-K1 cells transformed with two cDNAs encoding *PEX2* and PTS2-GFP [44]. The import deficiency of PTS2-protein is caused by a point mutation locating immediately upstream of the longer form of Pex5 (Pex5L)-specific 37-amino acid insertion sequence in ZPG231. These results provide the first evidence that disruption of Pex5L-Pex7 interaction completely abolish the PTS2-protein import (Fig. 4.1). Similarly, loss of *PEX5L* in CHO mutant ZPEG241 [45, 46] or patients' fibroblasts results in deficient transport of PTS2-protein but not PTS1 [23] as observed in the patients' fibroblasts with RCDP1.

4.2.3 EST Homology Search

From a homology search by screening the human EST database using *PEX* cDNAs obtained from several yeast species, including *Saccharomyces cerevisiae, Pichia pastoris, Hansenula polymorpha,* and *Yarrowia lipolytica*, several human orthologues *PEX* genes responsible for PBDs, including *PEX1, PEX3, PEX5, PEX6, PEX7, PEX10, PEX12, PEX13,* and *PEX16,* were identified [4]. Similarly, *PEX11β* was identified by EST database search using yeast *PEX11* [47], which led to the identification of homozygous nonsense mutation in the *PEX11β* in the patient with a defect of peroxisome division [16, 17].

4.3 Identification of *PEX* Mutation in Patients with Very Mild-Phenotype

Patients with ZS tend to carry mutation, such as nonsense mutation, frameshifts, which often causes a truncation of functional domain and/or large domain deletion in peroxins, resulting in the severe dysfunction of peroxins. In contrast, missense mutations are frequently found in patients with milder phenotypes of PBDs, NALD, and IRD [22, 48], which is likely owing to the residual activity of peroxisomal matrix protein import. Interestingly culturing PBD cells with mild defects in peroxisome biogenesis at 30 °C restores peroxisomal assembly as well as biochemical abnormalities [49, 50], while growing cells at 40 °C exacerbates the impaired peroxisomal assembly by judging with catalase immunostaining [51, 52]. The latter procedure is proposed to apply for the diagnosis of atypical PBD patients with nearly normal or very mild defects of peroxisomal functions.

Recent studies reveal that next-generation sequencing, such as WGS, is a powerful tool to identify the gene mutation. Indeed, mutation in *PEX1* or *PEX6* was identified in six patients with Heimler Syndrome, a rare recessive disorder showing sensorineural hearing loss, amelogenesis, nail abnormalities, and occasional or late-onset retinal pigmentation [53]. Next-generation sequencing is becoming more available, by which number of patients with mutation in *PEX* gene is increasing [16, 54–63]. It is noteworthy that biochemical and functional studies in the patients' fibroblasts are important to prove pathogenicity of the identified mutations in *PEX* genes.

By clinical exome sequencing, we recently identified an autosomal recessive missense variant, c.153C > A (p.F51L), in the peroxisome biogenesis factor 26 gene (*PEX26*) in a 19-yr-old female who had moderate to severe hearing loss otherwise normal prenatal and postnatal clinical courses and neurodevelopment [63]. Hearing deficit is one of the most consistent symptoms in patients manifesting ZSDs during adolescent to adulthood. Pex26 is a tail-anchored type II membrane protein in peroxisomal membrane and recruits the AAA ATPase peroxins, Pex1 and Pex6, which is essential process for peroxisomal matrix protein import [15]. (Chap. 1, Peroxisomes: Biological Functions and Biogenesis). Furthermore, we showed that import of peroxisomal matrix proteins, including catalase, is impaired in patient fibroblasts at 42 °C. Moreover, a rate of matrix proteins is significantly reduced in the patient's fibroblasts at 37 °C due to the less efficient interaction with Pex1-Pex6 complexes, although normal morphology and biogenesis of peroxisomes, including matrix protein import of both PTS1 and PTS2 proteins, are observed [63]. The observed phenotype of less efficient transport in the patient's fibroblast may cause a dysregulation of the quality control of peroxisomal matrix protein. These biochemical and functional studies in fibroblasts from the patient confirm the diagnosis caused by the mutation in *PEX26*. The analyses using fibroblasts from patients with mild phenotype are useful to study the severity of the defect as well as for proper clinical management of the patient.

4.4 Conclusions and Future Perspective

Identification of pathogenic genes responsible for PBDs of all 14 CGs is accomplished, and the

basic role of individual peroxins in the step of peroxisome biogenesis is uncovered. Unveiling the pathogenic mutation by means of next-generation sequencing together with biochemical and functional studies in the fibroblasts from patients with mild phenotypes will shed light on the understanding of the regulatory mechanism of peroxins during peroxisome biogenesis.

However, pathogenesis of PBDs still remains largely unknown. To date, pathogenesis of PBDs has been mainly explored by using fibroblasts from patients, CHO cell mutants defective in peroxisome biogenesis, and model organisms deficient of *PEX* gene, including *Caenorhabditis elegans*, *Drosophila melanogaster, Danio rerio*, and *Mus musculus* [64]. Impaired peroxisomal β-oxidation and synthesis of plasmalogens are commonly accepted defects in the patients. The pathogenesis caused by the impaired synthesis of plasmalogens is partially unveiled in mouse defective in plasmalogen synthesis. For instance, impaired myelination in plasmalogen-deficient mouse cerebellum [65] is likely caused by two distinct mechanisms, i.e., reduced transcription level of mRNAs encoding proteins required for the myelination, including myelin basic protein and proteolipid protein 1, which is caused by a compromised plasmalogen-mediated cholesterol homeostasis in cerebellum [66] and reduced ability of plasmalogen-mediated initiation of membrane wrapping of oligodendrocytes [67]. Furthermore, it is shown that impaired synthesis of plasmalogens exacerbates the pathology caused by VLCFA accumulation in testis and nervous tissue [68]. Similarly, defects in peroxisomal β-oxidation and plasmalogen synthesis are well-defined in the patients' fibroblasts as well as peroxisome assembly-defective CHO cell mutants. In addition to these biochemical defects, cytosolic localization of catalase is evident in those cells. Furthermore, mosaic catalase immunofluorescence pattern is shown in the fibroblasts from patients with mild phenotypes [69]. Importantly, catalase retains function in the cytosol in peroxisome assembly-defective cells [70]. Our studies using the CHO mutant cells show that a redox state in the cytosol is more reductive than that of the wild-type cells [71]. Nevertheless, we are still far from understanding how the catalase retained in the cytosol affects the cellular metabolic processes, except for the reductive redox state in the cytosol of cell mutants.

Our recent study implies that catalase localization is regulated by modulating the peroxisome membrane permeability in mammals [34], suggesting that localization of catalase seems to be tightly coupled with the cellular demand. The fact that the regulation of catalase localization is totally compromised in the mutant cells is implying that a more reductive redox balance in peroxisome-assembly defective cells affects multiple cellular metabolisms in addition to the impairment of typical peroxisomal metabolism. Therefore, comprehensive omics studies, including metabolomics, proteomics, and tanscriptomics, may be further required for fully understanding the pathogenesis of PBDs as shown by the earlier study presenting the perturbation of carbohydrate metabolism in liver from the mouse with hepatocyte-restricted elimination of *PEX5* [72]. These studies will unveil the pathogenesis of the patients with severe and mild PBD phenotypes, such as homozygous nonsense mutation in the *PEX11β* showing a mosaic catalase localization where catalase is localized in the cytosol of around 10% of the patient's fibroblast [16, 17] as well as mouse deficient *PEX11β* sharing several pathologic features by mouse models of ZS but lacking the several peroxisomal metabolic defects, including accumulation of VLCFAs and impaired synthesis of plasmalogens [73].

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5 Fatty Acid Oxidation in Peroxisomes: Enzymology, Metabolic Crosstalk with Other Organelles and Peroxisomal Disorders

Ronald J. A. Wanders, Frédéric M. Vaz, Hans R. Waterham, and Sacha Ferdinandusse

Abstract

Peroxisomes play a central role in metabolism as exemplified by the fact that many genetic disorders in humans have been identified through the years in which there is an impairment in one or more of these peroxisomal functions, in most cases associated with severe clinical signs and symptoms. One of the key functions of peroxisomes is the β-oxidation of fatty acids which differs from the oxidation of fatty acids in mitochondria in many respects which includes the different substrate specificities of the two organelles. Whereas mitochondria are the main site of oxidation of medium-and long-chain fatty acids, peroxisomes catalyse the β-oxidation of a distinct set of fatty acids, including very-long-chain fatty acids, pristanic acid and the bile acid intermediates di- and trihydroxycholestanoic acid. Peroxisomes require the functional alliance with multiple subcellular organelles to fulfil their role in metabolism. Indeed, peroxisomes require the functional interaction with lysosomes, lipid droplets and the endoplasmic reticulum, since these organelles provide the substrates oxidized in peroxisomes. On the other hand, since peroxisomes lack a citric acid cycle as well as respiratory chain, oxidation of the end-products of peroxisomal fatty acid oxidation notably acetyl-CoA, and different medium-chain acyl-CoAs, to CO_2 and H_2O can only occur in mitochondria. The same is true for the reoxidation of NADH back to NAD^+. There is increasing evidence that these interactions between organelles are mediated by tethering proteins which bring organelles together in order to allow effective exchange of metabolites. It is the purpose of this review to describe the current state of knowledge about the role of peroxisomes in fatty acid oxidation, the transport of metabolites across the peroxisomal membrane, its functional interaction with other subcellular organelles and the disorders of peroxisomal fatty acid β-oxidation identified so far in humans.

Keywords

Peroxisomes · Fatty acid oxidation · Peroxisomal diseases · Interorganellar crosstalk · Redox shuttles · Porins · ABC transporters · Bile acids · Genetic diseases · Fatty acids

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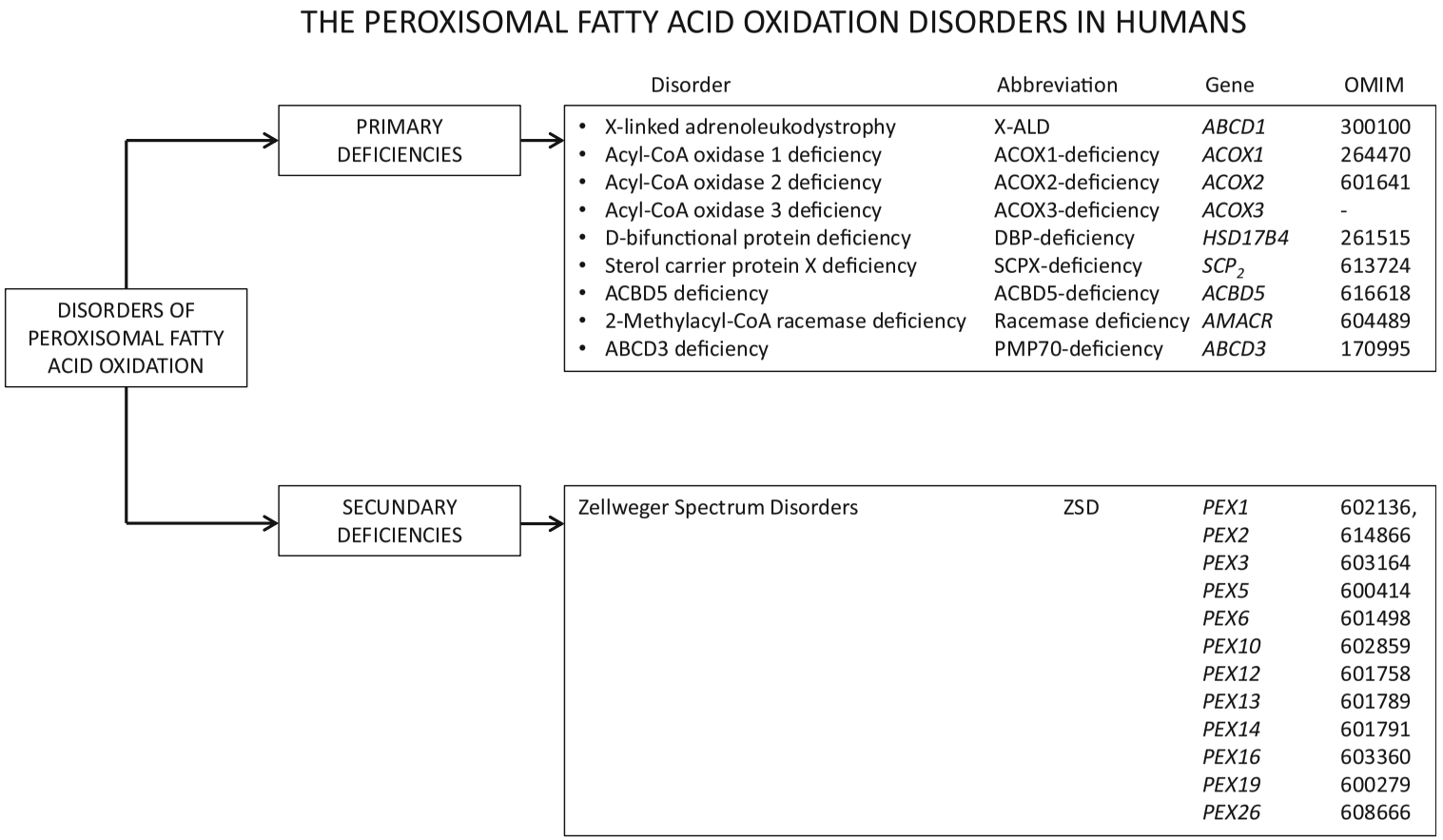
5.1 Introduction

Eukaryotic cells contain a large variety of subcellular organelles each playing their own role in cellular homeostasis. It is the complicated interaction between all these different organelles, each equipped with their own specific enzyme content, which allows cells to function properly under different conditions. Peroxisomes also belong to the group of subcellular organelles, are present in virtually all eukaryotic cells except the erythrocyte and were already identified morphologically in the early 1950s. Using differential and density gradient centrifugation methods, de Duve and coworkers were able to isolate peroxisomes in the 1960s. They performed the initial characterization of peroxisomes as organelles containing several oxidases generating H_2O_2 and catalase to decompose H_2O_2 back to molecular oxygen [1]. These findings did not elicit too much excitement in the scientific community, and the same was true for the discovery by Lazarow and de Duve of a fatty acid β-oxidation system in peroxisomes in 1976 [2], because the function of a second β-oxidation system, next to that in mitochondria, was not immediately evident. The crucial role of peroxisomes in human metabolism only became clear in the early 1980s thanks to the work on Zellweger syndrome (ZS), which in its severe form is a lethal, autosomal recessive disease characterized by the absence of peroxisomes. Detailed metabolic studies in blood samples from patients revealed a series of abnormalities which led to the identification of all the different metabolic functions of peroxisomes we know today. First, Moser and coworkers reported the accumulation of very-long-chain fatty acids (VLCFA) notably hexacosanoic acid (C26:0) in plasma from ZS patients with no abnormalities at the level of long-chain fatty acids, like palmitic, oleic, linolenic and linoleic acid [3]. This immediately suggested that the peroxisomal β-oxidation system would catalyse the β-oxidation of a distinct set of substrates different from the fatty acids oxidized by mitochondria which turned out to be true. At the same time, our group in Amsterdam discovered the deficiency of plasmalogens in erythrocytes from ZS patients which pointed to the crucial role of peroxisomes in ether phospholipid biosynthesis [4]. These findings were soon followed by other reports documenting additional metabolite abnormalities which led to the discovery of other metabolic functions of peroxisomes, including fatty acid α-oxidation, bile acid synthesis and glyoxylate detoxification [5]. The importance of all these different metabolic functions of peroxisomes is stressed by the existence of a large number of peroxisomal disorders, including the peroxisome biogenesis disorders as well as the single peroxisomal enzyme deficiencies, affecting these pathways (Fig. 5.1).

Peroxisomes are very much dependent on the interaction with other organelles, including lysosomes, mitochondria and the endoplasmic reticulum (ER), to perform their role in metabolism properly. This is immediately clear if it is realized, for example, that peroxisomes lack a citric acid cycle as well as a respiratory chain and require the participation of mitochondria to degrade the acetyl-CoA units produced in peroxisomes to CO_2 and H_2O and to allow reoxidation of NADH back to NAD^+. It is the purpose of this review to describe the role of peroxisomes in fatty acid oxidation, with particular emphasis on its functional organization within the context of the intracellular organellar metabolic network and the defects in peroxisomal fatty acid oxidation in humans. We will start with a brief description of the permeability properties of peroxisomes, because this aspect of peroxisomes is crucial for all metabolic functions of peroxisomes, including peroxisomal fatty acid oxidation.

5.2 Metabolite Transport Across the Peroxisomal Membrane

Early work by de Duve and coworkers established that peroxisomes are highly permeable, at least for small molecular weight (Mw) compounds. This was concluded from the equilibrium density of peroxisomes in sucrose gradients but more convincingly from the



Disorder	Abbreviation	Gene	OMIM
• X-linked adrenoleukodystrophy	X-ALD	*ABCD1*	300100
• Acyl-CoA oxidase 1 deficiency	ACOX1-deficiency	*ACOX1*	264470
• Acyl-CoA oxidase 2 deficiency	ACOX2-deficiency	*ACOX2*	601641
• Acyl-CoA oxidase 3 deficiency	ACOX3-deficiency	*ACOX3*	-
• D-bifunctional protein deficiency	DBP-deficiency	*HSD17B4*	261515
• Sterol carrier protein X deficiency	SCPX-deficiency	*SCP_2*	613724
• ACBD5 deficiency	ACBD5-deficiency	*ACBD5*	616618
• 2-Methylacyl-CoA racemase deficiency	Racemase deficiency	*AMACR*	604489
• ABCD3 deficiency	PMP70-deficiency	*ABCD3*	170995
Zellweger Spectrum Disorders	ZSD	*PEX1*	602136,
		PEX2	614866
		PEX3	603164
		PEX5	600414
		PEX6	601498
		PEX10	602859
		PEX12	601758
		PEX13	601789
		PEX14	601791
		PEX16	603360
		PEX19	600279
		PEX26	608666

Fig. 5.1 Disorders of peroxisomal fatty acid β-oxidation: see text for details

observation that peroxisomal enzymes, like D-amino acid oxidase, L-α-hydroxyacid oxidase and urate oxidase, failed to exhibit so-called structure-linked latency which means that the activity of these three enzymes was identical in intact peroxisomes when compared with sonicated or Triton X-100 treated peroxisomes [1]. These findings were corroborated by work from various authors showing that the permeability of the peroxisomal membrane for low-Mw compounds is actually catalysed by pore-forming proteins of which Pxmp2 is best characterized [6–10]. More recently, Mindthoff and coworkers reported that yeast *S. cerevisiae* Pex11 is a pore-forming protein sharing sequence similarity with TRPM cation-selective channels with an estimated channel size of ~0.6 nm. This would allow the permeation of solutes with an Mw of 300–400 Da [11]. Mammals, including humans, actually have three different PEX11 proteins called α, β and γ, and it remains to be established whether they are pore-forming proteins as well.

In contrast to the free permeability of low-Mw compounds through the peroxisomal membrane, there is ample evidence showing that peroxisomes are impermeable to larger hydrophilic solutes, such as the various nucleotides, including NAD^+, NADH, $NADP^+$ and NADPH, as well as ATP, and coenzyme A (CoA) either acylated or not. This is true for yeast as shown for: (1) acetyl-CoA and NAD(H) [12], (2) acyl-CoAs [13] and (3) NADP(H) [14] but also for mammalian peroxisomes [15, 16]. Only few peroxisomal solute carriers have been identified and characterized in mammalian peroxisomes so far. These include the three peroxisomal membrane proteins (PMP) which belong to the family of ATP-binding cassette (ABC) proteins named ABCD1, 2 and 3. Studies in the yeast *S. cerevisiae* have shown that the Pxa1-Pxa2 heterodimer, which is the yeast variant of the human ABCD1 homodimer, catalyses the ATP-driven transport of acyl-CoA esters across the peroxisomal membrane [17]. Wiesinger and coworkers showed the same for the ATP-dependent transport of C26:0-CoA by ABCD1 in human skin fibroblasts [16]. Thus, human ABCD1 has been shown to transport saturated very-long-chain acyl-CoAs across the membrane, but the substrate specificity of human ABCD2 has remained more enigmatic. However, there is evidence suggesting that ABCD2 may catalyse the transport of saturated and especially unsaturated very-long-chain acyl-CoAs [18, 19]. ABCD3, which is better known as PMP70, has been claimed to catalyse the transport of dicarboxylyl-CoAs [20] and more recently has also been implicated in the transmembrane transport of phytanoyl-CoA, pristanoyl-CoA and the CoA-esters of the bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA) [21]. One of the few other true mammalian PMPs supposed to be involved in transmembrane transport of solutes is PMP34 (*SLC25A17*) which belongs to the mitochondrial carrier family (MCF). Human PMP34 was expressed and incorporated into liposomes by Palmieri and coworkers and was found to show highest affinity for CoA, FAD and NAD^+ [22]. The function of PMP34 was recently studied in vivo in a mouse model lacking Pmp34 by Van Veldhoven and coworkers [23]. Mutant mice did not exhibit a phenotype nor did they show any abnormality in one or more of the peroxisomal biomarkers. However, when challenged with a diet high in phytol, a precursor of phytanic acid, the mice accumulated phytanic and pristanic acid. The authors concluded that PMP34 is not so much involved in the homeostasis of FAD and/or NAD^+ but instead of CoA. Finally, the conclusion that peroxisomes are open to low-Mw solutes but not to solutes of higher Mw is supported by elegant in vivo work by DeLoache and coworkers who developed an equally ingenious and powerful method to study the permeability of peroxisomes under in vivo conditions [24]. These authors made clever use of β-glucosidase (BGL) from *Neurospora crassa*, a promiscuous enzyme known to be able to hydrolyse a wild variety of glycosides. They reasoned that if the hypothesis of the peroxisomal membrane being permeable towards low-Mw metabolites but not high-Mw metabolites would be true, *N. crassa* β-glucosidase targeted to peroxisomes in *S. cerevisiae* would have activity with small substrates but not larger ones. They

selected three structurally similar, size-diverse substrates for testing; all of which oligosaccharides conjugated to a dye with Mws of 409, 571 and 733 Da, respectively. For the smaller two substrates, rates of hydrolysis were equal in cells whether BGL was peroxisomal or not. However, this was different for the high-Mw substrate which was hydrolysed at a much reduced rate (23% of control) in cells expressing BGL in peroxisomes versus cells with BGL in the cytosol [24]. These results are very important, because many of the conclusions drawn about the permeability properties of peroxisomes have come from studies with isolated peroxisomes and homogenates but not intact cells.

5.3 Peroxisomal Fatty Acid β-Oxidation

Peroxisomes contain a fatty acid β-oxidation system just like mitochondria. Although the mitochondrial and peroxisomal β-oxidation systems are identical in chemical terms which implies four sequential steps of dehydrogenation, hydration, dehydrogenation and thiolytic cleavage, there are major differences between the two systems. These include: (1) peroxisomes and mitochondria have different substrate specificities as detailed below; (2) the four reactions of the mitochondrial and peroxisomal β-oxidation systems are catalysed by different enzymes encoded by distinct genes; (3) fatty acids are transported across the peroxisomal membrane as acyl-CoAs and in mitochondria as acylcarnitines; (4) mitochondria are able to degrade fatty acids all the way to CO_2 and H_2O, whereas peroxisomes can only chain-shorten fatty acids to acetyl-CoA, propionyl-CoA and medium-chain acyl-CoAs, which all need to be shuttled to mitochondria for full oxidation to CO_2 and H_2O; and (5) the role of carnitine: in mitochondria carnitine plays a central role as obligatory component of the carnitine cycle; in peroxisomes it is required for the export of the end products of peroxisomal β-oxidation as acylcarnitine esters (for reviews see [5, 25, 26]).

The mitochondrial and peroxisomal β-oxidation systems serve different purposes in cellular metabolism with the medium- and long-chain fatty acids predominantly oxidized in mitochondria, whereas the peroxisomal system is especially important for the oxidation—or rather chain shortening—of a series of special fatty acids, including VLCFAs (for example, C26:0), pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), the C27-bile acid intermediates DHCA and THCA and some mono- and poly-unsaturated fatty acids, such as tetracosahexaenoic acid (C24:6n-3). Peroxisomes also contribute to the oxidation of long-chain fatty acids albeit to a limited extent [27]. β-oxidation of fatty acids in peroxisomes is mediated by three different acyl-CoA oxidases (ACOX 1, ACOX2 and ACOX3), two bifunctional enzymes with 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities (L-bifunctional protein (LBP encoded by *EHHADH*) and D-bifunctional protein (DBP encoded by *HSD17B4*) and two thiolases (3-ketoacyl-CoA thiolase *(ACAA1)* and sterol carrier protein X (SCPx encoded by *SCP2*)). Much of our knowledge about the physiological role of each of these enzymes has come from studies in patients affected by a defect in one of these enzymes (see Fig. 5.1). Indeed, patients with ACOX1 deficiency, for instance, only accumulate VLCFAs, whereas in patients with ACOX2 deficiency, there is only accumulation of the bile acid intermediates DHCA and THCA. We recently identified a third human ACOX named ACOX3 which accepts pristanoyl-CoA as substrate but not DHC-CoA and THC-CoA [28]. ACOX3 dysfunction was recently described as a potential cause of recurrent spontaneous vasospasm of the internal carotid artery via a yet unknown mechanism [29]. Studies in patients affected by DBP deficiency as well as in mutant mice have revealed that DBP is involved in the oxidation of VLCFAs, pristanic acid as well as DHCA and THCA, with LBP only involved in the oxidation of dicarboxylic acids [30, 31]. Finally, in patients with SCPx deficiency the oxidation of DHCA, THCA and pristanic acid is deficient. Peroxisomal 3-ketoacyl-CoA thiolase deficiency has not yet been described in humans.

Peroxisomes are not only able to oxidize saturated fatty acids but also mono- and poly-unsaturated fatty acids as well as 2-(R)-methyl-branched chain fatty acids and 2-hydroxy-fatty acids. In order to β-oxidize all these different fatty acids, peroxisomes are equipped with a set of auxiliary enzymes, including 2-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase, to remove the double bond present in mono- and poly-unsaturated fatty acids. Furthermore, peroxisomes contain the enzyme 2-methylacyl-CoA racemase (AMACR) which is able to convert (2R)-methylacyl-CoAs into the corresponding (2S)-methylacyl-CoAs which is an obligatory step in the oxidation of (2R)-methylacyl-CoAs like (2R)- pristanoyl-CoA among other (2R)-acyl-CoAs. The underlying reason is that the different peroxisomal acyl-CoA oxidases only accept (2S)-methylacyl-CoAs as substrate and not (2R)-methylacyl-CoAs (for reviews see [5, 25, 26]).

5.4 Peroxisomal Fatty Acid β-Oxidation and the Crosstalk with Other Organelles

Fatty acid oxidation in peroxisomes is very much dependent on the interaction with other organelles. The first interaction is with the ER, since at least some of the substrates oxidized in peroxisomes originate from the ER. This is true for the VLCFAs, because most of the VLCFAs are not derived from dietary sources, as originally believed, but are in fact synthesized from shorter-chain fatty acids via the fatty acid synthase (FAS) complex followed by the chain-elongation system localized in the ER. Chain-elongation of fatty acids is a complicated process which involves the stepwise addition of an acetyl unit to an acyl-CoA ester via a set of four different reactions which mimics the four reactions of the fatty acid beta oxidation spiral but operating in the reverse direction. The first step of fatty acid chain-elongation is mediated by a series of seven different so-called elongases (ELOVL1-7), and it has been shown that ELOVL1 is the predominant elongase involved in VLCFA formation [32]. The VLCF-CoAs synthesized in this way can be incorporated into different lipid species or will undergo β-oxidation in peroxisomes in order to keep the VLCF-CoA levels in check. Recent evidence suggests that the transfer of VLCF-CoA from the site of synthesis, i.e. the ER, to the peroxisome is dependent on a protein named ACBD5 which was already identified years ago by Warscheid and coworkers [33] as part of a large proteomic study of human peroxisomes. ACBD5 is a tail-anchored protein present in the peroxisomal membrane with the bulk of the protein exposed to the cytosol and is equipped with both an acyl-CoA-binding site as well as a so-called FFAT motif (two phenylalanines [FF] in an acidic tract). ACBD5 was found to bind to the resident ER protein vesicle-associated membrane protein-associated protein B (VAPB) which contains a major sperm protein (MSP) domain able to bind to FFAT-like motifs. Together ACBD5 and VAPB mediate the close association between the ER and peroxisomes [34, 35]. The finding that patients with a genetic deficiency of ACBD5 have elevated VLCFA levels [36] suggests that intracellular VLCFA homeostasis requires the direct physical interaction between the ER and peroxisome. Although not yet rigorously proven, our current concept is that the C26:0-CoA synthesized at the ER via chain-elongation is in part used for the synthesis of various lipid species at the ER, whereas the other part is shuttled to peroxisomes for β-oxidation in order to preserve cellular VLCFA homeostasis and that it is the ACBD5-VAPB interaction which actually brings the two organelles together (Fig. 5.2).

The mitochondrion is another organelle with which peroxisomes interact heavily. The reason is that the end products of peroxisomal β-oxidation, the shortened acyl-CoAs, require the mitochondrion for full oxidation to CO_2 and H_2O. Transfer of the different acyl-CoAs from peroxisomes to mitochondria can occur via two distinct routes, a carnitine-mediated route and a thioesterase-mediated pathway. In the carnitine-mediated route the different acyl-CoAs are converted into the corresponding acylcarnitines by the two intraperoxisomal enzymes carnitine

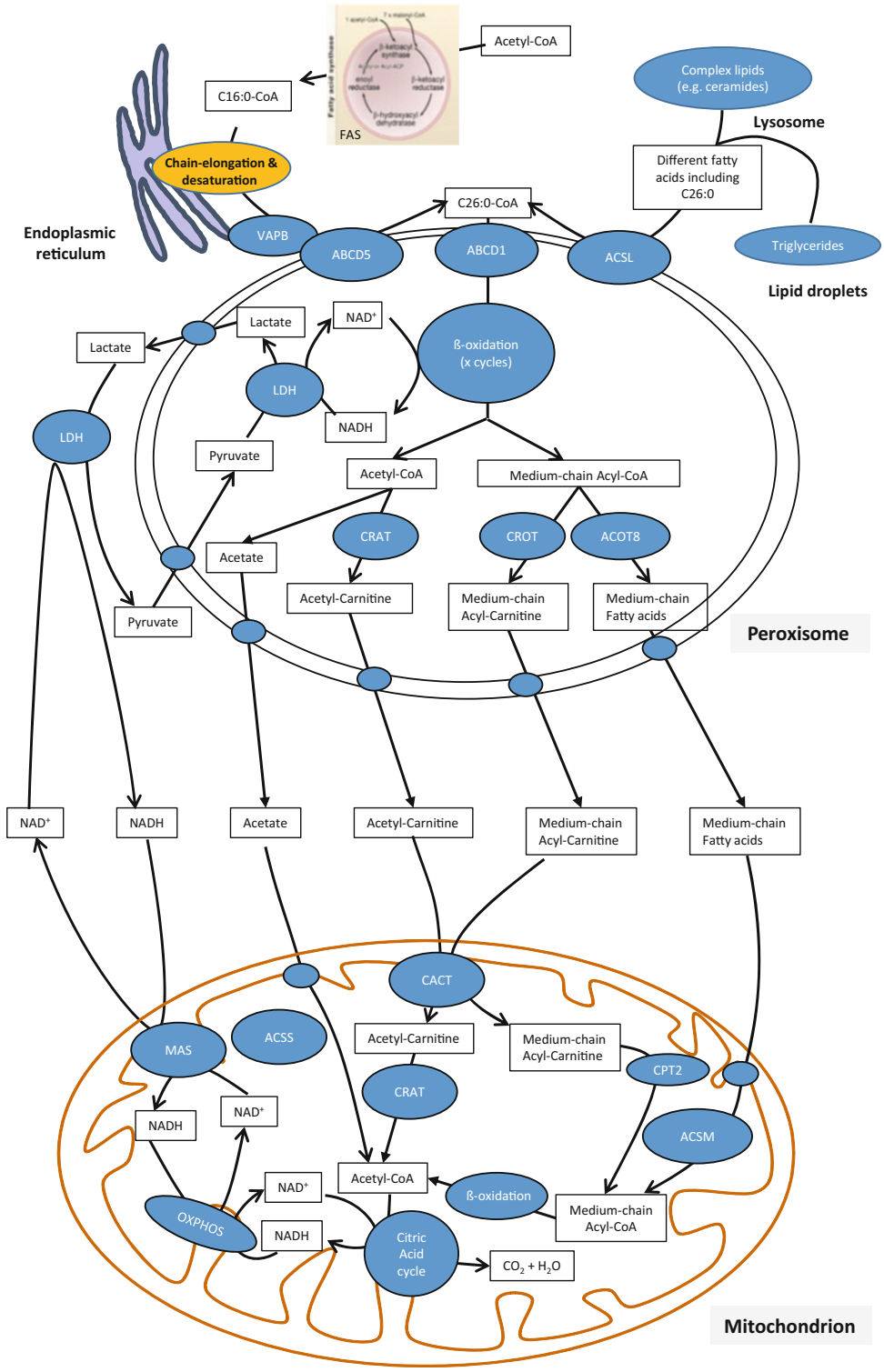


Fig. 5.2 Peroxisomal fatty acid β-oxidation and its functional interaction with multiple subcellular organelles, including mitochondria, lysosomes, lipid droplets and the endoplasmic reticulum: see text for background information

acetyltransferase (CRAT) and carnitine octanoyl transferase (CROT). They then leave the peroxisome to enter the mitochondrion via carnitine acylcarnitine translocase (encoded by *SLC25A20*) which catalyses the uptake of acylcarnitines into mitochondria in exchange for free carnitine. Once inside, the acylcarnitines are reconverted back into acyl-CoAs so that further oxidation to CO_2 and H_2O can occur. Recent evidence suggests that the interaction between peroxisomes and mitochondria is also mediated by tethering proteins which bring the organelles closer together to facilitate transfer of metabolites [37].

Another product of fatty acid β-oxidation in peroxisomes is NADH which needs to be reoxidized back to NAD^+ in order to allow β-oxidation to continue. Reoxidation of intraperoxisomal NADH is brought about by so-called redox-shuttles analogous to the redox-shuttles involved in the reoxidation of cytosolic NADH via the malate-aspartate and glycerol-3-phosphate shuttle. In the yeast *S. cerevisiae,* reoxidation of intraperoxisomal NADH occurs via two different NAD(H)-redox-shuttles, including a malate dehydrogenase-based shuttle [12] and a glycerol-3-phosphate dehydrogenase-based shuttle [38]. In mammalian peroxisomes, reoxidation of NADH is catalysed by a different NAD(H)-redox-shuttle, which involves lactate dehydrogenase as first shown by Baumgart et al. [39]. The principle of this shuttle system is that the NADH produced during β-oxidation in peroxisome is reconverted back to NAD^+ by peroxisomal lactate dehydrogenase (LDH) thereby generating lactate from pyruvate (Fig. 5.2). Subsequently, lactate is transported out of the peroxisome and is reconverted back into pyruvate by cytosolic lactate dehydrogenase which is coupled to the formation of NADH from NAD^+. The net result of this LDH-based redox-shuttle is that the NADH produced in the peroxisome is reoxidized at the expense of cytosolic NAD which is reduced to NADH. The ultimate site of reoxidation of NADH is the oxidative phosphorylation system of mitochondria which implies that cytosolic NADH needs to be shuttled across the mitochondrial membrane, since complex I of the respiratory chain reacts with intramitochondrial NADH but not cytosolic NADH. Transfer of the reducing equivalents of NADH across the mitochondrial membrane is mediated by the malate/aspartate shuttle and the glycerol-3-phosphate shuttle.

The identity of the peroxisomal LDH activity has long remained mysterious until Thoms and coworkers discovered that the peroxisomal LDH is not the product of a distinct gene but is instead produced from the same gene as the one coding for cytosolic LDHB via a unique mechanism called translational read-through [40]. This mechanism ensures production of two transcripts, one larger than the other with at the carboxy-terminal end a typical peroxisome targeting signal of the PTS1-type (-SRL), which was shown to target this extended LDHB to peroxisomes [41].

Peroxisomes do not only interact with mitochondria and the ER but also with lysosomes. Indeed, lysosomal degradation of different lipid species will generate fatty acid substrates, including VLCFAs, which are released from the lysosome to be degraded in peroxisomes. Interestingly, lysosome-to-peroxisome contact sites have been identified, and it has been claimed that this contact is actively mediated by lysosomal synaptotagmin VII binding to the lipid $PI(4,5)P_2$ at the peroxisomal membrane [42].

5.5 The Peroxisomal Disorders of Fatty Acid β-Oxidation

Figure 5.1 lists the different peroxisomal disorders in which there is an impairment in the peroxisomal fatty acid oxidation system as subdivided in two groups. These include the primary peroxisomal enzyme deficiencies caused by mutations in genes coding for the different peroxisomal enzymes and the secondary disorders of peroxisomal β-oxidation due to a peroxisome biogenesis disorder.

The identification of all these different disorders has been greatly helped by the availability of a set of peroxisomal biomarkers which can be measured in blood. These biomarkers include:

(1) VLCFAs, notably C26:0 and the C26:0/C22:0-ratio (in plasma) and C26:0-lysophosphatidylcholine (C26:0-lysoPC); (2) pristanic acid (in plasma); (3) the bile acid synthesis intermediates DHCA and THCA (in plasma); (4) pipecolic acid (plasma); and (5) plasmalogens (in erythrocytes).

5.6 Primary Peroxisomal Fatty Acid Oxidation Disorders

5.6.1 X-Linked Adrenoleukodystrophy (X-ALD)

X-ALD is the most frequent peroxisomal disorder and is caused by mutations in the *ABCD1* gene encoding the peroxisomal transmembrane protein adrenoleukodystrophy protein (ALDP). ALDP transports very-long-chain acyl-CoAs across the peroxisomal membrane as discussed above. The clinical spectrum of X-ALD in males ranges from isolated adrenocortical insufficiency and a slowly progressive myelopathy to devastating cerebral demyelination. X-ALD patients were initially classified into distinct groups each representing a distinct phenotype, but it is now generally agreed that X-ALD represents a disease spectrum and should be considered a progressive neurodegenerative disease [43]. In male patients, adrenal failure is often the first symptom which can occur at different ages ranging from (early) childhood to even adulthood. The lifetime prevalence is about 80% in males [44]. A progressive gait disorder and incontinence due to spinal cord disease develops on average in the third decade in males and the fifth decade in females [45, 46]. Some 60% of affected males will develop a progressive leukodystrophy of whom 40% in childhood [47].

Importantly, hematopoietic stem cell transplantation (HCT) using an HLA-matched donor is available as treatment for the leukodystrophy, at least when performed at an early stage of the disease with few lesions on MRI. More recently, autologous HCT after ex vivo lentiviral gene therapy has been developed with encouraging results [48].

X-ALD is characterized by the accumulation of VLCFAs notably C26:0 in plasma and tissues as a consequence of mutations in *ABCD1* which render ALDP inactive. For many years the laboratory diagnosis of patients was based on the analysis of C26:0 and the C26:0/C22:0-ratio usually by means of gas chromatography, but in more recent years, measurement of C26:0-lysoPC by means of liquid chromatography-tandem mass spectrometry (LC-MS/MS) has turned out to be more reliable [49, 50]. Furthermore, this new LC-MS/MS-based method allows high-throughput analysis which has opened the way towards inclusion of X-ALD in neonatal screening programs, especially now that realistic treatment options have become available for X-ALD. The superiority of C26:0-lysoPC compared with previous biomarkers has been demonstrated by Huffnagel and coworkers who performed a study in 46 women with ALD [51]. Out of 46 women with ALD, 6 had normal values for C26:0 and the C26:0/C22:0 ratio in plasma which corresponds to an 87% sensitivity for plasma VLCFA analysis—in line with literature data—whereas all women had C26:0-lysoPC levels outside the normal range thus giving rise to a sensitivity of 100%. This finding obviously has great benefit for the laboratory diagnosis of ALD in women.

5.6.2 Acyl-CoA Oxidase 1 (ACOX1) Deficiency

ACOX1 deficiency was first reported in 1988 by Poll-The and coworkers in three patients showing all signs and symptoms described for neonatal adrenoleukodystrophy. However, whereas neonatal adrenoleukodystrophy was known for its deficiency of peroxisomes with abnormalities in all peroxisomal biomarkers as listed above, peroxisomes were found to be abundant upon morphological investigations of peroxisomes in the liver. Furthermore, peroxisomal abnormalities were restricted to the accumulation of VLCFAs in plasma from the three patients which explains why these patients were originally described under the name “pseudo-NALD” [52]. These

puzzling findings were resolved when ACOX1, the first enzyme in the peroxisomal oxidation of the CoA-esters of VLCFAs, like C26:0, was found to be deficient. In subsequent years, many additional patients have been identified and in 2007 we reported on the clinical, biochemical and mutational spectrum of ACOX1 deficiency in 26 patients [53]. All 26 patients had psychomotor retardation, but some did acquire limited skills, including the ability to sit and stand unsupported with voluntary control of hand function, and limited speech. In most patients (83%), however, there was loss of motor achievements with a mean age of regression of 28 months. Brain imaging (MRI and/or CT) revealed cerebral and/or cerebellar white matter abnormalities in all 12 patients in whom this was investigated. Three of these patients showed neocortical dysplasia, a feature also described in ZS [54]. Other abnormalities documented in these 26 patients include: hypotonia (92%), seizures (91%), visual system failure (78%), impaired hearing (77%), facial dysmorphism (50%), hepatomegaly (50%) and failure to thrive (38%). The mean age of death was 5 years with a range from 4 to 10 years. More recently, ACOX1 deficiency has been reported in two adult patients who only developed progressive neurological symptoms in later childhood but were diagnosed at the age of 52 and 55 years [55].

Of the various peroxisomal biomarkers in blood, only the plasma VLCFA levels are abnormal in ACOX1 deficiency. Definitive diagnosis of patients requires enzyme analysis in fibroblasts and/or genetic analysis of the *ACOX1* gene [53].

5.6.3 Acyl-CoA Oxidase 2 (ACOX2) Deficiency

Recently, several patients have been reported with an isolated deficiency of acyl-CoA oxidase 2 (ACOX2), the first enzyme in the peroxisomal oxidation of the CoA-esters of the bile acid intermediates, DHCA and THCA, and pristanic acid although oxidation of the latter is shared with ACOX3 [28]. Vilarinho et al. identified ACOX2 deficiency in an 8-year-old male with intermittently elevated transaminases, liver fibrosis, mild ataxia and cognitive impairment. Exome sequencing revealed homozygosity for a variant, leading to a premature stop codon and a truncated protein in line with the absence of ACOX2 upon immunoblot analysis [56]. Biomarker analysis in a blood sample from the patient revealed markedly elevated levels of DHCA and THCA. A second patient with ACOX2 deficiency and accumulation of DHCA and THCA was described by Monte et al. in an adolescent male with persistently elevated transaminases of unknown origin without other symptoms. Sequence analysis of the candidate genes revealed a homozygous mutation in the *ACOX2* gene, and subsequent expression studies showed that the identified variant renders the ACOX2 enzyme inactive [57]. Finally, we reported ACOX2 deficiency in a much more severely affected patient, a girl with arthrogryposis, neuropathy and myopathy in whom bile acid analysis was performed because of mild conjugated hyperbilirubinaemia [28]. The patient died at 6 months of age. The marked difference in clinical signs and symptoms between the patients reported by Vilarinho et al. and Monte et al. compared with the patient of Ferdinandusse et al. raises the question whether the ACOX2 deficiency in the latter patient is really causal or not. As discussed in more detail by Ferdinandusse et al., the answer to this question may well be no for two reasons. First, the patient described by Ferdinandusse et al. was from a consanguineous marriage which implies that she may well have suffered from another genetic disease especially since only a peroxisomal gene panel was tested. Secondly, the truncating mutation identified in the patient of Ferdinandusse et al. turned out to be present in a relatively high frequency in the population (0.21% ExAc, 0.37% ESP and 0.1% 1000 Genome database) and has even been observed homozygously. This implies that there are probably a number of unidentified ACOX2-deficient individuals with no or only mild clinical signs and symptoms, suggesting that ACOX2 deficiency may well be a relatively benign disease.

5.6.4 D-Bifunctional Protein (DBP) Deficiency

DBP deficiency was first described by Suzuki et al. and ourselves in a few patients showing many of the hallmarks of ZS [58, 59] but lacking the characteristic deficiency of peroxisomes. In retrospect, the first patient with DBP deficiency was already reported many years earlier by Goldfischer et al. in a patient with a Zellweger-like presentation in whom peroxisomes were found to be abundantly present upon morphological examination of the liver [60]. The true defect in this patient was only resolved many years later by Ferdinandusse et al. [61] following the discovery of DBP deficiency caused by mutations in the *HSD17B4* gene, by several groups of investigators. Since those early days, much has happened, and DBP deficiency has actually been delineated as one of the more frequent peroxisomal disorders. In 2006 we reported the collective results of 126 patients with genetically confirmed DBP deficiency [62]. In short, the clinical presentation of DBP deficiency is dominated by neonatal hypotonia (98%) and seizures within the first months of life (93%). Failure to thrive was observed in 43% of the patients. Visual system failure, including nystagmus, strabismus and/or failure to fixate objects, was also frequent. In addition, a progressive loss of vision and hearing was noted in 35% and 46% of the patients, respectively. Virtually none of the 126 patients acquired any psychomotor development, and the few patients who did improve subsequently showed progressive loss of motor achievements. External dysmorphism was also a frequent finding. Remarkably, the dysmorphism observed in DBP patients resembles that of patients with ZS in many respects as exemplified by the high forehead, large open fontanelles, long philtrum, epicanthal folds, hypotelorism, microcephaly, supraorbital ridges, retrognathia and low-set ears. Brain imaging showed dilatation of the ventricles in 29% of the patients and neocortical dysplasia in 27%. Furthermore, there was delayed maturation of the white matter before 1 year of age in 16/47 patients, and demyelination of the cerebellar and cerebral hemispheres occurred in 7% and 17% of the patients, respectively.

More recently, a number of patients with much milder phenotypes have been described who were identified upon whole exome sequencing (WES). Indeed, WES revealed mutations in *HSD17B4* in four patients with a relatively indolent, juvenile-onset condition characterized by cerebellar ataxia, hearing loss, peripheral neuropathy and premature ovarian failure suggestive of Perrault syndrome [63, 64]. Furthermore, again using WES analysis, Lines et al. reported the identification of *HSD17B4* as the mutant gene in three adult siblings with a slowly progressive, juvenile-onset phenotype comprised of cerebellar atrophy, ataxia, intellectual decline, hearing loss, hypogonadism, hyperreflexia, demyelinating sensory motor neuropathy and—in two of the three—supratentorial white matter changes. Importantly, prior investigations had revealed no clue about the underlying defect in these patients [65]. To verify the causal role of the mutations in *HSD17B4* identified in these patients, detailed functional analyses in cultured fibroblasts of the patients were done in our laboratory in Amsterdam, in fact. The importance of such functional studies also in these days of next generation sequencing has been emphasized in literature [66]. In plasma most DBP patients accumulate VLCFAs, pristanic acid (diet- and age-dependent) and DHCA and THCA.

5.6.5 Peroxisomal Alpha-Methylacyl-CoA Racemase (AMACR) Deficiency

AMACR deficiency has so far been documented in a few patients only, but it is already clear that the phenotypic spectrum is wide. There is a childhood form of AMACR deficiency which presents with the typical features reminiscent of a defect in bile acid biosynthesis as dominated by: (1) variable liver abnormalities which may progress into liver failure if untreated; (2) fat malabsorption with steatorrhea and growth retardation and (3) fat-soluble vitamin deficiency which may

lead into coagulation defects and rickets [67]. Most patients, however, do not show this early-onset phenotype dominated by liver involvement but present later in life. The first two patients with AMACR deficiency which we reported in 2000 [68] showed an adult-onset sensory motor neuropathy initially judged as adult Refsum disease, whereas a different phenotype was reported by Haugervoll and coworkers in two brothers with a complex, adult-onset phenotype consisting of peripheral neuropathy, epilepsy, relapsing encephalopathy, bilateral thalamic lesions, cataract, pigmentary retinopathy and tremor [69]. We have recently identified quite a few AMACR-deficient patients in adults with retinitis pigmentosa and polyneuropathy as dominant features but also in three children with liver abnormalities but without clinical symptoms. Taken together, it may well be that AMACR deficiency is more frequent than previously anticipated.

The laboratory diagnosis of AMACR deficiency involves the analysis of bile acids in plasma which can best be done using MS/MS and specifically analysis of the stereoisomers of DHCA and THCA. In AMACR deficiency, only the (24R) form of DHCA and THCA accumulate, whereas in all the other disorders in which DHCA and THCA accumulate, both stereoisomers accumulate [70]. Since enzymatic analysis of AMACR is cumbersome, requiring specific synthesis of the substrate required, the finding of (24R)-DHCA and (24R)-THCA combined with elevated levels of phytanic acid and especially pristanic acid (diet- and age-dependent) can best be followed up by genetic analysis of the *AMACR* gene.

5.6.6 Sterol Carrier Protein X (SCPX) (Peroxisomal Branched-Chain β-Ketothiolase) Deficiency

Only two patients with SCPX deficiency caused by mutations in the *SCP2* gene have been described in literature so far [71, 72]. The first patient presented with torticollis and dystonic head tremor, slight cerebellar signs with intention tremor, nystagmus, hyposmia and azoospermia. Laboratory investigations revealed the accumulation of pristanic acid in plasma and excretion of bile alcohol glucuronides which ultimately led to the finding of SCPX deficiency. The second patient presented with adult-onset spinocerebellar ataxia, deafness and thalamic and pontine T2 hypointensity on MRI with brain iron accumulation. The SCP2 gene has two transcription start sites, thereby generating two transcripts of which the larger one codes for an enzymatically active protein of 58 kDa with β-ketothiolase activity specific for branched-chain 3-ketoacyl-CoA esters [73–75]. The shorter one codes for a 13 kDa SCP2 protein which lacks thiolase activity.

5.6.7 ACBD5 (Peroxisomal ACYL-CoA Binding Domain Protein Type 5) Deficiency

ACBD5 deficiency was first described in 2013 in a study aimed to determine the genetic basis of retinal dystrophy in a large cohort of patients, using WES. In three siblings with retinal dystrophy in combination with severe white matter disease and spastic paraparesis, a homozygous splice site was identified, but no functional studies were done [76]. The proband was described in more detail later [77]. We identified ACBD5 deficiency in a girl with progressive leukodystrophy, syndromic cleft palate, ataxia and retinal dystrophy due to a homozygous indel mutation in *ACBD5*, causing the complete loss of the ACBD5 protein in the patient [36]. Detailed functional studies in plasma and fibroblasts from the patient showed that the loss of ACBD5 was associated with the impaired oxidation of VLCFAs in the patient's fibroblasts, whereas the oxidation of pristanic acid as well as phytanic acid was fully normal. In agreement with these results, all peroxisomal biomarkers were normal in the patient's blood except from the accumulation of VLCFAs. As discussed above, current knowledge holds that ACBD5 interacts with VAPB present in the ER-membrane, thereby allowing close physical contact between the ER

and peroxisomes to facilitate the transfer of metabolites, including C26:0-CoA from the ER to the peroxisome (Fig. 5.2).

5.6.8 ABCD3 (ATP Binding Cassette Subfamily D Member 3) Deficiency

Deficiency of ABCD3, which is better known as peroxisomal membrane protein 70 (PMP70), has so far been described in a single patient who presented with hepatosplenomegaly and severe liver disease that remained unexplained until metabolite analyses revealed the accumulation of the bile acid intermediates DHCA and THCA. Detailed biochemical studies in fibroblasts from the patient revealed the absence of the ABCD3 (PMP70) protein. Subsequent genetic studies identified a homozygous deletion in the *ABCD3* gene predicted to result in a truncated ABCD3 protein at least lacking the C-terminal 24 amino acids. The liver disease in the patient progressed with time which prompted liver transplantation at 4 years of age. The patient expired shortly after transplantation [21].

5.7 Secondary Disorders of Peroxisomal Fatty Acid β-Oxidation

Peroxisomal fatty acid β-oxidation is also deficient in peroxisomal disorders caused by a defect in the biogenesis of peroxisomes. The prototype of this group of disorders is ZS, which is a multisystem disorder clinically characterized by typical craniofacial features, profound developmental delay and additional abnormalities in multiple organs. Apart from ZS with its neonatal onset and early fatal course, milder variants have been described originally named neonatal adrenoleukodystrophy and infantile Refsum disease. The realization that we are dealing with a disease spectrum rather than distinct, well-defined phenotypes has led to the collective term Zellweger Spectrum Disorders (ZSD). Roughly, three main phenotypes can be discerned among ZSD patients. These include: (1) a neonatal form, essentially involving severe hypotonia, epileptic seizures, failure to thrive, liver dysfunction and craniofacial dysmorphia; (2) a childhood form characterized by milder symptoms, including mild hypotonia, developmental delay, failure to thrive, adrenal insufficiency and mild craniofacial features; and (3) a much milder, adolescent-adult presentation with cerebellar ataxia only in the mildest affected patients [78–80]. In principle all peroxisomal biomarkers as measured in a blood sample are abnormal in ZSD patients, but in milder affected patients peroxisomal biomarkers can be minimally abnormal to even completely normal which surely can hamper fast and correct diagnosis of patients. This will not be covered here but is amply discussed in literature.

5.8 Concluding Remarks

Taken together, much has been learned in recent years about the fatty acid oxidation system in peroxisomes, including its role in health and disease and its functional interaction with other subcellular organelles notably the mitochondrion and ER. Much of this knowledge has been obtained from studies in patients affected by a defect in peroxisomal fatty acid oxidation or transport.

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Zellweger Syndrome Disorders: From Severe Neonatal Disease to Atypical Adult Presentation

6

David Cheillan

Abstract

Zellweger syndrome disorders (ZSD) is the principal group of peroxisomal disorders characterized by a defect of peroxisome biogenesis due to mutations in one of the 13 *PEX* genes. The clinical spectrum is very large with a continuum from antenatal forms to adult presentation. Whereas biochemical profile in body fluids is classically used for their diagnosis, the revolution of high-throughput sequencing has extended the knowledge about these disorders. The aim of this review is to offer a large panorama on molecular basis, clinical presentation and treatment of ZSD, and to update the diagnosis strategy of these disorders in the era of next-generation sequencing (NGS).

Keywords

Peroxisome · Peroxisome biogenesis disorder (PBD) · Zellweger syndrome disorder (ZSD) · *PEX* genes · Very-long-chain fatty acids (VLCFAs) · Next-generation sequencing (NGS) · Prenatal diagnosis

6.1 Introduction

Peroxisomal biogenesis disorders (PBD) represent a large group of autosomal recessive disorders affecting the formation of peroxisomes. All these defects present a large clinical continuum from the most severe antenatal/neonatal presentations to milder phenotypes in adult patients. Historically, they were divided into two distinct groups: The Zellweger syndrome disorders (ZSD) composed by Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD), and the rhizomelic chondrodysplasia punctata (RCDP) [1]. However, these disorders are characterized by a common defective peroxisome biosynthesis due to mutations in the different *PEX* genes coding for peroxin proteins implicated in the peroxisome assembly [2]. As a consequence of the absence of functional peroxisomes, all the metabolic pathway specifics of these organelles are defective; in particular the β-oxidation of very-long-chain fatty acids (VLCFAs) which is a very useful biomarker for their diagnosis. Recently, the development of whole exome sequencing (WES)/whole genome sequencing (WGS) has allowed the description of milder phenotypes in adult patients

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[3, 4]. Moreover, new defects in genes implicated in the fission of peroxisomes and mitochondria have extended the spectrum of PBD to neuromuscular disorders and inherited mitochondrial diseases [5, 6]. This review propose an overview of the knowledge on clinical manifestation of ZSD, their management, and will focuses on the diagnosis of this complex group of disorders.

6.2 Molecular Basis of ZSD

ZSD are caused by mutations in one of the 13 *PEX* genes encoding proteins named peroxins involved in peroxisome formation and/or peroxisomal protein import and 2 other genes coding for proteins associated with a fission/fusion pathway in common with mitochondria (Table 6.1) [7]. These genes are essential for the assembly of functional peroxisomes. The genes *PEX5* and *PEX7* encode the cytosolic receptors PTS1 and PTS2 necessary for targeting protein inside the matrix of the peroxisomes. The other peroxins were localized in the peroxisomal membrane and constitute a complex machinery leading to internalize specifically these cytosolic protein complexes (Fig. 6.1) [8]. A defect of one peroxin is associated with a global defect of all this macromolecular system and responsible of a PBD. *PEX1*, *PEX6*, and *PEX26* genes are the most commonly associated with ZSD (~80%) and are forming the recycling system of PTS receptors [9]. Three other genes, *PEX2*, *PEX10*, and *PEX12*, encode the translocation membrane system and are mutated in at least 10% of ZSD. Other *PEX* genes are less frequently associated with PBD with an exception to *PEX7* found in more than half of RCDP patients. More recently, new insights in the formation of peroxisomes have been discovered with some fission/fusion process in common with mitochondria [10]. Interestingly, the rare patients described with mutations in *PEX11β* or *DLP1/DNML1* genes experiment a hybrid phenotype between peroxisomal disorders and mitochondrial deficiencies [5, 6].

The common biochemical consequence of ZSD is a global dysfunction of the catabolic and anabolic peroxisomal pathways, including α-oxidation, β-oxidation, plasmalogens synthesis, biliary acid synthesis, catalase, etc.

6.3 Clinical Presentations of ZSD Patients

The most recent estimation of ZSD incidence, provided by newborn screening experiments in North America and by in silico exploitation of WES databases, is close to 1 in 90,000 births [11, 12]. ZSD are generally defined as a continuum of three phenotypes described before the comprehension of their molecular bases: ZS, NALD, and IRD. Phenotypic features are closely related to age of the patients. Antenatal presentation and neonatal/infancy death are common in ZS, while children with NALD may live to school age and late infancy, or adult presentation are more often associated with IRD. Actually, the most appropriate terms to categorized patients should be: severe, intermediate, or milder ZSD.

6.3.1 ZS or Severe ZSD

ZS is the most severe form of PBD and is a classical polymalformative syndrome, also historically called cerebro-hepato-renal syndrome. Patients present, since birth, profound hypotonia, seizures, hepatocellular dysfunction, and facial dysmorphia (flat face, large anterior fontanelle, and micrognathia). Renal cysts are often apparent but are rarely clinically significant. Abnormalities of long bones/chondrodysplasia could be noted due to plasmalogen synthesis defect [13]. Brain MRI shows polymicrogyria, pachygyria, and germinolytics cysts, reflecting the neuronal migration defects. Neurosensorial deafness and visual defects (including cataracts, optic atrophy, and pigmentary retinopathy) are commonly associated with ZS. Life expectancy for children presenting this syndrome is not above 1 year. The dysfunctions of the fission machinery (*DNM1L/DLP1*) could be responsible of a severe phenotype combining the consequences of altered mitochondrial respiratory chain and the absence of

Table 6.1 List of *PEX* genes associated with ZSD and estimated frequencies based on literature and our experience [7]

Genes	OMIM Number	Estimated frequency
PEX1	602136	> 50%
PEX6	601498	15–20%
PEX12	601758	5–10%
PEX26	608666	1–5%
PEX10	602859	1–5%
PEX2	170993	1–5%
PEX5	600414	1–5%
PEX13	601789	1–5%
PEX16	603360	1–5%
PEX3	603164	<1%
PEX19	602279	<1%
PEX14	601791	<1%
PEX11β [a]	603867	< 1%
FIS1[a]	609003	<1%
DNM1L[a]	603850	<1%

[a]These genes are implicated in the process of fission of peroxisome/mitochondria

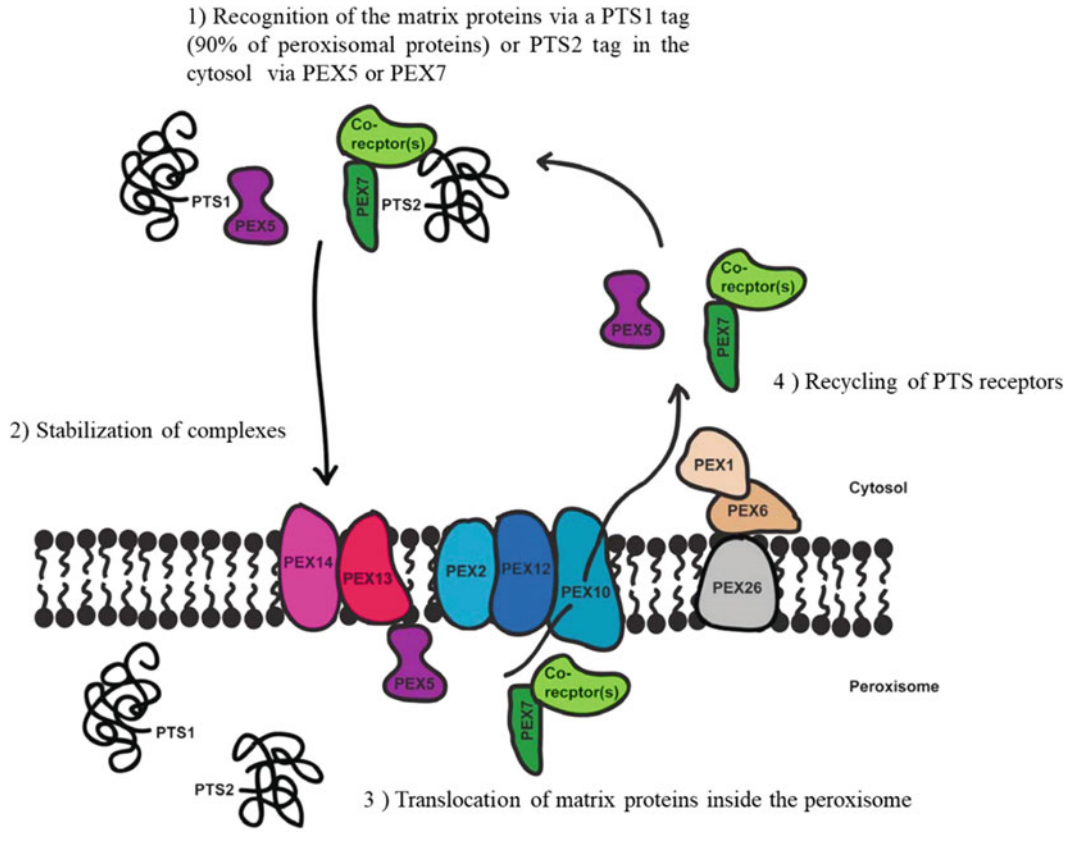

Fig. 6.1 Simplified scheme of peroxisomal biogenesis machinery in humans adapted from the review of Ronald Wanders [8]

functional peroxisomes. This phenotype included microcephaly, optic atrophy, lactic academia, and brain gyration abnormalities or Leigh syndrome phenotype [14, 15].

6.3.2 NALD and IRD or Intermediate/ Milder ZSD

These patients may present various clinical signs since the newborn period with hypotonia, failure to thrive, and attenuated facial dysmorphism, but they could have a degree of psychomotor development. In some individuals, a leukodystrophy develops during infancy which may lead to loss of previously acquired skills. Liver dysfunction may lead to bleeding episodes due to vitamin K-responsive coagulopathy, and some older children could develop adrenal insufficiency [16]. In later childhood, the common manifestation of this group of patients is the development of sensorineural hearing loss and retinitis pigmentosa. The children with milder presentations have a longer life, and children who survive the first year could reach the school age and sometimes survive until adulthood [17].

6.3.3 New Clinical Descriptions of ZSD in Adult Patients

Some atypical ZSD have been described with adult clinical presentation marked by cerebellar ataxia, peripheral neuropathy, and cataracts but without psychomotor retardation [18, 19]. The diagnosis of these milder forms could be a challenge because of the normal biochemical profile observed in some patients. Moreover, mitochondria/peroxisome fission defect caused by *PEX11β* mutations could also give some milder phenotype with cataract, gastrointestinal problems, migraine-like episodes, and mild intellectual disability. Recently, WES has allowed to associate *PEX1*, *PEX6*, and *PEX26* variants with Heimler syndrome which is a rare autosomal recessive disorder characterized by sensorineural hearing loss, amelogenesis imperfecta, nail abnormalities, and sometimes retinal pigmentation [3, 4].

6.3.4 Antenatal Manifestation of ZSD

Some inborn error of metabolism (IEM) can be suspected in the antenatal period when abnormal ultrasound findings are observed, usually during the third trimester of pregnancy. ZSD can be suspected on ultrasound scan with enlarged cerebral ventricles, shortening long bones, and Binder syndrome (midface hypoplasia reflecting the facial dysmorphism). In complement of ultrasound screening, fetal brain magnetic resonance imaging (MRI) is very useful to confirm cerebral gyration anomalies, with polymicrogyria and germinolysis cysts [13, 20].

6.4 Biological Diagnosis of ZSD

Peroxisomes are characterized by an increasing number of specific biochemical pathways, and clinical suspicion of ZSD requires the demonstration of a defect in more than one of these functions. The first step in diagnosis of ZSD and other peroxisomal disorders is generally to measure several metabolites in body fluids (blood and urine), then the confirmation is based nowadays on NGS approach by gene panel. A proposed flow chart for the diagnosis of ZSD is described on Fig. 6.2.

6.4.1 Biomarkers of ZSD

In general, ZSD biomarkers include plasmatic measurement of VLCFAs and branched chain fatty acids (phytanic and pristanic acids) associated with plasmalogens levels in erythrocytes [21]. Elevation of C26:0 fatty acids and the ratio C26:0/C22:0 are consistent in the classical form of ZSD but could also be observed in other peroxisomal defects (such as X-linked adrenoleukodystrophy (X-ALD) and several isolated enzyme deficiencies). Increased levels of methyl-branched fatty acids (phytanic and

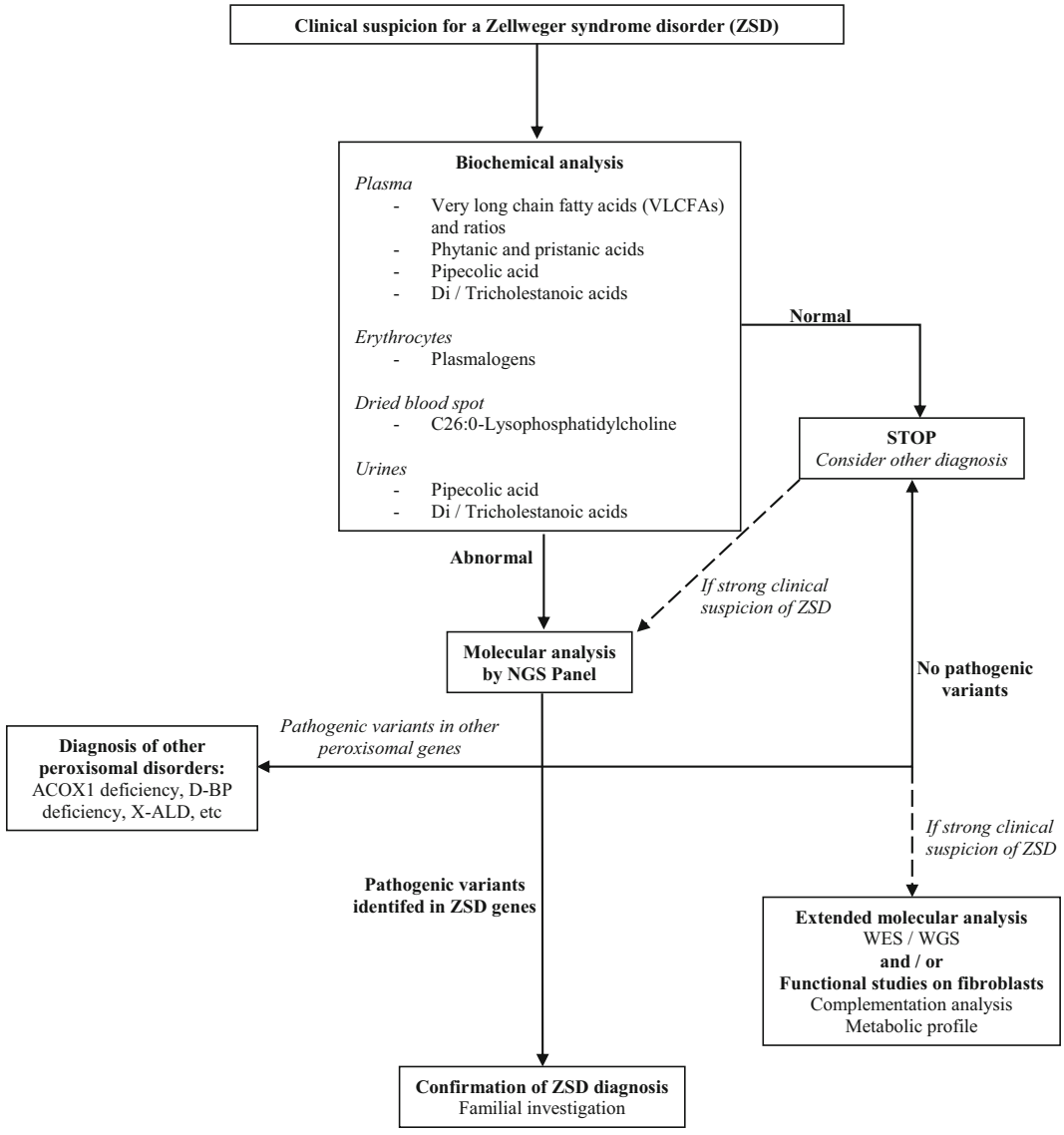

Fig. 6.2 Flow chart for diagnosis of Zellweger syndrome disorder (ZSD)

pristanic acids) and decreased levels of plasmalogens demonstrate a multiple pathway defects, typical of ZSD and confirm this diagnosis. Nonfasting and hemolyzed specimens or patient under ketogenic diet should be responsible of a nonspecific elevation of VLCFAs and a source of false positive results [22]. Additionally, phytanic and pristanic acids may be normal in newborns who are not consuming dairy products or derivates. Sometimes, an isolated elevation of C24:0/C22:0 could be observed in patients with hepatic insufficiency [23]. On the other hand, it should be noted that false negative results or very slight abnormal profile may be encountered in adult patients, and extensive investigations have to be considered in case of strong clinical suspicion [24].

Additional biochemical investigations in plasma and/or urine, including pipecolic acid and C27-bile acids intermediates (dihydroxycholestanoic acid (DHCA) and trihydroxycholestanoic acid (THCA)), could offer some

elements of confirmation in case of atypical VLCFAs profile.

Moreover, the research for new biomarkers adapted to newborn screening has shown the interest of C26:0 lysophosphatidylcholine (C26:0-lysoPC) which is elevated in ZSD and other peroxisomal disorders [25, 26]. This interesting lipid is easily measurable in dried blood spots and is already used in newborn screening program for X-ALD; however, its utility for diagnosis of peroxisomal disorders is still under evaluation in comparison with the classical VLCFAs profile [27].

On a technical point of view, all these parameters are easily measured with classical analytical systems, such as gas chromatography coupled or not with mass spectrometry (GC or GC-MS) and tandem mass spectrometry (MS/MS).

6.4.2 Molecular Diagnosis

Nowadays, for patients with a suggestive clinical and biochemical pattern, the diagnosis of ZSD is generally established with the identification of biallelic pathogenic variants in one of the gene implicated in these diseases, including *PEX* genes. The first line of genetic testing is based on gene-targeted panel by NGS technologies [28]. These panels could cover easily all the genes coding for membrane or matrix peroxisomal proteins (approximately 50 genes for humans). As described previously, three *PEX* genes are responsible for more than 80% of these disorders (*PEX1*, *PEX6*, and *PEX26*, Table 6.1). WES or WGS may be considered for patient with negative peroxisomal gene panel test and with a very strong suspicion of ZSD. During the last years, an increasing number of patients are identified using these new tools in first line screening test. However, functional investigations (blood sample or fibroblasts studies) are often useful to ascertain that the variants identified are causatives [29]. Recent study from Falkenberg et al. reports some patients with allelic expression imbalance, promoting an overrepresentation of a heterozygous mutation in *PEX6* mimicking a dominant presentation of this classical autosomal recessive disorder [30]. This finding is of great importance for a correct interpretation of WES/WGS data, as heterozygous variants inherited from parents are generally filtered out by bio-informatic tools.

6.4.3 Functional Studies

Historically, biochemical testing of skin fibroblasts was useful to confirm the defect suspected with plasma and urine profile. The assays were based on measurement of multiple enzyme activities or more global pathway explorations (VLCFAs β-oxidation for example) associated with complementation studies to pinpoint the defective *PEX* gene. Nowadays, these explorations have moved from confirmatory test to post genomic test to characterization of variants of unknown significance (VOUS) frequently identified by the NGS tools.

6.4.4 Prenatal Diagnosis

In the vast majority of cases, the prognosis is very poor for the proband, and consequently, genetic counselling and antenatal diagnosis should be offered to parents of affected children. Before prenatal testing, it is of a great importance to characterize the pathogenic mutations and to confirm their segregation inside family. Classically, prenatal diagnosis for ZSD could be performed in the first or second trimester in chorionic villus material and amniocytes directly or on cultured cells. As we have described above, some presentations of ZSD should be revealed antenatally by ultrasound screening (see Sect. 6.3.4) and need biochemical investigations on cultured cells [20]. As for functional studies, it is possible to quantitate VLCFAs or plasmalogens and to measure some enzymatic activities (in general, dihydroxyactetone-phosphate acyl transferase (DHAP-AT), the second enzyme of plasmalogens synthesis) to explore the peroxisomal pathways and to try to identify the disease. More recently, some works describe the application of NGS

(panel or WES) to prenatal diagnosis, including peroxisomal diseases [31]. However, until now, the important delay to obtain genomic results (several weeks/months) has limited the development of this approach in this particular screening.

6.4.5 Differential Diagnosis

The differential diagnosis of ZSD varies with age of presentation and the most important clinical feature of the presentation. During neonatal period, hypotonia is the most important symptom of ZSD patients and could be confused with other conditions, resulting in profound hypotonia, such as spinal muscular atrophy, chromosomal abnormalities, or congenital myopathies. Older children could experiment sensorineural defects, cataract, or adrenal insufficiency, and specific etiologies for each of these symptoms should be explored. For adult patients, it seems that cerebellum is most often affected, and the discussion is around the differential diagnosis of cerebellar ataxia [32, 33]. At least, most of ZSD clinical signs should be encountered in single peroxisomal enzyme deficiencies and X-ALD; D-bifunctional enzyme deficiency, Refsum disease, and other rare peroxisomal disorders have to be considered.

6.5 Clinical Management and Treatment

ZSD have no curative therapy, and the clinical management is mainly based on supportive care regarding the affected organs. Some dietary interventions have been tried in the past year but without clear benefits for the patients or main clinical improvements: Docosahexaenoic acid supplementation (DHA;C22:6 ω3); plasmalogens compensation, or phytanic acid restricted diet [34, 35]. Lorenzo's oil (mix of glyceryl trioleate and glyceryl trierucate) originally developed for treatment of X-ALD have shown some efficiency to reduce plasma VLCFAs accumulation but had no effect on disease progression [36]. Some recent experiments have tried to reduce the accumulation of C27-bile acid intermediates DHCA/THCA by providing cholic acid, the final C24-bile acid product. This supplementation reduces the hepatic production of bile acids by a feedback inhibition, in particular the C27-intermediates but with moderate effect on the disease [37]. Liver transplantation was also tested with some improvement in three mild ZSD patients, but this alternative needs a very early intervention, before onset of the sensorineural defect, to be efficient [38]. Finally, some trials have been performed using chaperone molecule therapy in mild ZSD patients, using arginine. This approach have shown that peroxisome biogenesis could be partially restored in fibroblasts of patient harboring mild missense mutations in *PEX1*, *PEX6*, or *PEX12* and may offer some improvement in neurophysiological (deafness) defects [39, 40]. Conceptually, gene therapy might be a potential treatment for ZSD but have never been tested in human due in part of the difficulty to reverse a complex developmental disorder.

6.6 Concluding Remarks

ZSD is a complex group of peroxisomal disorders caused by numerous genes; thus, the challenge for the physicians is to consider this diagnosis in a wide spectrum of clinical presentations. The revolution of NGS has profoundly transformed the diagnostic strategy for rare disease placing genomic investigations sometime before classical biologic screening. This shit of paradigm have allowed us to identify unsuspected presentation of ZSD but raise the challenge to confirm the diagnosis with functional tests that are more and more complex. Whereas treatment is still symptomatic for ZSD, a confirmed diagnosis is of a great importance to initiate specific therapy, to improve the quality of life for patients and to offer the best management for their family.

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Heimler Syndrome

7

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Abstract

Heimler syndrome is a rare syndrome associating sensorineural hearing loss with retinal dystrophy and amelogenesis imperfecta due to *PEX1* or *PEX6* biallelic pathogenic variations. This syndrome is one of the less severe forms of peroxisome biogenesis disorders. In this chapter, we will review clinical, biological, and genetic knowledges about the Heimler syndrome.

Keywords

Heimler syndrome · Hearing loss · Retinal dystrophy · Amelogenesis imperfecta · PEX1–PEX6

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7.1 Heimler Syndrome's Characteristics

Heimler syndrome (HS) is a combination of sensorineural hearing loss (SNHL), retinal dystrophy (RD), and amelogenesis imperfecta (AI) due to *PEX1* or *PEX6* biallelic pathogenic variations. HS is one of the less severe forms of peroxisome biogenesis disorders (PBDs).

HS is probably underdiagnosed due to misdiagnosis of enamel defects.

HS (MIM#234580; MIM#616617) is a rare syndrome with an unknown prevalence. It was first described by Heimler et al. in [1] as a combination between sensorineural hearing impairment (SNHI), progressive macular dystrophy, and AI without any cerebral or liver dysfunction [1]. More recently, pathogenic biallelic variations in peroxisomal biogenesis factors 1 and 6 (*PEX1* MIM#602136 and *PEX6* MIM#601498) have been described as the etiology of HS. To date, 23 cases of HS from 13 unrelated families have been described.

7.2 Clinical Signs of HS

Sensorineural hearing loss: Hearing loss is the most penetrant clinical sign of HS even in early childhood. All patients presented with bilateral prelingual or congenital hearing impairment. If described, the hearing defect at the onset is severe (10 cases) or profound (11 cases). Only one patient has been described with a unilateral hearing loss and one with a moderate hearing impairment but in both cases the diagnosis of HS has not been confirmed by whole exome sequencing (WES) analysis. There is no description to date of neither involvement of vestibular function nor evolution of the hearing loss when it is not profound at birth.

Amelogenesis imperfecta: AI is one of the most specific clinical signs of HS, even if it has been described in few infantile Refsum cases [2] and complex PBD [3]. Amelogenesis imperfecta is a heterogeneous group of genetic rare diseases presenting with enamel defects (hypoplasia, hypomineralization, and hypomaturation) that usually affect (but not always) both deciduous (primary) and permanent dentitions [4]. To date, almost all the reported HS cases suggest that enamel hypoplasia recognized as hypoplastic/hypomineralized AI affects only the permanent secondary dentition with posterior (premolar and molar) teeth more severely affected than anterior (incisor) teeth [1, 5–7] (Fig. 7.1a). However, this clinical sign is difficult to recognize on the primary dentition, and a clinical reevaluation of one patient has revealed affected primary molars [8, 10]. Smith et al. also describe the AI as affecting both the primary and permanent dentitions with a generalized reduced enamel volume (hypoplasia) and variable hypomineralization. Moreover, AI is little known by general public, and its diagnosis is usually made by specialized health professionals.

Retinal dystrophy (RD): RD was not associated with HS in the first original publication [1]. The RD has been first described as part of HS features in 2011 after the clinical reevaluation of one of the original patient at 29 years of age [11]. The prevalence of the retinal defect could depend on the age of patients. In the two publications of HS cohorts, the prevalence of RD is 7/12 and 9/9 [8, 9]. A childhood onset of RD with slow progression is usually described. For example, in Ratbi et al. the mean age of RD diagnosis is 16.8 years (12y–21y) [9]. However earlier diagnoses have been described [7, 10]. There are very few phenotypic descriptions of the ophthalmological defect of HS. The description of the RD phenotype seems to range from retinitis pigmentosa to macular dystrophy, including cone-rod dystrophy; the very case reports urge for collecting more cases to identify the retinal phenotype according to the age. Wangtiraumnuay has noted cone-rod dystrophy associated with macular intraretinal cystoid spaces on optical coherence tomograpy (OCT) in two previously reported cases [8, 10]. A cystoid macular edema has been also reported in a case of a 14 months of age [7] (Fig. 7.1b).

Nail Abnormalities: Different nail abnormalities have been described in HS; Beau's lines, leukonychia, and onychoschizia

can be associated [1, 9]. Both finger and toe nails can be affected. Nail abnormalities are described in almost half of the patients. However, those features could appear progressively with a higher prevalence in older cases [9] (Fig. 7.1c).

7.2.1 Molecular and Biochemical Aspects

Even if pathogenic variations of *PEX1* and *PEX6* have been implicated in HS [7, 9], different reports highlight *PEX6* as the most common etiology of HS. However, this predominance has to be confirmed, as in the 13 reported families, 6 had *PEX1* biallelic pathogenic variations and 7 *PEX6* pathogenic variations (Fig. 7.2). *PEX1* seems to be more frequently responsible of more severe PBDs forms [12]. Eleven *PEX6* pathogenic variations and nine *PEX1* have been described in HS.

Several studies of PBDs have reported evidences for genotype-phenotype correlations [13] with the distinction between two types of PEX pathogenic variations: class I leading to residual PEX protein levels and function, implicated in milder phenotypes and class II almost abolishing PEX protein functions, resulting in more severe phenotypes. In all but one family, patients have at least one missense variation. A homozygous stop mutation has been reported in one HS family [7, 9]. This pathogenic variation is located one base pair away from the last exon-exon boundary, and it is not expected to create mRNA decay. The recurrent UK/US p. Arg601Gln *PEX6* HS variation has been demonstrated to have a hypomorphic nature, using DNA transfection complementation assay [8]. Other complementation assays in *PEX1*- and *PEX6*-null cells with variants from HS patients demonstrated that all affected individuals had at least one PEX variant with residual activity in peroxisomal biogenesis [9]. Some *PEX1* and *PEX6* pathogenic variations have been identified in either HS or severe forms of Zellweger syndrome spectrum disorder (ZSSD). However, in HS those variations were always associated with a missense variation *in trans* [7–9, 12, 14–16].

Peroxisomal parameters in plasma, erythrocytes, and cultured fibroblasts of affected patients with *PEX1* and *PEX6* pathogenic variations are within normal ranges [7]. However some studies have reported "mosaic" peroxisome patterns: cells with normal peroxisomal staining, cells with a reduced number of peroxisomes, and "ghosts" with only peroxisomal membrane remnants [9], previously described for hypomorphic *PEX1* and *PEX6* variations [17, 18]. Consistent with this functional peroxisomal mosaicism, electromicroscopy of cultured fibroblasts has identified ultrastructural abnormalities of peroxisomes only at elevated temperature [9].

Very few are known about the role of peroxisomes in hearing process. However, a peroxisomal role has been supposed in the pathophysiology of some forms of human hearing loss [19, 20].

In murine retina, PEX6 is preferentially expressed in ciliary region, the inner segment and the outer plexiform layers [3] but also in the ganglion cell layers and external limiting membranes [8]. Retinal dystrophy has been investigated in the PEX1-G844D mice, a mouse model of mild ZSSD forms [21]. In early stages, cones have shown attenuated function and abnormal morphology, with gradually decreasing rod function. In later stages, structural defects at the inner retina occurred in the form of bipolar cell degradation, inner segment disorganization, and enlarged mitochondria, while other inner retinal cells appeared preserved.

In mouse molar teeth, peroxisomes are expressed during amelogenesis and in particular PEX6 which is present in ameloblast Tome's processes during the secretory stage [22].

PEX26 has been implicated in two patients sharing clinical signs with HS [23]. At 14 years, the first patient presents with clinical signs of HS (profound hearing loss associated with retinitis pigmentosa and enamel dysplasia). However, he has also a hepatosplenomegaly and abnormal hepatic enzymes dosages. The second patient presents at 4 years old with SNHL and AI localized in deciduous teeth. No other feature is described. No Cerebral magnetic resonance

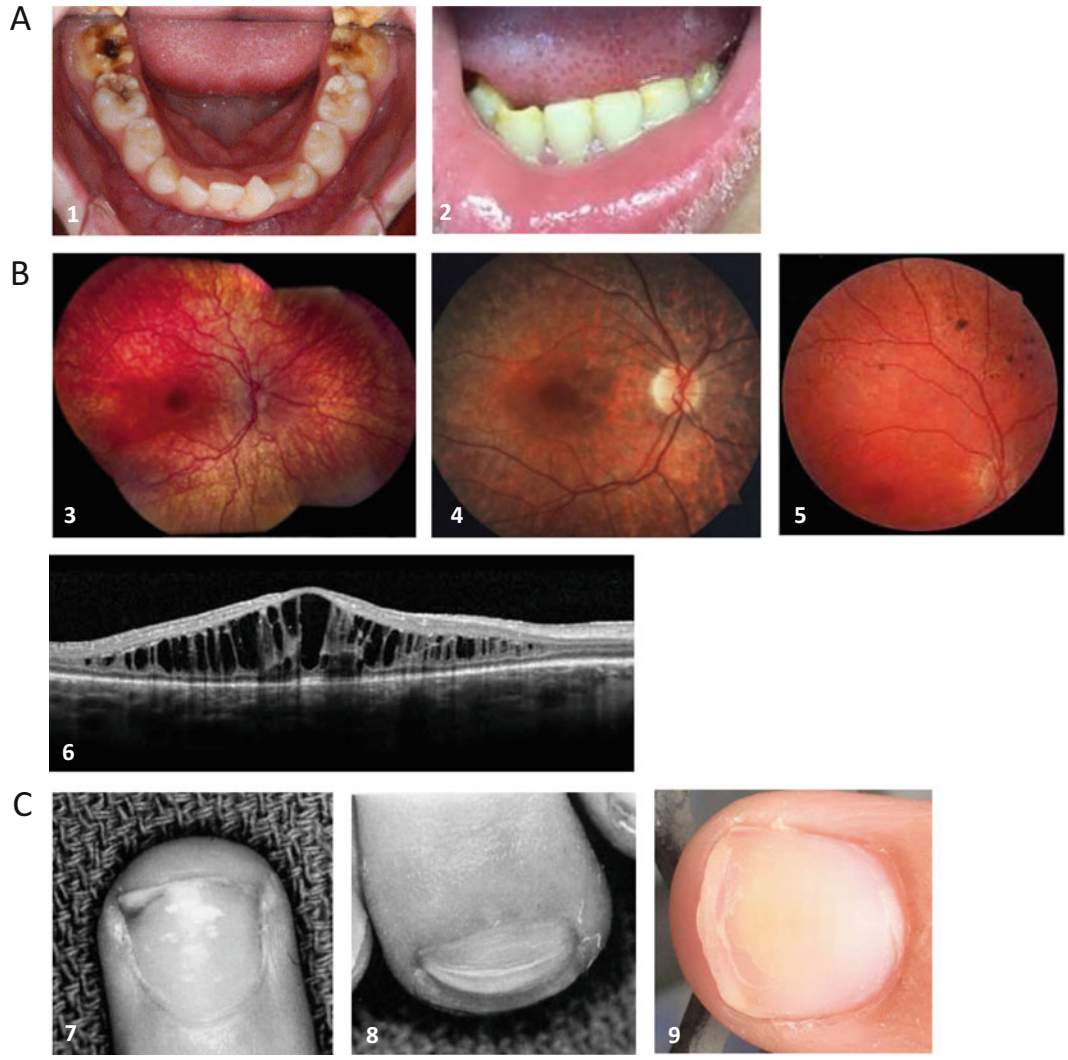

Fig. 7.1 Clinical examination of reported HS cases. (**a**) Dental abnormalities. Respectively 11 and 19 years old patients' permanent dentition (*PEX6* mutations); enamel hypoplasia of the pre-molars and molars more severely affected (1. [5]) than incisor teeth. (2.) (**b**) Retinal abnormalities. Patients' fundus (*PEX6* mutations); pigmentary maculopathy and mild retina vascular attenuation (20 years old; 3. [8]), marked mottling of the retinal pigment epithelium (4. [9]), and hyperpigmented lesions (12 years old; 5. [10]). 12 years old patient's optical coherence tomography (OCT); Cystoid spaces in the external plexiform layer causing distorted lamination (6. [10]). (**c**) Nail abnormalities. Respectively 9, 11, and 20 patients' finger of toenails photographies; leukonychia on fingernails (7. [1]), Beau's line on toenails (8. [1]), and onychoschizia and Beau's lines on fingernails (9. [9])

imaging (MRI) or biochemical investigation has been performed for those two cases. No *PEX1* or *PEX6* variations have been found using Sanger sequencing, and WES identified in both patients a known pathogenic *PEX26* missense variation already described in infantile Refsum disease (IRD), neonatal adrenoleukodystrophy (NALD), and Zellweger syndrome (ZS) [24] *in trans* with an unknown missense variation in one and a variation of the initiation site in the other.

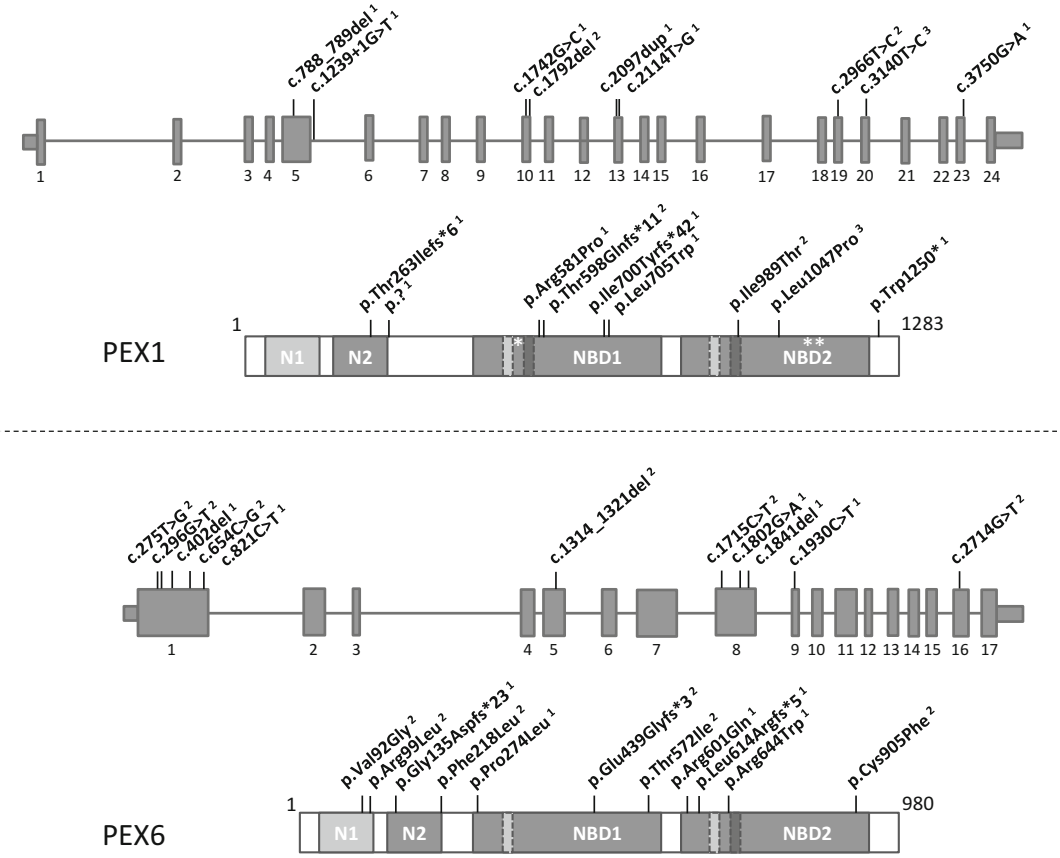

Fig. 7.2 Location of HS-associated variants in schematic representation of human PEX1 and PEX6, showing predicted motifs and protein domains with the corresponding schematic *PEX1* and *PEX6* genomic structure. N-terminus (N1, N2), nucleotide-binding domain 1 (NBD1), nucleotide-binding domain 2 (NBD2), Walker A-loop (light grey bars), Walker B (dark grey bars), and Arginine fingers (white stars). *PEX1*: NM_000466.2; *PEX6*: NM_000287.3. Schematic structure of proteins adapted from Saffert et al. 2017, and location of HS-associated variants adapted from: 1 [9]; 2 [8]; 3 [7]

Recently a new homozygous *PEX26* missense variation has been described as implicated in a non-syndromic form of hearing loss in an Ashkenazi Jewish family. However no clinical investigation has been reported in this publication [25]. For the moment, the implication of *PEX26* in HS remains to be confirmed.

7.3 Diagnosis and Differential Diagnosis

Most of the published HS molecular diagnoses have been established using WES, as the diagnosis is rarely evocated on clinical signs [8, 9]. This misdiagnosis is probably due to the underdiagnosed AI in early-age patients and to the existence of a prevalent differential diagnosis: Usher syndrome which is the most frequent form of association between hearing loss and retinal dystrophy [26]. This justifies checking for AI in patients suspected for Usher syndrome and also including *PEX1–PEX6* molecular analysis in next-generation sequencing (NGS) panels used for hearing impairment, RD, and deafblindness. This systematic sequencing appears to be more efficient than routine biochemical analysis, as most of HS patients have normal peroxisomal

plasma screening [9]. Jalili syndrome associating RD with AI is also a possible differential diagnosis, although deafness is not a manifestation [27].

Heimler syndrome is one of the less severe forms of peroxisome biogenesis disorders. Better phenotype and genotype characterization studies should identify therapeutic endpoints for future preclinical trials. Indeed, even if the vital prognosis of the patients is not engaged, HS is responsible for a severe disability affecting hearing and vision.

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Part III

Potential Roles of Peroxisomes in Major Neurodegenerative Diseases

8 Potential Involvement of Peroxisome in Multiple Sclerosis and Alzheimer's Disease

Peroxisome and Neurodegeneration

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Abstract

Peroxisomopathies are rare diseases due to dysfunctions of the peroxisome in which this organelle is either absent or with impaired activities. These diseases, at the exception of type I hyperoxaluria and acatalasaemia, affect the central and peripheral nervous system. Due to the significant impact of peroxisomal abnormalities on the functioning of nerve cells, this has led to an interest in peroxisome in common neurodegenerative diseases, such as Alzheimer's disease and multiple sclerosis. In these diseases, a role of the peroxisome is suspected on the basis of the fatty acid and phospholipid profile in the biological fluids and the brains of patients. It is also speculated that peroxisomal dysfunctions could contribute to oxidative stress and mitochondrial alterations which are recognized as major players in the development of neurodegenerative diseases. Based on clinical and in vitro studies, the data obtained support a potential

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role of peroxisome in Alzheimer's disease and multiple sclerosis.

Keywords

Alzheimer's disease · Multiple sclerosis · Peroxisomal dysfunction

Abbreviations

ACOX1	acyl-CoA oxidase 1
AD	Alzheimer's disease
ADHAPS	alkyldihydroxyacetonephosphate synthase
AMACR	2-methyl-CoA racemase deficiency
CNS	central nervous system
DAT	dementia of the Alzheimer's type
DHAPAT	dihydroxyacetonephosphate acyltransferase
D-PBE deficiency	D-peroxisomal bifunctional enzyme deficiency
IRD	infantile Refsum disease
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MFP2 deficiency	peroxisomal multifunctional enzyme type 2 deficiency
MS	multiple sclerosis
NALD	neonatal adrenoleukodystrophy
PBDs	peroxisome biogenesis deficiencies
PLP	proteolipid protein
RCDP type I	rhizomelic chondrodysplasia type I
RNS	reactive nitrogen species
ROS	reactive oxygen species
VLCFA	very-long-chain fatty acids
X-ALD	X-linked adrenoleukodystrophy
ZS	Zellweger syndrome

8.1 Hypothesis: Peroxisome and Neurodegeneration

The pathophysiology of neurodegenerative and neuropsychiatric diseases is still poorly understood, and for most of these diseases, there are no treatments to cure them. It is currently well-accepted that these diseases are associated with mitochondrial dysfunction and with a break in the Redox equilibrium which results in an increase in oxidative stress [1]. Cytokine inflammation is also often observed that may result from activation of glial (astrocytes) and microglial cells [2]. These various dysfunctions can lead to the activation of various pathways involved in the cell death/survival equilibrium, including survival or lethal autophagy and/or cell death by apoptosis of brain cells (neurons, oligodendrocytes). In the context of frequent neurodegenerative diseases, such as Alzheimer's disease (AD) and multiple sclerosis (MS), the previously described dysfunctions are often observed [3]. Moreover, in certain rare neurodegenerative diseases of genetic origin, called peroxisomopathies, the neurodegeneration is associated with peroxisomal deficiencies; these organelles are either absent or nonfunctional [4]. Due to recent studies in cultured cells or in genetically modified animals [5, 6], it is well established that peroxisomal deficiencies can affect mitochondrial morphology and activity, disrupt myelination, and promote oxidative stress and inflammation [7]. Based on clinical and experimental studies realized to better understand the physiopathology of peroxisomopathies, it has been hypothesized that peroxisomal dysfunction that may contribute to enhance oxidative stress, to trigger mitochondria dysfunctions, and to favor inflammation may also promote neurodegeneration and participate to the development of frequent neurodegenerative diseases, like AD and MS (Fig. 8.1). The arguments in favor of this hypothesis are discussed.

8.2 Peroxisomopathies

Peroxisomes are small organelles from 0.1 to 1 μm diameters [8]. In humans, they are present in all cells at the exception of erythrocytes. They embrace several metabolic pathways with their large panel of enzyme activities, including cholesterol and bile acids biosynthesis, fatty acid α-

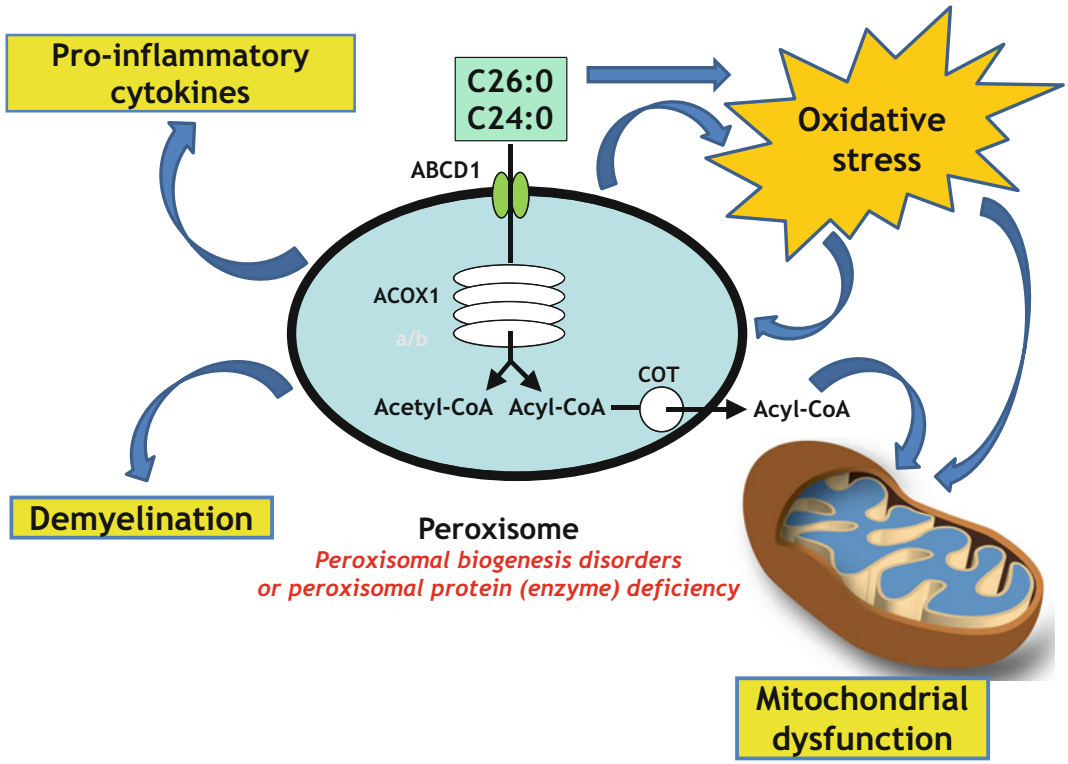

Fig. 8.1 Potential impact of peroxisomal deficiency on oxidative stress, inflammation, demyelination, and mitochondrial dysfunctions. Peroxisomal deficiency, such as peroxisomal transporter and enzyme deficiency (ABCD1 deficiency associated with X-linked adrenoleukodystrophy (X-ALD) and ACOX1 deficiency, respectively), can favor very-long-chain fatty acid (VLCFA) accumulation which can also contribute to enhance oxidative stress which in turn can amplify mitochondrial and peroxisomal dysfunction. Peroxisomal dysfunction can also favor pro-inflammatory cytokines secretion, mitochondrial dysfunction, and demyelination. These data established in the context of peroxisomopathies (X-ALD, ACOX1 deficiency) lead to suggest that peroxisomal biogenesis disorders or peroxisomal protein (transporter or enzyme) deficiency could also contribute to neurodegeneration and to the development of major neurodegenerative diseases, such as Alzheimer's disease (AD) and multiple sclerosis (MS)

and β-oxidation, plasmalogen biosynthesis, and purine degradation [9, 10]. This key role of peroxisomes in cell metabolism is underlined by the consequences of the functional peroxisome defects leading to severe human peroxisomal disorders which are associated with severe consequences in multiple organs, including the central and peripheral nervous system. Such peroxisomal disorders are classified into two groups: (a) diseases associated with a defect in peroxisome biogenesis and (b) diseases caused by a deficiency of a single protein (transporter or enzyme) [4, 11].

(a) Diseases associated with a defect in peroxisome biogenesis (PBDs) are divided into two subgroups. The first is known as the Zellweger Spectrum and includes Zellweger syndrome (ZS), which is the most severe form of peroxisomal diseases, neonatal adrenoleukodystrophy (NALD), and infantile Refsum disease (IRD), the least severe form of this subgroup. For the Zellweger Spectrum, many mutations affecting 12 different *PEX* genes, *PEX* 1, 2, 3, 5, 6, 10,12, 13, 14, 16, 19, and 26 (each encoding for a different peroxin) were described. Peroxins are essential proteins for the biogenesis of

peroxisomes and peroxisomal proteins import. The second subgroup of PBDs includes punctuated rhizomelic chondrodysplasia type I (RCDP type I), a disease linked to mutations in the gene encoding PEX7, a receptor for peroxisomal targeting signal 2 (PTS2) proteins [9].

(b) Diseases caused by a deficiency of a peroxisomal enzyme or transporter are associated with severe clinical abnormalities, and different phenotypes are observed depending on the nature of the deficient transporter or enzyme and its function in the peroxisome [4]. In this group, four diseases are associated with impairment of peroxisomal fatty acid β-oxidation: X-linked adrenoleukodystrophy (X-ALD), acyl-CoA oxidase 1 deficiency (ACOX1 deficiency, also called pseudo-neonatal adrenoleukodystrophy (P-NALD)), D-peroxisomal bifunctional enzyme deficiency (D-PBE deficiency; also known as peroxisomal multifunctional enzyme type 2 (MFP2) deficiency), and 2-methyl-CoA racemase deficiency (AMACR). X-ALD is due to deficiency of the ABCD1 (ATP-binding cassette transporter 1) gene that encodes the transporter ALDP (adrenoleukodystrophy protein), believed to be responsible for transporting CoA-esters of VLCFA through the peroxisomal membrane [11, 12]. This disease is characterized by an accumulation of C26:0 and C24:0 cholesterol esters and a 60% reduction in the oxidation of VLCFA sets and their activation. There are currently 1058 mutations of the ABCD1 gene described [13]. Depending on the age of onset of the disease and the organs affected; different clinical phenotypes exist. Among them are the following: (i) the cerebral form or CCALD (35% of cases) which occurs between 4 and 8 years of age and is characterized by progressive inflammatory demyelination of the central nervous system and (ii) adrenomyeloneuropathy (AMN) which manifests in adulthood and is characterized mainly by non-inflammatory peripheral demyelination [14].

ACOX1 deficiency is characterized by an accumulation in plasma and tissues of VLCFA and a severe reduction in the oxidation of their acyl-CoA esters. Patients have symptoms and clinical signs similar to those seen in children with NALD except that in the case of ACOX1 deficiency, peroxisomes are present in the cells [15, 16].

D-PBE deficiency/MFP2 deficiency has severe clinical symptoms similar to those observed for ZS. In patients deficient in D-PBE, peroxisomal β-oxidation of all major substrates is disrupted as a result of the involvement of this enzyme in the oxidation of VLCFA, pristanic acid, DHCA, and THCA that accumulate in plasma and tissues [17, 18]. Deficiency in methyl-acyl-CoA racemase (AMACR) has moderate clinical signs, resembling those observed in Refsum disease. It is characterized by an accumulation of pristanic acid, DHCA, and THCA, since AMACR converts the substituted fatty acids of configuration 2R into 2S [19, 20].

Peroxisomal diseases caused by deficiency of other peroxisomal enzymes include RCDP of type 2 or type 3, Refsum disease, type I hyperoxaluria, and acatalasaemia. RCDP type 2 is due to mutations of the DHAPAT (or GNPAT) gene encoding dihydroxyacetonephosphate acyltransferase (DHAPAT). RCDP type 3 is associated with mutations in ADHAPS gene (or AGPS) encoding the alkyldihydroxyacetonephosphate synthase (ADHAPS). Patients with these deficiencies have a defect in the synthesis of phospholipid ethers, including plasmalogens [21]. Refsum disease is due to a deficiency of phytanoyl-CoA hydroxylase, an enzyme in the pathway of oxidation leading to the accumulation of phytanic acid [22]. Type I hyperoxaluria is a disease linked to a mutation in the AGAT gene encoding alanine/glyoxylate aminotransferase, an enzyme that converts the glyoxylate formed in peroxisome into glycine. The accumulation of glyoxylate leads to the formation of oxalate, which precipitates calcium oxalate into tissues and causes loss of kidney function [9]. Acatalasaemia is a very rare disease associated with a mutation in the gene encoding catalase and leading consequently to a defect in the metabolism of H_2O_2 [9].

Based on the side effects of peroxisomal deficiencies on the peripheral and central nervous system, the contribution of the peroxisome in the physiopathology of frequent neurodegenerative

diseases (AD and MS) has been studied. In this context, emerging studies suggest that peroxisomal function may be altered with aging and contribute to the pathogenesis of a variety of diseases, including neurodegenerative disorders [23]. In addition, the interest for the peroxisome in neurodegeneration is supported by several data reporting an impact of peroxisome activity on the axon functionality and myelin synthesis [24–26].

8.3 Peroxisome and Myelin

Ultrastructural and cytochemical studies have shown the presence of peroxisomes in all cell types of the nervous system which can impact directly or indirectly myelin production: astrocytes, microglia, oligodendrocytes, Schwann cells, and neurons [27, 28]. Peroxisomes of the nervous system are smaller (150–200 nm in diameter) than those present in the liver or kidney (0.1–1 μm in diameter) and are therefore called "microperoxisomes" [27, 28]. Peroxisomes have been identified in the myelin sheath of the white and grey matter [27, 28]. The myelin sheath, which surrounds the axons of neurons, is a lipoproteic complex synthesized by the oligodendrocytes in the central nervous system and by the Schwann cells in the peripheral nervous system. The myelin sheath is essential for an efficient transmission of the nerve impulse which depends on the composition of myelin and of the structure of the myelin sheath [29]. Myelin contains 70% lipids (cholesterol, phospholipids, plasmalogens (etherphospholipids), and glycolipids) and 30% proteins (mainly in the central nervous system: Proteo Lipid Protein (PLP), Myelin Basic Protein (MBP), and Myelin Associated Glycoprotein (MAG)) [29, 30]. In humans, approximately 25% of the total amount of the cholesterol is localized in the brain and is a product of local synthesis [31]. About 70% of the cholesterol within the brain is in myelin, the remaining 30% of brain cholesterol is divided between glial cells (20%) and neurons (10%), mainly located in the cellular membrane [32]. The peroxisome is involved in cholesterol biosynthesis and catabolism (degradation of cholesterol in bile acid) [9]. In addition, among complex lipids, we find plasmalogens (representing 20–30% of phospholipids): the first two steps in plasmalogen biosynthesis take place in the peroxisome via the DHAPAT (dihydroxyacetonephosphate acyltransferase) and ADHAPS (alkyldihydroxyacetonephosphate synthase) peroxisomal enzymes located on the luminal side of the peroxisomal membrane [9, 21, 33].

The presence of peroxisomes in different nerve cells has been linked to the preservation of axonal integrity and normal brain development through the use of knocked out (KO) mice for peroxisomal proteins [27, 28, 34]. Thus, in mouse models, several peroxisomal dysfunctions have been relied to the alteration of the myelin sheath and have permitted to link the peroxisome to the synthesis and degradation of myelin. The importance of the peroxisome in myelin formation has been demonstrated when using KO mice for the peroxisomal protein peroxin 5 (PEX5). By selectively inactivating the import factor PEX5 in myelinating glia, the generated mutant mice developed normally but within several months showed ataxia, tremor, and premature death [24]. Absence of functional peroxisomes from oligodendrocytes caused widespread axonal degeneration and progressive subcortical demyelination but did not interfere with glial survival. Moreover, it caused a strong pro-inflammatory milieu and, unexpectedly, the infiltration of B and activated CD8+ T cells into brain lesions [24]. These data support that peroxisomes provide oligodendrocytes with an essential neuroprotective function against axon degeneration and neuroinflammation, which is relevant for human demyelinating diseases [24]. The conditional KO Nes-PEX5 (Nestin-PEX5) mice have lesions in the white and grey matter of the brain, with significant defects in the myelinated axons of the white matter of the corpus callosum and cerebellum. In the absence of a normally functioning peroxisome, the myelin sheath formed is completely disorganized protein content/protein content [34]. The absence of plasmalogens is compensated by esterphospholipids; this replacement can not only change the ratio of polyunsaturated fatty acids to the disadvantage of DHA but

also have a consequence on lipid rafts and their functioning, where lipids linked to ethers have an important role [34–36].

In addition, peroxisomes in myelin can contribute to the proper functioning of axons. Axons have very few peroxisomes, but they need the lipid components produced by the peroxisomes to maintain their functional membranes. So, a transport of peroxisomal metabolites would occur between peroxisome-rich areas, myelin, and axons, supporting that myelin lipids may be an energy reservoir that could be mobilized by myelin peroxisomes to support axonal function [25]. Interestingly, a plasmalogen deficient mouse model presents a decreased level of neurotransmitters and an impaired neuronal release after a strong chemical or electrical stimulus [37]. This plasmalogen deficient mouse model manifests a motor coordination defect as well as a hyperactivity and impaired social interaction [38]. Deficits in contextual and cued fear conditioning indicate severe memory deficits linking plasmalogen deficiency to AD [38, 39].

At the moment, whereas peroxisomal dysfunctions are well-established in peroxisomopathies (ZS, X-ALD, ACOX-1 deficiency, and MFP-2 deficiency) and could consequently contribute to demyelination, it is suspected that peroxisomal alteration, which could occur in frequent neurodegenerative diseases (MS and AD) as a consequence of the disease via various side effects (oxidative stress and inflammation) might further favor demyelination and neurodegeneration [40].

8.4 Pivotal Role of Peroxisome in Neurodegeneration

8.4.1 Impact on Mitochondria

There are several evidences of metabolic connections existing between peroxisomes and mitochondria [41–43]. In yeast, it has been shown that peroxisomes are implied in the regulation of necrosis. Thus, *S. cerevisiae* PEX6 deleted cells display hallmarks of necrosis and strongly elevated formation of ROS [44]. In *H. polymorpha* methylotrophic yeast, deletion of PMP20 leads to pronounced induction of necrosis [45]. In PEX5 knockout mice, the absence of peroxisomes in hepatocytes causes endoplasmic reticulum and mitochondrial abnormalities [46, 47]. Thus, in PEX5 deficient mice, the mitochondria were characterized by (i) reduced activity in complex I, III, and V, (ii) differentially affected respiratory complexes (reduction in complexes I and III, incomplete assembly of complex V, non-significant reduced activity of complexes II and IV), (iii) depleted mitochondrial DNA, and (iv) increased permeability and fluidity in mitochondrial membranes leading to a collapse of the mitochondrial inner membrane potential [47, 48]. These observations are in agreement with several studies which support the concept of peroxisomal interactions and crosstalk between peroxisomes and other subcellular compartments [49] and key connections between peroxisomes and mitochondria for biogenesis and activity [50]. Noteworthy, in aging and neurodegeneration, it is suggested that peroxisomal dysfunctions could favor secondary mitochondrial alterations which could contribute to cell death [40, 51].

8.4.2 Impact on Oxidative Stress

There are several evidences that peroxisomes have the capacity to impact redox-linked physiological processes. Thus, it is known that peroxisomes counteract the side effects of ROS by antioxidant enzymes. Among these are peroxisomal catalase, glutathione peroxidase, peroxiredoxin I, and peroxiredoxin Pmp20p to degrade hydrogen peroxide and Cu/ZnSOD and MnSOD to detoxify superoxide anions [52]. Peroxisomes also cooperate with other organelles, especially mitochondria, to contribute to Redox homeostasis, and it has been shown that these two organelles are tightly connected to regulate oxidative stress [41]. The use of the genetically encoded photosensitizer KillerRed in the peroxisome of mouse embryonic fibroblast allowed gaining a better insight into the interplay between peroxisomes and cellular oxidative stress

[53]. The phototoxic effects of peroxisomal KillerRed induce mitochondria-mediated cell death and this process can be counteracted by targeted overexpression of a select set of antioxidant enzymes, including peroxisomal glutathione S-transferase kappa 1, superoxide dismutase 1, and mitochondrial-targeted catalase. These data indicate that mitochondria may act as dynamic receivers, integrators, and transmitters of peroxisome-derived mediators of oxidative stress. In addition, on 158 N murine oligodendrocytes, SiRNA knockdown of *Abcd1* or *Acox1* increased ROS and reactive nitrogen species (RNS) production even in the absence of VLCFA and especially potentiate VLCFA-induced ROS overproduction [54]. These data demonstrate that *Abcd1* or *Acox1* knockdown contribute to disrupt Redox equilibrium supporting a link between oxidative stress and the deficiency of peroxisomal proteins ABCD1 or ACOX1.

8.4.3 Impact on Inflammation

At the moment, the impact of peroxisomal dysfunction on inflammation linked to neurodegeneration has been mainly studied in X-ALD which is characterized by ABCD1 deficiency. Under its severe form, X-ALD is associated with brain demyelination and inflammation. In demyelination, a potential role of pro-inflammatory cytokines, including TNF-α, has been suggested [55]. The most aggressive phenotypes of X-ALD are accompanied by inflammatory changes in the white matter of the brain [56]. The inflammation in X-ALD is triggered by an impaired plasticity and pro-inflammatory skewing of macrophage [57]. It has been hypothesized that TNF-α, which has been shown to get increased upon accumulation of VLCFA in C6 cells, could contribute to induce cell death on various neural cells. In addition the blood-brain barrier, whose permeability may vary according to environmental factors, such as inflammatory processes [58], might influence the delivery of VLCFA towards the brain cells. Silencing of *Abcd1* and *Abcd2* genes also sensitizes rat astrocytes for inflammation [59]. The up-regulation of pro-inflammatory genes as *Trem2* (triggering receptor expressed on myeloid cells 2), which is involved in microglial polarization and phagocytosis control, was also observed in CRISPR/cas9-induced acyl-CoA oxidase 1 (ACOX1)-deficient BV-2 microglial cells [60]. Similarly, a MFP2 deficiency in mouse brain microglia provokes intrinsic dysregulation of their pro-inflammatory and proliferative phenotypes but preserves their appropriate responses to inflammatory stimuli [61]. The use of mice with peroxisome deficiency in neural cells also shows that peroxisome inactivity also trigger a fast neuroinflammatory reaction [62, 63].

Based on the three hit hypothesis [64], inflammation constitutes the second hit and would be activated by oxidative stress which constitute the first hit; the third hit characterized by general peroxisomal dysfunction would trigger cell death and neurodegeneration. According to data mainly obtained in peroxisomopathies, it can be supposed that peroxisomal dysfunction contributes to neuroinflammation, which can further favor neurodegeneration. This hypothesis is reinforced by several in vitro and in vivo studies in other fields that neurodegeneration which have contributed to identify the part taken by the peroxisome in the regulation of immunity [65].

8.5 Abundance of Lipids on Peroxisomal Dysfunctions and Neurodegeneration

The most abundant lipid species identified in the CNS are cholesterol (approximately 25% of the total amount of cholesterol present in humans is localized in the brain), phospholipids (phosphatidylcholine, phosphatidylethanolamine, plasmalogens, and sphingomyelin), ceramides, glucosylceramides, and sulfatides [67]. Beside these major lipids, cholesterol oxide derivatives (also named oxysterols) are present as well as neuroactive steroids and derivatives of polyunsaturated fatty acids, including prostaglandins, leukotrienes, neuroprotectins, and resolvins [67]. These different lipids can be identified in

the different compartments of the major cells of the CNS: glial cells (astrocytes, oligodendrocytes), microglial cells, and neurons. The major lipid components of myelin are cholesterol, glycerophospholipids, and glycosphingolipids [67]. In several neurodegenerative diseases, important modifications of lipid profile have been described leading to suspect dysfunctions of lipid metabolism. Enhanced or decreased levels of several oxysterols formed by auto-oxidation enzymatically or both have been reported, including in X-ALD patients [68]. In peroxisomopathies and in patients with dementia, enhanced levels of VLCFA have also been described [69, 70]. Based on these observations, the incidence of these lipids (oxysterols, VLCFA) was studied on different types of nerve cells, taking into account the impact on the organelles and in particular on the peroxisome. In dementia, based on in vitro experiments, it is hypothesized that enhanced VLCFA levels could favor mitochondrial dysfunctions and oxidative stress, which could in turn contribute to peroxisomal dysfunctions [40, 54, 71]. When nerve cells (158 N murine oligodendrocytes, microglial BV-2 cells) were treated with various oxysterols found at enhanced levels in the plasma and/or brain of patients with several neurodegenerative diseases (mainly 7-ketocholesterol (7KC) and 7-β-hydroxycholesterol), there are also evidences that high concentrations of these molecules are cytotoxic, leading to an oxiapoptophagic mode of cell death, which is associated with functional modifications of the peroxisome as well as with quantitative and qualitative peroxisomal changes (reduced number of peroxisomes per cell which can be associated with topographical modifications and presence of peroxisome with different sizes and shapes revealed by transmission electron microscopy (TEM)) [66, 72] (Fig. 8.2).

8.6 Peroxisomal Dysfunction in Multiple Sclerosis

MS is characterized by immune-mediated demyelination and the loss of axons in the central nervous system. This disease often has a severe course. According to the brain regions affected, the signs are variable [73, 74]. The causes of MS are multiple and involve genetic and environmental factors, leading to immune processes and lipotoxicity that could vary according to the form of MS considered. In the relapsing-remitting (RR) form, the role played by inflammation is important. In progressive MS (primary progressive MS (PP-MS) or secondary progressive MS (SP-MS)), the impaired myelination and the altered conduction of the nervous influx, which results from oligodendrocyte dysfunction and from energy loss at the axonal level, can be predominant [75, 76]. Various studies support an involvement of peroxisome in the development of MS. Indeed, in the MS grey matter neurons, ABCD3 immuno-labelling was significantly lower compared to control. In addition, calibration of ABCD3 gene expression with reference to glyceraldehyde 3-phosphate dehydrogenase revealed overall decreases in expression in MS compared to controls [77]. In the MS grey matter, this decrease of peroxisomes was associated with an increase in the C26:0 level [77]. In the serum of patients with RR-MS, SP-MS, and PP-MS, Senanayake et al. showed that the ratio of phosphatidylethanolamine, containing DHA (C22:6 n-3), to phosphatidylethanolamine containing its precursor (C18:3 n-3) was higher than in controls [78]. This increase is more significant in the early stages of the RR-MS form. DHA synthesis occurs via elongation and desaturation of (C18:3 n-3) to (C24:6 n-3) in the endoplasmic reticulum followed by β-oxidation to (C22:6 n-3) in the peroxisome [9, 41]. These observations suggest that enhanced flux through this pathway occurs early in the MS process [78]. With the plasmalogens, which are in part synthesized in the peroxisome, increased levels of all plasmalogen species measured in the serum were significantly elevated in SP-MS compared with the controls [78]. In an experimental model of MS (experimental autoimmune encephalomyelitis (EAE) in the rat), peroxisomal functions were decreased; lower degradation of VLCFA leading to their accumulation was observed; decrease in the activity of the first enzyme

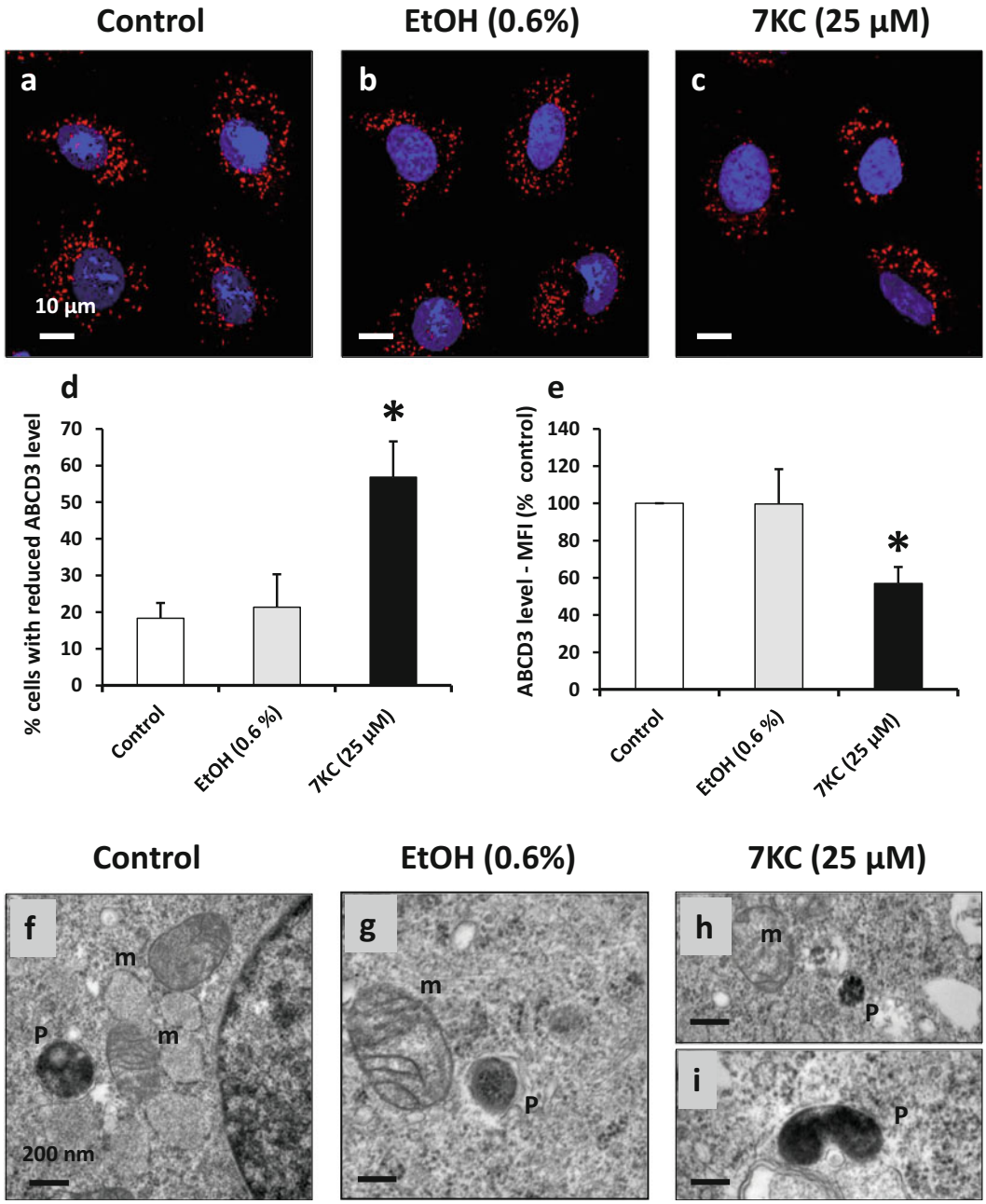

Fig. 8.2 Impact of 7-ketocholesterol on peroxisome topography and morphology. 7-ketocholesterol (7KC), which is mainly formed by cholesterol auto-oxidation, is often enhanced in the plasma, cerebrospinal fluid, and/or brain of patients with neurodegenerative diseases, such as AD and MS. In vitro, the impact of 7KC on peroxisome was determined by various methods, including indirect immunofluorescence microscopy (using a fluorescent microscope coupled to an Apotome system (Imager M2, Zeiss)) and flow cytometry on murine oligodendrocytes 158 N cultured for 24 h with or without 7KC (25 µM). The peroxisomes were detected with a rabbit polyclonal antibody raised against ABCD3 [66] in untreated (control) (**a**), vehicle control-(EtOH 0.6%) (**b**) and 7KC (25 µM)-treated cells (**c**). The quantity of peroxisomes in 7KC-treated-cells determined by flow cytometry was lower than in untreated and vehicle-treated cells (**d**–**e**). By transmission electron microscopy (**f**–**i**), in staining condition with diaminobenzidine (DAB) allowing to detect the peroxisomes [66], DAB-positive structures, with various sizes and shapes, which evocate abnormal peroxisomes, were detected in 7KC-treated cells (**h**–**i**), whereas round and regular peroxisomes were observed in the control (**f**) and vehicle control (**g**) cells

responsible for plasmalogen synthesis (DHAPAT) associated with a decrease in PLGN levels and decrease in catalase activity was revealed [79]. These changes in peroxisomal activity result in a decrease in myelin integrity and repair [79]. Lovastatin, a cholesterol-lowering and anti-inflammatory statin, administered during EAE induction provided protection against loss/down-regulation of peroxisomal functions [79]. Studies on brain material from autopsies show selective loss of ethanolamine plasmalogen in the affected white matter and in the myelin sheath [80].

8.7 Peroxisomal Dysfunctions in Alzheimer's Diseases

Peroxisomes are involved in the synthesis of specific fatty acids, such as docosahexaenoic acid (DHA, C22:6 n-3), which is essential for the brain and retina, and of plasmalogens, which play crucial roles in neural cells and are essential components of myelin [81]. In humans, quantitative modifications of DHA level as well as qualitative and quantitative modification of PLGN levels support a potential role of peroxisome in AD and in dementia of the Alzheimer's type (DAT) [81]. In addition, several studies conducted in animal models and in humans provided evidence for a role of DHA in preventing brain degeneration; significantly lower levels of PLGN were also observed in patients with severe dementia, and a decreased activity of carnitine acetyltransferase, an enzyme present in peroxisome (but also detected in mitochondria, the endoplasmic reticulum, and nucleus) was reported in AD patients [81]. Altogether, these different observations suggest a potential role of peroxisomal dysfunction in AD and DAT. This hypothesis is supported by data obtained in AD patients. Thus, lipid analyses of cortical regions of AD patients revealed accumulation of C22:0 and VLCFA (C24:0 and C26:0), all substrates for peroxisomal β-oxidation, in cases with stages V-VI pathology compared with those modestly affected (stages I–II) [69]. Conversely, the level of PLGN, which need intact peroxisomes for their biosynthesis, was also decreased in affected tissues. In addition, the peroxisomal volume density was increased in the soma of neurons in gyrus frontalis at advanced AD stages. Interestingly, the decrease in plasmalogens and the increase in VLCFA and peroxisomal volume density in neuronal somata all showed a strong association with neurofibrillary tangles [69]. During normal aging the levels of lysophosphatidylcholine, choline plasmalogen, and lyso-platelet-activating factor increase significantly; similar but more pronounced changes were observed in AD patients [40]. As several lipid metabolism alterations have been observed in the brain and plasma of AD patients, suggesting a relation between lipid metabolism alteration and dementia, the fatty acid profiles were established using gas chromatography with or without mass spectrometry on matched plasma and red blood cells (RBCs) of patients diagnosed with AD, vascular dementia, or other dementia, and compared with a control group of elderly individuals. The variations of fatty acid levels and the accumulation of C26:0 in the plasma and RBCs (which may constitute a convenient blood biomarker of dementia) highlight an alteration of fatty acid metabolism in demented patients, including AD patients, and point toward possible peroxisomal dysfunction [70].

8.8 Conclusion

There are now several arguments in favor of the implication of peroxisomal dysfunction in major neurodegenerative diseases, such as AD, DAT, and MS which could be triggered at least in part by cytotoxic oxysterols [81, 82] (Fig. 8.3). In these patients, the lipid metabolism is disturbed, and enhanced level of VLFCA and lower PLGN level have been observed. These data suggest that some of these lipids could constitute suitable biomarkers of neurodegeneration and/or used as therapeutic tools (PLGN). In addition, here are now several arguments that peroxisomal dysfunction favors oxidative stress, leading to altered mitochondrial activity which are recognized as key parameters in neurodegeneration.

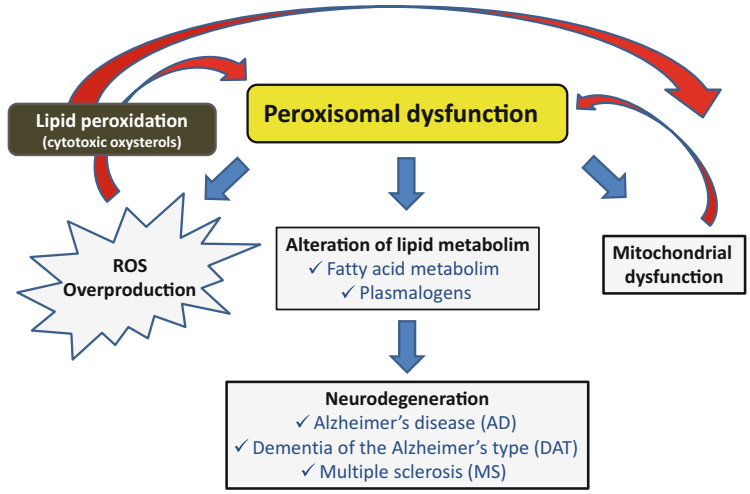

Fig. 8.3 Incidence of peroxisomal dysfunction on neurodegeneration. Several data support that peroxisomal dysfunctions are involved in major neurodegenerative diseases (AD, DAT, and MS). On various in vitro and in vivo models, as well as in clinical studies, it has been established that peroxisomal dysfunction favors ROS overproduction, alter lipid metabolism leading to increased level of VLCFA and plasmalogens, and favors mitochondrial dysfunction which could contribute to decrease the activity of nerve cells and favors cell death. In turn, as previously suggested by Trompier et al. [40], ROS overproduction and mitochondrial dysfunction could amplify peroxisomal dysfunction and favor neurodegeneration. In X-ALD, ROS overproduction was considered as the first hit; inflammation and cell death, associated with mitochondrial dysfunction, were considered as the second and third hit, respectively [8, 30]. It is also known that ROS overproduction favors lipid peroxidation and leads to the formation of cytotoxic oxysterols. This has led to suggest that some oxysterols formed by cholesterol auto-oxidation could act as second messenger and favor mitochondrial and peroxisomal dysfunction leading to cell death by oxiapoptophagy [66]

Consequently, several studies suggest that the peroxisome could contribute to the physiopathology of major neurodegenerative diseases and could constitute a new therapeutic target.

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9 Developmental and Degenerative Cerebellar Pathologies in Peroxisomal β-Oxidation Deficiency

Stephanie De Munter and Myriam Baes

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, preceded 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 nonautonomous mechanisms.

Keywords

Peroxisomes · β-Oxidation · Multifunctional protein-2 · Ataxia · Cerebellum · Mouse model

9.1 Introduction

Peroxisomal disorders can be categorized in two major classes based on the molecular defect [1]. 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 [1]. It is quite striking that not only in PBD but also in the SED cerebellar pathology is a common feature [2]. Here, we

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briefly summarize the cerebellar pathology in these patients and the corresponding mouse models. For a more thorough review on this matter, the reader is referred to [2].

PBD encompass a spectrum of disease manifestations, ranging from early postnatal death hallmarked by brain malformation and hypotonia, known as the Zellweger syndrome, to survival into adulthood [3]. Peroxisome biogenesis in mammals relies on the collaboration of 14 peroxin proteins (PEX), and defects can give rise to the 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 [4]. 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 the cerebral white matter [5]. Cerebellar atrophy without white matter changes was reported in a few but not in all patients with the mildest forms of PBD [3]. 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 [2].

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 [6]. This is always related to white matter anomalies and linked to the loss of plasmalogens resulting in both developmental and degenerative pathologies. 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 [7].

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, which 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 [8]. 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 [2]. 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.

9.2 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 [9].

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 [9]. The

second and third steps of the β-oxidation are catalyzed by two multifunctional proteins that each executes 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 and 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 [9].

9.3 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 [10]. 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 [10].

Information on the expression pattern of peroxisomal β-oxidation enzymes in the brain is rather scarce. ABCD1 is expressed more highly in nonneuronal 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) [11] 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 and ependymal cells and neurons of the cortex, thalamus, and hippocampus were shown to be MFP2 immunoreactive.

9.4 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 [1].

The most frequent peroxisomal disorder, X-linked adrenoleukodystrophy (X-ALD) due to a defective ABCD1 transporter, has many clinical presentations [12]. A minority of patients (<2% worldwide but 9% in Japan) has a cerebello-brainstem dominant phenotype, characterized by cerebellar ataxia and gait and speech disturbances [13]. Histological investigation in one of these patients revealed torpedoes on PC axons and patchy PC loss [13]. 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 [14, 15] exhibit strong similarity with the PBD patients, both with respect to the severe, intermediate, and mild end of the spectrum [5]. Two mildly affected ACOX1 siblings developed cerebellar atrophy leading to ataxia in their teens, and

they survived into their 50s. 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 [2]). Still, of all the 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 [14]. 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 [5]. These patients achieve some developmental milestones, but then deteriorate, sometimes rapidly [16]. Notably, autoantibodies to MFP2 can be the cause of the Stiff-man syndrome, indicating the importance of MFP2 for cerebellar functioning.

9.5 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 [17]. Besides global knockouts in which the phenotype is influenced by peripheral sources [18], also a neural-selective MFP2 knockout was generated (*Nestin-Mfp2*$^{-/-}$) [19]. 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* promoter driving CRE expression is less selective than originally expected. It indeed also gives rise to gene deletion in the peripheral organs such as the kidney and pancreas. Finally, a PC-restricted MFP2 knockout (*L7-Mfp2*$^{-/-}$) was generated which was instrumental to distinguish between cell autonomous and nonautonomous and between developmental and degenerative mechanisms [20]. The cerebellar phenotypes of these mouse models are summarized in Table 9.1.

9.5.1 CNS Deletion of MFP2 Induces Ataxia

For unknown reasons, the total ablation of MFP2 in mice does not cause severe neurodevelopmental abnormalities, and *Mfp2*$^{-/-}$ mice are indistinguishable at birth from wild-type mice [18]. This is in striking contrast to the severe pathology in neonates with MFP2 deficiency [14]. However, already from the age of 3 weeks, *Mfp2*$^{-/-}$ mice display limb dyskinesia rapidly evolving in an unsteady gait with coordination problems [18]. 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 [18]. 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 [19] strongly deviates from global *Mfp2*$^{-/-}$ mice. At the histological level, global *Mfp2*$^{-/-}$ mice 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 [21]. 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 the studies in *Pex2*$^{-/-}$ mice in which bile acid feeding partially corrected the cerebellar developmental defects [4]. A second divergence is that in

Table 9.1 Comparison of the phenotype and cerebellar histology of mouse models with elimination of MFP2 from all cells (*Mfp2*$^{-/-}$), all neural cells (*Nestin-Mfp2*$^{-/-}$), or from PCs only (*L7-Mfp2*$^{-/-}$)

	Mfp2$^{-/-}$	*Nestin-Mfp2*$^{-/-}$	*L7-Mfp2*$^{-/-}$
MFP2 loss in	*All cells*	All neural cells	PCs
Phenotype			
Life span	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[a]	Yes	Yes	Not determined
Onset axonal swellings	<2 months[b]	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[c]	5 months	6 months	Not determined

[a]Altered cerebellar development in the *Mfp2*$^{-/-}$ model refers to a delay in cerebellar foliation. In *Nestin-Mfp2*$^{-/-}$ mice, mild disturbances in PC afferent innervation and spine morphology occur. [b]Earliest age investigated. [c]Myelin loss in cerebellar branches of (*Nestin*)-*Mfp2*$^{-/-}$ mice occurs after the appearance of PC axonal swellings

Mfp2$^{-/-}$ mice an extensive neuroinflammation spreads over the brain with age, although the cerebellum is relatively spared [19, 22]. This is much less pronounced in *Nestin-Mfp2*$^{-/-}$mice, likely leading to their increased survival (>10 months) [23]. Hence, the latter mouse model was better suited to perform a longitudinal study of the cerebellar pathology [24].

The PC-selective MFP2 knockout (*L7-Mfp2*$^{-/-}$) mice allowed to demonstrate that, in addition to developmental anomalies, the 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 [20]. The discordance between the motor phenotypes in *L7-Mfp2*$^{-/-}$ and *Nestin-Mfp2*$^{-/-}$ mice might have several origins that will be explained below.

9.5.2 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 [24]. 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 [24]. 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.

9.5.3 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 [24]. This can in part be caused by an increase in membrane capacitance, although we cannot exclude that additional mechanisms might play a role. The clear-cut increase in PC membrane capacitance was not associated with an

increased size of the cell soma, nor with a more elaborate dendritic arborization [24]. 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.

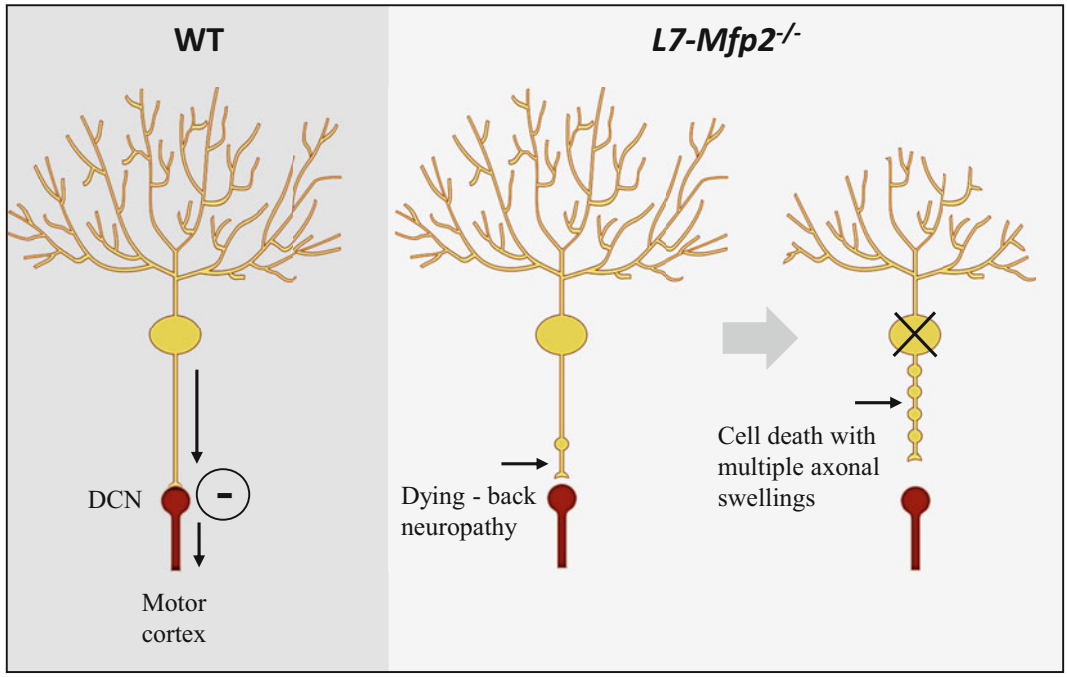
Detailed analysis of the action potentials of *Nestin-Mfp2*$^{-/-}$ PCs did not reveal alterations in action potential amplitude and shape, suggesting that Na^+, K^+, or Ca^{++} channels operate properly. Not unimportant is the fact that peroxisomes were shown to be more crowded in the PC axon hillock, 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.

9.5.4 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*$^{-/-}$ [19], in *Nestin-Mfp2*$^{-/-}$ [24], and in the PC-selective *L7-Mfp2*$^{-/-}$ mice [20]. 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 (Fig. 9.1) [20]. Since cerebellar ataxia in *L7-Mfp2*$^{-/-}$ mice was observed well before extensive PC death [20], the motor disabilities of these mutants are due to the defects in PC functioning.

However, in *Mfp2*$^{-/-}$ and in *Nestin-Mfp2*$^{-/-}$ mice, axonal swellings were observed at a much earlier age [24], indicating that the 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, and the number of swellings progressively increased thereafter. Among 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, the 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 [25]. 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 [19]. Motor dyscoordination and cerebellar neurodegeneration have been described in both glia-specific and BG-specific knockout mouse models mainly linked to the 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-nonautonomous contribution to axonal atrophy is not related to the absence of MFP2 in a particular cell type (cerebellar neurons,



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.

Fig. 9.1 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 6–8 months and worsening with time. This is followed by the degeneration and loss of PCs. Reproduced from (9) with permission from John Wiley and Sons

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-oxidation 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 [13]. Additional pathological studies in peroxisomal β-oxidation patients are warranted to validate the findings in mice.

9.5.5 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 [24]. 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 [20].

9.5.6 Postdevelopmental PC Degeneration in MFP2 Deficiency

The *L7-Mp2*$^{-/-}$ mice not only clarified that the 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 onward and is not fully established until 2–3 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* promoter 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 nonrandom manner since PCs in the caudal cerebellum survive longer [20]. 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 [26]. 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 (MAP 1A), neuroplastin, and neurogranin [27].

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.

9.5.7 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-month-old *L7-Mfp2*$^{-/-}$ mice, the age at which motor learning was assessed.

9.5.8 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 the BG of the *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 [18]. 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 [18]. The most likely other metabolic culprit in the 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 [28]. 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 [19]. 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 [29]. 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 [30]. It should also be emphasized that in mild MFP2 patients, no or borderline metabolic changes are detectable in the 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 the cerebellar tissue. It remains also unexplained why among adult X-ALD patients who all have increased levels of VLCFA, only a few present with a cerebellar phenotype.

9.6 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 the cerebellar folia which likely has a systemic origin, as it was not seen in *Nestin-Mfp2*$^{-/-}$ mice. In the latter, the absence of MFP2 from all neural cells affected the electrophysiological properties of PCs, 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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Part IV

Cell and Animal Model Systems

A Mouse Model System to Study Peroxisomal Roles in Neurodegeneration of Peroxisome Biogenesis Disorders

10

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Abstract

Fourteen *PEX* genes are currently identified as genes responsible for peroxisome biogenesis disorders (PBDs). Patients with PBDs manifest as neurodegenerative symptoms such as neuronal migration defect and malformation of the cerebellum. To address molecular mechanisms underlying the pathogenesis of PBDs, mouse models for the PBDs have been generated by targeted disruption of *Pex* genes. Pathological phenotypes and metabolic abnormalities in *Pex*-knockout mice well resemble those of the patients with PBDs. The mice with tissue- or cell type-specific inactivation of *Pex* genes have also been established by using a *Cre-loxP* system. The genetically modified mice reveal that pathological phenotypes of PBDs are mediated by interorgan and intercellular communications. Despite the illustrations of detailed pathological phenotypes in the mutant mice, mechanistic insights into pathogenesis of PBDs are still underway. In this chapter, we overview the phenotypes of *Pex*-inactivated mice and the current understanding of the pathogenesis underlying PBDs.

Keywords

Peroxisome biogenesis disorders · *Pex* (*in italic*) gene · Knockout mouse · Neurodegeneration · Neuronal migration defect · Dysmyelination

Abbreviations

Abcd1	ATP-binding cassette family D1
ADAPS	alkyl-dihydroxyacetone phosphate synthase
BA	bile acid
BDNF	brain-derived neurotrophic factor
BM	brain mutant
Cnp	2′,3′-cyclic nucleotide phosphodiesterase
CNS	central nervous system
DHAPAT	dihydroxyacetone phosphate acyltransferase
DKO	double knockout
EGL	external granule layer

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GSK3β	glycogen synthase kinase 3 β
IRD	infantile Refsum disease
IZ	intermediate zone
KO	knockout
MBP	myelin basic protein
MEF	mouse embryonic fibroblast
ML	molecular layer
NALD	neonatal adrenoleukodystrophy
PBD	peroxisome biogenesis disorder
PNS	peripheral nervous systems
PTS	peroxisomal targeting signal
RCDP	rhizomelic chondrodysplasia punctata
RING	really interesting new gene
TrkB	tropomyosin-related kinase B
vGlut2	vesicular glutamate transporter 2
VLCFA	very-long-chain fatty acid
VZ	ventricular zone
X-ALD	X-linked adrenoleukodystrophy
ZS	Zellweger syndrome
ZSD	Zellweger spectrum disorder

10.1 Introduction

The peroxisome, a single membrane-bounded subcellular organelle, serves as a platform for various catabolic and anabolic reactions, including β-oxidation of very-long-chain fatty acids (VLCFAs), degradation of hydrogen peroxide, and ether-phospholipid biogenesis [1]. The physiological consequence of peroxisomal functions is highlighted by the pathogenesis of peroxisome biogenesis disorders (PBDs), autosomal recessive diseases manifesting as progressive disorders of the central nervous system (CNS) and various organs [2, 3]. PBDs, including Zellweger spectrum disorders (ZSDs), rhizomelic chondrodysplasia punctata type 1 (RCDP1) [4–6], and RCDP5 [7], are caused by mutations of *PEX* genes encoding peroxins, Pexs, required for peroxisome assembly [8–10]. The primary defects of RCDP1 and RCDP5 are the loss of *PEX7* and the long isoform of *PEX5*, respectively, whereas mutations in any of the other *PEX* genes give rise to the ZSD.

ZSDs, counting about 80% of the PBD patients [2], are classified into three groups according to their clinical severity: Zellweger syndrome (ZS or the cerebro-hepato-renal syndrome), neonatal adrenoleukodystrophy (NALD), and infantile Refsum disease (IRD) [3]. Patients with ZS, the most severe ZSDs, are characterized by craniofacial abnormalities, eye abnormalities, neuronal migration defects, hepatomegaly, and chondrodysplasia punctata. Affected children present profound hypotonia, seizures, and inability to feed and generally die before reaching the age of 1 year old. The CNS pathological features of patients with ZS include disturbance of neuronal layer structure in cerebral cortex, heterotopia of Purkinje cells in cerebellum, and dysplastic alterations of inferior olivary nuclei [3, 11–13]. The biochemical abnormalities, including marked reduction of plasmalogens, accumulation of VLCFAs, and reduction in the level of docosahexaenoic acid [2], are thought to be relevant to manifestation of malformations in the CNS. However, pathogenic mechanisms of PBDs are largely unknown.

Molecular mechanisms of peroxisome biogenesis have been unveiled by functional analyses of Pex proteins. The proteins destined for peroxisome matrix harbor two types of peroxisomal targeting signals (PTSs), PTS type 1 (PTS1) at C-terminal tripeptide motif [14–16] or PTS type 2 (PTS2) at N-terminal cleavable presequence (R/K)(L/V/I)X_5(H/Q)(L/A) [17–19]. Newly synthesized PTS1- and PTS2-containg proteins are recognized by Pex5 [20–22] and Pex7 [4, 23–25], respectively, and then transported into peroxisomal matrix through the interaction with Pex13–Pex14 docking complex [26–30]. After transit to the RING (really interesting new gene) complex consisting of Pex2, Pex10, and Pex12 [31–33], Pex5 is exported from peroxisomes by AAA ATPase Pex1 and Pex6 [34] as well as Pex1–Pex6 recruiter, Pex26 [35]. The defect of any of these peroxins impairs peroxisomal matrix protein import. Pex3, Pex16, and Pex19 mediate the targeting of peroxisomal membrane proteins [36–39]. The defect of Pex3, Pex16, or Pex19 leads to the absence of peroxisomal membrane.

To study the pathogenesis of PBDs, a number of mice with generalized or tissue-specific inactivation of the *Pex* genes have been established. Here, we summarize the phenotypes in the PBD model mice, mainly focusing on neurodegenerative pathologies and pathogenic mechanisms of PBDs.

10.2 Mouse Deficient in PTS1 and PTS2 Peroxisomal Protein Import: A ZS Model Mouse

10.2.1 *Pex* Knockout Mice

The deletion of individual *Pex* genes causes the deficiency of peroxisomal matrix protein import and abnormal morphology of the CNS [40, 42, 46]. The mice with inactivation of *Pex2* [42], *Pex5* [40], *Pex10* [47], *Pex13* [46], and *Pex14* [48] have been generated (Table 10.1). The characteristics of *Pex* knockout (KO) mice are summarized below.

10.2.1.1 *Pex5*$^{-/-}$ Mouse

Pex5 (*Pxr1*)-deficient, *Pex5*$^{-/-}$, mouse as a ZS model mouse was reported by Baes and colleagues [40]. Pex5 is a cytosolic receptor for PTS1 proteins and Pex7-PTS2 protein complexes. Cargo-loaded Pex5 targets the peroxisome membrane via the docking complex comprising Pex13 and Pex14 [9]. Peroxisomal matrix protein import was deficient in the *Pex5*$^{-/-}$ mouse, and thus only peroxisomal remnants, "ghost," were detectable. The *Pex5* mutant mouse showed biochemical abnormalities such as the marked reduction of ether-phospholipids in the liver and brain and accumulation of VLCFA in the liver and plasma.

10.2.1.2 *Pex2*$^{-/-}$ Mouse

Pex2 is a RING domain-containing 35-kDa peroxisomal integral membrane protein [31, 59]. RING-peroxin complexes including Pex2, Pex10, and Pex12 are required at a step (s) downstream of Pex5 docking to Pex14, most likely during the translocation of matrix proteins across the membrane [32, 33]. The mouse deficient in *Pex2* gene, *Pex2*$^{-/-}$ mouse, was generated by elimination of exon 5 that contained the translation initiation site and entire coding sequence for the gene [42]. Peroxisomal matrix protein import was completely defective in the fibroblasts derived from *Pex2*$^{-/-}$ mouse. VLCFA was accumulated in plasma, and erythrocytic plasmalogens were barely detectable in the mutant mouse.

10.2.1.3 *Pex13*$^{-/-}$ Mouse

Pex13 and Pex14 comprise a docking complex, serving as the target of Pex5-cargo complex on peroxisomal membrane [9]. Pex13 is suggested to be involved in the import of folded and oligomeric proteins such as catalase [60]. Maxwell and colleagues generated a targeted mouse with a *loxP*-modified *Pex13* gene to enable conditional Cre recombinase-mediated inactivation of *Pex13* [46]. By crossing with transgenic mice that express Cre recombinase in all cells, mice with ubiquitous disruption of *Pex13*, *Pex13*$^{-/-}$ mouse, were generated [46]. The *Pex13*$^{-/-}$ mouse showed the defect of peroxisomal biogenesis, including the impairment of peroxisome matrix protein import, VLCFA accumulation, and the reduction of plasmalogens.

10.2.1.4 *Pex10*$^{CY/CY}$ Mouse

A mouse deficient in *Pex10* was generated by a forward genetic screen of ethylnitrosourea-mutated mutagenesis [61] as a progressive loss of limb movement from embryonic day 17.5 (E17.5) to birth [47]. The mutant mouse possesses C294Y mutation in the second zinc-finger motif of RING domain of Pex10, thus termed *Pex10*$^{CY/CY}$ mouse. *Pex10*$^{CY/CY}$ mouse embryonic fibroblasts (MEFs) showed the defect of peroxisomal matrix protein transport [47].

10.2.1.5 *Pex14*$^{\Delta C/\Delta C}$ Mouse

Pex14 is the target of Pex5-cargo complex on the peroxisomal membrane. *Pex14*$^{\Delta C/\Delta C}$ mouse expresses C-terminal region-deleted Pex14 and shows a mild defect of peroxisomal matrix protein import [48]. The mutation in *Pex14*$^{\Delta C/\Delta C}$ mouse resembled that in the patient with ZS lacking C-terminal region of Pex14

Table 10.1 Mouse models of peroxisome biogenesis disorders

			Macroscopic phenotypes		Neurodegenerative phenotypes			
Disrupted gene	Mutation	Corresponding human disease	Life span	Others	Cortex	Cerebellum	Others	References
Pex5	Exons 7–10 deletion	ZS	~3 d	Growth retardation Hypotonia	Neuronal migration defect		Abnormal segmentation of the inferior olivary nuclei	[40, 41]
Pex2	Exon 5 deletion	ZS	~12 h (C57BL/6J) ~18 d (SW/129)	Hypotonia Growth delay Spasticity Microcephaly	Neuronal migration defect	Abnormal foliation Migration defect of granule cells Abnormal dendrites of Purkinje cells	Lipid accumulation in the inferior olivary nuclei	[42–45]
Pex13	Exon 2 deletion (*loxP* flanked)	ZS	~12 h	Hypotonia Hypoactivity Growth retardation	Neuronal migration defect			[46]
Pex10	c.881G > A (p.C294Y)	ZS	~Few h, <2% of pups survive over 6 w	Locomotor defect Ataxia			Defect of axon bundling by Schwann cells Defect of the hind limb synapse	[47]
Pex14	Exons 6–8 deletion p.R129Kfs*36	ZS	~1 d (C57BL/6J) ~14 d (BL/ICR)	Growth retardation	Neuronal migration defect	Abnormal foliation Migration defect of granule cells Abnormal dendrites of Purkinje cells		[48]
Pex7	Exon 3 deletion	RCDP1	~0.5 d (50%) ~Weaning (20%) >18 mo (30%)	Dwarfism Hypotonia Eye cataracts Ossification defect	Neuronal migration defect	Astrogliosis Demyelination (DKO)* Microgliosis (DKO)*	Myelination defect at PNS Degeneration of myelinated axon in PNS	[49–51]
Pex11α	Exons 2–3 deletion	–	Normal	Higher body weight	Normal	Normal	Enlargement of the lateral ventricle Microgliosis	[52–54]

Pex11β	Exons 1–3 deletion	Mild ZSD	~1 d	Hypotonia	Neuronal migration defect Oxidative stress Catalase reduction	Oxidative stress	Enhancement of neuronal apoptosis	[55, 56]
Pex1	c.2531G > A (p.G844D)	Mild ZSD	~20 d (80%) >50 d (20%)	Growth retardation				[57, 58]

*: *Pex7/Abcd1* double knockout mouse
–: No distinct phenotype

[62]. Plasmalogen biosynthesis and β-oxidation of VLCFA were partially defective [48].

10.2.2 Macroscopic Phenotype of ZS Model Mouse

The patients with ZS, most severe PBDs, show craniofacial abnormalities, severe hypotonia, and generally die before reaching the age of 1 year old [3].

The ZS model mice of C57BL/6 strain show intra-utero growth retardation without embryonic lethality [40, 42, 46, 48]. However, the body weight of mutant mice at birth was apparently lower than that of control mice. A majority of ZS model mice hardly feed and die several hours after birth. The pups rarely showed suckling movement and survived up to 1 or 2 days after birth. The mutant mouse maintained a contracted "C" posture that appeared to particularly affect the hind limbs, indicating hypotonia. However, ZS model mouse manifested no obvious dysmorphia such as craniofacial abnormality seen in the patients with ZS. The aberrant bone formation was observed in the calvarium of the ZS mutant mice showing a delay in membranous ossification [42]. The macroscopic phenotypes in ZS model mice appear to reflect those in the patients with ZS, including severe hypotonia and short life span.

10.2.3 Neurodegenerative Phenotype of ZS Model Mouse

10.2.3.1 Neuronal Migration Defect in Cerebral Cortex

During cerebral development, pyramidal glutamatergic neurons are born sequentially in the ventricular zone (VZ) and migrate toward the pial surface [63]. In this process, late-born neurons pass early-born neurons and form a neuronal layer on top of the older layer in an inside-out manner. Finally, a six-layered laminar structure is formed in the cortex. In the patient with ZSDs, the laminar structure of the cerebral cortex is severely disturbed [11, 12], and microgyric and pachygyric phenotypes are observed [11, 13, 64].

ZS model mice also showed abnormal cortical plate with apparent reduced thickness of the neocortical plate [40, 46]. Distribution of cells within the developing cortical plate was altered with an increased cellular density in intermediate zone (IZ) (Fig. 10.1a and b) [40, 42, 48]. At the cortical layer region, an immature appearance of neurons with enlarged nuclei and round cytoplasm emerged, and the boundary between layers IV and V was obscured [40, 42, 48]. This was most likely due to the migration delay of cortical neurons during embryonic development (E13.5–18.5), as shown by the BrdU pulse-chase experiments. Therefore, the disturbance of cortical laminar structure was the common phenotype between ZS model mice and the patients with ZS.

10.2.3.2 Abnormal Cerebellar Development in ZS Model Mice

Abnormal cerebellar development in the patients with ZSDs is characteristic phenotype, including dysmorphology of the Purkinje cell arborization, heterotopia of Purkinje cells in the white matter, and granule cell clustering between the Purkinje cells [11–13, 65, 66]. Cerebellar development requires integration of afferent-target interactions between multiple neuronal populations and migratory patterns established by neuron-glial interactions.

The formation and circuitry of a mouse cerebellum develop by a complicated process during a couple of postnatal weeks. Therefore, the short life span of *Pex*-KO mice on C57BL/6 background [42, 46–48] made it difficult to analyze the cerebellar development. Intriguingly, it was reported that replacement of the *Pex2*-null allele in a C57BL/6 × 129 Sv background to Swiss Webster × 129SvEv genetic background prolonged life span [43, 44]. This life span elongation was also reported in $Pex14^{\Delta C/\Delta C}$ mouse, where C57BL/6 background mice were mated with ICR Swiss background, $Pex14^{\Delta C/\Delta C}$ BL/ICR mouse [48]. Both Sw/129 $Pex2^{-/-}$ and $Pex14^{\Delta C/\Delta C}$ BL/ICR mice showed significant postnatal survival, with approximately 20–30%

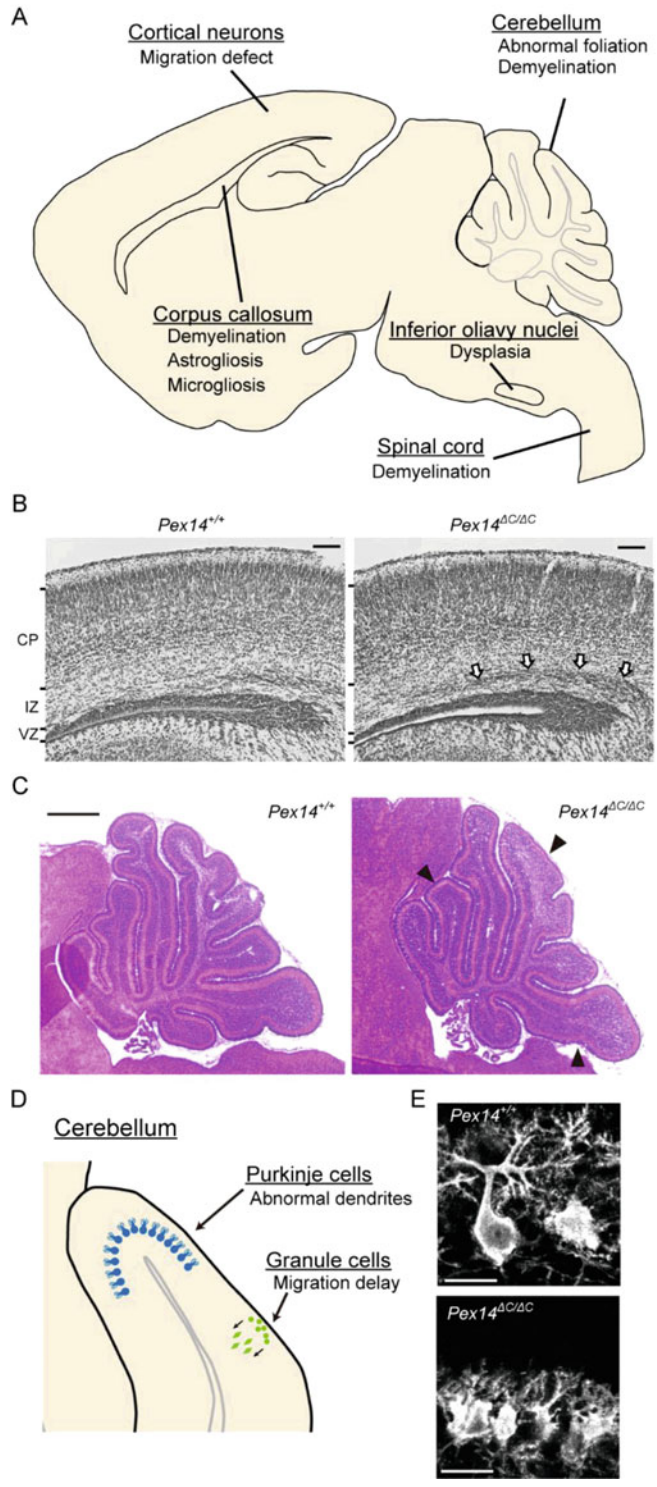

Fig. 10.1 Neurodegenerative pathologies in ZS model mouse. (**a**) Distinct neurodegenerative phenotypes are indicated in the respective brain regions of ZS model mouse. (**b**) Cresyl violet staining of a coronal section of the cortex at P0.5 from wild-type ($Pex14^{+/+}$) and ZS model ($Pex14^{\Delta C/\Delta C}$) mice is shown. The open arrows indicate accumulated neurons in the intermediate zone (IZ). CP, cortical plate; VZ, ventricular zone. Scale bar, 100μm. (**c**)

of mice surviving for 7–10 days, and a lower rate of survival to postnatal day 12–13. The mutant mice are severely delayed in growth. Such prolongation of life span enabled the assessment of postnatal development in *Pex*-KO mice.

The size of cerebellum in *Pex*-KO mice was apparently smaller than that in wild-type mice (Fig. 10.1c) [43, 44, 48]. The folial development was delayed, and folial pattern within the vermis is markedly altered (Fig. 10.1c, arrowheads). The layer structure of the cerebellum was also impaired, i.e., the external granule layer (EGL) was thicker, and the molecular layer (ML) and internal granule layer (IGL) were thinner [44]. Generally, granule cells proliferate in the EGL and then start migration to the IGL across ML along radial glia. BrdU incorporation analysis revealed that granule cell migration was delayed, resulting in the thick EGL and thin IGL [44].

Purkinje cells in the cerebellum develop their highly branched dendrites during a couple of postnatal weeks. In the cerebellum of the patients with ZS, Purkinje cells show abnormal arborization and heterotopia in the white matter [11–13, 65, 66]. At postnatal day 0 (P0), a majority of Purkinje cells in mutant mice reached a normal position, while a slight delay was observed in the migration and differentiation of Purkinje cells [42]. These findings suggested that the migrational defect in peroxisome-deficient mouse Purkinje cells was much less severe than that in the patients with ZS. After the positioning of Purkinje cells, they begin to develop their dendrite [67]. The overall size and degree of branching of the Purkinje cell dendrites were markedly reduced in mutant mice, *Pex2*$^{-/-}$ [44] and *Pex14*$^{\Delta C/\Delta C}$ mice [48] (Fig. 10.1d and e). Multiple dendritic processes emanated at random angles from the Purkinje cell soma of *Pex*-KO mouse. Purkinje cell somas and proximal dendrites were studded with the spines in the mutants.

From the inferior olivary nuclei in the brain stem, climbing fiber projects toward the cerebellum and forms vesicular glutamate transporter 2 (vGlut2)-positive inhibitory synapse at Purkinje cell [68, 69]. At P3, climbing fiber reaches at IGL region of the cerebellum and forms vGlut2-positive synapse at Purkinje cell body. In wild-type mouse, the synapse shifts to the dendritic compartment of Purkinje cells together with synaptic pruning [70, 71]. In *Pex*-KO mice, the number of vGlut2-positive around Purkinje cells was reduced and remained on the IGL side of cell soma at P7 [48]. Climbing fibers reached to IGL region of the cerebellum [44], suggesting that synapse formation and synapse pruning were impaired in the mutant mouse.

Taken together, the cerebellum of ZS model mice represented various morphological abnormalities, such as cerebellar atrophy, granule cell migration defect, malformation of Purkinje cells, and impairment of climbing fiber-Purkinje cell synapse formation. These phenotypes resemble those in the cerebellum of patients with ZS. However, heterotopia of Purkinje cells as observed in the patients [11–13] was barely detectable in the mutant mouse [42].

10.2.3.3 Dysplasia of Inferior Olivary Nuclei

In the olivary nucleus of the patients with ZS, dysplastic changes are seen with an abnormal serpiginous course, laminar discontinuities, and condensation of neurons around the periphery of the nuclear islands [11, 13, 72]. The principal olivary nucleus in rodents is a simple u-shaped structure devoid of the convolutions seen in humans. Malformation of inferior olivary nuclei was reported only in the *Pex5*$^{-/-}$ mouse, where a grossly malformed olivary complex lacked its typical segmentation [40]. This phenotype was inconsistent with that in *Pex2*$^{-/-}$ and *Pex14*$^{\Delta C/}$

Fig. 10.1 (continued) Hematoxylin and eosin staining of the sagittal sections of the cerebellum at P7 is shown. Arrowheads indicate the shallow cerebellar folia in the cerebellum of the *Pex14*$^{\Delta C/\Delta C}$ mouse. Scale bar, 500μm. (**d**) Schematic view of cerebellar cells illustrating developmental impairments in ZS model mouse. (**e**) Abnormal dendritic development of Purkinje cells in the cerebellum of *Pex14*$^{\Delta C/\Delta C}$ mouse at P7 is shown. Sagittal sections of the cerebellum were labeled with an antibody to calbindin-D28k. Scale bar, 10μm. Figures b, c, and e are adopted and modified from [48]

$^{\Delta C}$ mice, where no obvious defects in gross morphology of the inferior olivary nuclei were observed [42, 44, 48]. Lipid inclusions were accumulated in the neural cytoplasm and scattered in the neuropil throughout the inferior olivary nuclei of *Pex2*$^{-/-}$ mouse [43]. Although a discrepancy remains in dysmorphogenesis of the inferior olivary nuclei, the severity appears much lower than that observed in the patients with ZS.

10.2.3.4 Elevation of Neuronal Cell Death

In *Pex*-KO mice, cells undergoing apoptotic cell death were elevated in cerebral cortex [40, 41] and EGL region of the cerebellum [44] as revealed by staining with TUNEL or anti-cleaved caspase-3 antibody. In the cell culture, cell proliferation is not affected by the deficiency of peroxisome biogenesis. Therefore, the peroxisome biogenesis is most likely involved in the cell survival in vivo.

10.2.3.5 Dysmyelination in CNS of *Pex*-KO Mice

In the patients with ZS, less stainable myelin is shown to be present throughout the brain and spinal cord [11, 12]. However, the morphological defect of myelin in *Pex*-KO mice is not reported. This may be owing to the short life span of the mutant mice, i.e., the majority of *Pex*-KO mice die before the development of myelination. The dysmyelination was implicated by the decrease in expressions of myelin basic protein (MBP) and proteolipid protein 1 in the cerebellum at P7 of *Pex14*$^{\Delta C/\Delta C}$ mouse [73]. The decrease of myelin in the cerebellum was apparent in CNS-specific *Pex5*$^{-/-}$ mouse at 2 weeks, and then further diminished in 3- and 6-week-old mutant mouse (see Sect. 10.6.1.1) [74, 75]. There seems to be both developmental and stability problems for cerebellar myelin in peroxisome-deficient mouse.

10.2.3.6 Neurological Defects in the Peripheral Nervous System

Pex10$^{CY/CY}$ mouse was obtained by ethylnitrosourea-mutated mutagenesis as a progressive loss of embryonic limb movement [47]. In the peripheral nervous systems (PNS), *Pex10* $^{CY/CY}$ mutants displayed prenatal pathology including defects in axonal integrity, decreased number of Schwann cells, and defective neuromuscular interface, thereby suggesting that peroxisome biogenesis is essential for the development of PNS.

10.2.4 Involvement of Hepatic Peroxisomes in CNS Development

10.2.4.1 Tissue-Selective Reconstitution of Peroxisomes in *Pex5*$^{-/-}$ Mice

To investigate whether peroxisomal dysfunction in a tissue affects the morphogenesis of other tissues, tissue-selective overexpression of Pex5 in *Pex5*$^{-/-}$ mouse was introduced [41]. To reconstitute peroxisomes in brain-specific *BR:Pex5*$^{-/-}$ mouse and liver-specific *LR:Pex5*$^{-/-}$ mouse, the *nestin-TK-mycPex5* transgene and *α-fetoprotein* (*AFP*)-*mycPex5* transgene were used, respectively. Double-rescued mice, termed *LBR:Pex5*$^{-/-}$ mice, were also generated. These mice showed the partial reconstitutions of peroxisome formation in respective target tissue. Interestingly, in both *BR:Pex5*$^{-/-}$ and *LR:Pex5*$^{-/-}$ mice, significant improvement of the neuronal migration process was observed. In double-rescued *LBR:Pex5*$^{-/-}$ mice, neuronal migration appeared to be restored. These results suggested that normal cortical development depends on functional peroxisomes in other organs, especially in the liver.

10.2.4.2 Treatment with Bile Acid Improves Morphology of Purkinje Cells in *Pex2*$^{-/-}$ Mouse

Peroxisomal β-oxidation in the liver is essential for the biosynthesis of bile acid (BA) that is important for the digestion and absorption of lipid nutrients [76]. In PBDs, BA intermediates, dihydroxycholestanoic acid (DHCA) and trihydroxycholestanoic acid (THCA), are accumulated [1, 2]. Because of the absence of BA biosynthesis, *Pex*-KO mice appear to suffer from malnutrition caused by the lipid

malabsorption in the intestine. Oral administration of BA to *Pex2*$^{-/-}$ mice prolonged postnatal survival and moderated intestinal malabsorption, while the degree of hepatic steatosis was exacerbated [77]. Interestingly, in the cerebellum of BA-treated *Pex2*$^{-/-}$ mice, arborization of Purkinje cells was partly improved as compared with the untreated mutant mouse [45]. Taken together, malabsorption in *Pex*-KO mice is likely involved in the malformation of Purkinje cells.

10.2.5 Summary of ZS Model Mouse Phenotypes

In the ZS model mice, *Pex5*$^{-/-}$, *Pex2*$^{-/-}$, *Pex13*$^{-/-}$, *Pex10*$^{CY/CY}$, and *Pex14*$^{\Delta C/\Delta C}$ mice, neurodegenerative phenotypes are largely in good agreement with those in the patients with ZS, such as hypotonia, ataxia, migrational defects of cortical neurons and cerebellar granule neurons, abnormal dendrites of Purkinje cells, and dysmyelination. However, dysplasia of the inferior olivary nuclei and defect of Purkinje cell migration in *Pex*-KO mice are milder than those observed in the patients with ZS.

As demonstrated by tissue-specific reconstitution of peroxisomes and BA administration experiment in *Pex*-KO mouse, hepatic peroxisome functions are essential, but not sufficient, for the CNS development. For the elucidation of the molecular mechanisms underlying neurodegeneration of PBDs, inactivation of *Pex* gene could not exclude the effects of peroxisome dysfunction in other tissues. Alternatively, Cre-*loxP*-mediated conditional *Pex* knockout system has been used to unravel the pathological mechanism of PBDs, as described in the Sect. 6.

10.3 Mouse Defective in PTS2 Protein Import: RCDP1 Model *Pex7*$^{-/-}$ Mouse (*Pex7*$^{-/-}$)

RCDP is an autosomal recessive peroxisomal disorder. The patients with RCDP represent the stippled foci of calcification within hyaline cartilage, dwarfism, congenital cataracts, hypotonia, and severe psychomotor retardation [78, 79]. The biochemical abnormalities in the patients with RCDP represent a marked reduction of ether-phospholipids. To date, five distinct genotypes of RCDP have been identified [64, 80]. Genes responsible for RCDP1 and RCDP5 encode cytosolic PTS2 receptors, Pex7 [4–6] and the long isoform of Pex5, Pex5L [7], respectively. Since the defect of Pex7 or Pex5L leads to the impairment of import of several peroxisomal matrix proteins harboring PTS2, RCDP1 and RCDP5 are classified as PBDs [64, 81, 82]. The remaining RCDPs, including RCDP types 2, 3, and 4, are single-enzyme deficiency disorders caused by the defect of the enzymes involved in biosynthesis of ether-phospholipids, i.e., dihydroxyacetone phosphate acyltransferase (DHAPAT) [83], alkyl-dihydroxyacetone phosphate synthase (ADAPS) [84], and fatty acyl-CoA reductase 1 (FAR1) [85]. We summarize the neurodegenerative phenotypes of RCDP1 model mouse, *Pex7*$^{-/-}$ mouse (Table 10.1).

Pex7 transports PTS2 proteins to peroxisomes, including ADAPS, phytanoyl-CoA hydroxylase (PHAX), and 3-oxoacyl-CoA thiolase (thiolase). Owing to the failed import of ADAPS, the enzyme involved in ether-phospholipids biosynthesis [86], and PHAX catalyzing phytanic acid α-oxidation [87, 88], patients with RCDP1 manifest marked reduction of ether-phospholipids and age- and diet-related accumulation of phytanic acid [89]. Although thiolase involved in peroxisomal β-oxidation is not localized in peroxisomes, VLCFA is not accumulated in the plasma of the patients with RCDP1 [78, 90, 91]. Since sterol carrier protein x (SCPx), a PTS1 protein, is most likely substituted for the thiolase activity [92], peroxisomal β-oxidation is normal in the patients with RCDP1.

Pex7$^{-/-}$ mice were born alive, exhibited a variable degree of dwarfism and hypotonia with decreased motility, hampering them feeding [49]. Approximately 50% of the homozygous mutant pups died on P0.5, 20% before weaning, and the remaining *Pex7*$^{-/-}$ mice survived beyond 18 months. In the *Pex7*$^{-/-}$ mouse cortex, increased density of neurons in the IZ was

observed, while migration abnormality appeared to be less severe than that observed in *Pex5*$^{-/-}$ mice [40, 49]. Neonatal *Pex7*$^{-/-}$ mouse showed not only depletion of plasmalogens but also VLCFA accumulation in the brain. However, VLCFA levels became normal in 2-month-old *Pex7*$^{-/-}$ mouse. These suggested that thiolase is predominant in peroxisomal β-oxidation pathway in a neonate and mislocalization of thiolase in neonatal *Pex7*$^{-/-}$ mouse leads to the defect of peroxisomal β-oxidation. In adulthood, upregulation of SCPx may contribute to the decreased level of VLCFA in the brain. The mutant mouse also showed a defect in ossification of distal bone elements of the limbs as well as parts of the skull and vertebrae. The demyelination in the PNS was also observed in *Pex7*$^{-/-}$ mouse, where Schwann cell-mediated myelination of axon was suppressed together with the depletion of MBP [50].

Abcd1, ATP-binding cassette family D1, functions as a transporter of straight-chain VLCFA-CoA on the peroxisomal membrane; thus deletion of Abcd1 results in the VLCFA accumulation [93, 94]. The patients with X-linked adrenoleukodystrophy (X-ALD) suffer from demyelination in the white matter [95, 96]. To generate *Pex7*:*Acbd1* double KO (DKO) mouse, *Pex7*$^{-/-}$ mice were mated with *Abcd1*-KO mouse, X-ALD model mouse [51, 97–99]. *Pex7*:*Acbd1* DKO mouse showed the accumulation of VLCFA and depletion of plasmalogens in the brain [49]. Morphological abnormalities in DKO mice such as astrogliosis, microgliosis, and demyelination were more evident than *Pex7*- or *Acbd1*-single KO mice. Therefore, the marked depletion of plasmalogens and accumulation of VLCFA coordinately aggravated the pathological phenotype of ZSDs.

10.4 Mice Deficient in *Pex11* Family

After the import of various newly synthesized proteins from the cytosol, the proliferation of peroxisomes takes place, followed by division [100]. Peroxisome division is proposed to involve three stages: elongation, constriction, and fission [101–103]. Pex11 is a peroxisome-specific division factor that is present in cells as three isoforms, Pex11α [52, 104], Pex11β [55, 103, 105], and Pex11γ [52, 106]. To date, the mice with inactivation of *Pex11α* or *Pex11β* have been generated (Table 10.1) [52, 53, 55].

10.4.1 *Pex11α*$^{-/-}$ Mouse

Pex11α is abundantly expressed in the liver and inducible by the treatment with a hypolipidemic agent, fibrate, in rodents [52]. Fibrate binds to peroxisome proliferator-activated receptor (PPAR) α and upregulates the Pex11α expression, followed by proliferation of peroxisomes and increase in peroxisome number in the liver [103, 104].

Pex11α$^{-/-}$ mice were generated independently by two groups and displayed distinct phenotypes [52, 53]. Li et al. [52] showed neither macroscopic phenotype nor obvious defect in division of constitutive peroxisomes in *Pex11α*$^{-/-}$ mice. On the other hand, Weng et al. [53] reported that the number and size of peroxisomes were lower in the livers of *Pex11α*$^{-/-}$ mice than in those of wild-type mice. Neurodegenerative phenotypes in *Pex11α*$^{-/-}$ were shown as an enlargement of the lateral ventricles and microgliosis characterized by F4/80 staining [54]. It is not clear why *Pex11α*$^{-/-}$ mice generated by two different groups show different phenotypes.

10.4.2 *Pex11β*$^{-/-}$ Mouse

Pex11β is ubiquitously expressed and engaged in the elongation of peroxisome membrane [103, 104]. Homo-oligomerization of Pex11β induces the elongated and constricted morphology of peroxisomes [107–110]. Dynamin-like protein 1 (DLP1) is recruited to the constricted peroxisomes by mitochondrial fission factor (Mff) and then polymerizes to cleave the membrane using the energy of GTP hydrolysis [108, 109]. Three reports have been published for the patients with homozygous or heterozygous nonsense mutations in *PEX11β* [111–113]. The patients with *PEX11β* mutation manifested as

cataract, hypotonia, polyneuropathy, and mild intellectual disability [111].

The mice lacking Pex11β died shortly after birth on day 1 and showed hypotonic and poor suckling [55]. *Pex11β*$^{-/-}$ mouse showed the neuronal migration defect [55] and the elevation of apoptotic cells in the cerebral cortex and cerebellum [56]. These phenotypes resembled the unique characteristics of ZS such as impaired laminar structure of the cortex and elevation of TUNEL-positive neuronal cells [40, 42, 46, 48]. In addition to ZS-like phenotypes, defects of synaptogenesis and induction of oxidative stress were observed in the *Pex11β*$^{-/-}$ mouse brain [56]. *Pex11β*$^{-/-}$ mouse showed metabolic abnormalities such as slight elevation of VLCFA in the liver and subtle reduction of plasmalogens in the brain, while the mutant MEFs with elongated and enlarged peroxisome membrane showed normal peroxisomal matrix protein import [55]. In the *Pex11β*$^{-/-}$ mouse, *Pex11γ* mRNA was elevated [56], thereby pointing to a compensatory function of Pex11γ in the absence of Pex11β [114]. Interestingly, cell-cultured fibroblasts from the patient with *PEX11β*$^{-/-}$ nonsense mutation at an elevated temperature at 40°C decreased *Pex11γ* mRNA and impaired the catalase import to peroxisomes [111], suggesting that the depletion of both Pex11β and Pex11γ impaired peroxisome matrix imports. Since the expression of Pex11γ is not ubiquitous [52], it is likely that mild alternations of peroxisome metabolites in *Pex11β*$^{-/-}$ mouse reflect the impairment of peroxisome matrix protein import in the cells with lower expression of Pex11γ. This partially impaired peroxisomes may give rise to the ZS-like phenotype of *Pex11β*$^{-/-}$ mouse [55], similar to *Pex14*$^{\Delta C/\Delta C}$ mouse, in which peroxisomal matrix protein import is partially defective [48].

10.5 Mild ZSD Model Mouse: Pex1-G844D Mutation

Mutations in *PEX1* are the most common cause, accounting for approximately 60–70% of known ZSD patients. The one-point mutation, *PEX1*-c.2528G > A (p.Gly843Asp or G843D), represents around 30% of all ZSD alleles [115–117]. *PEX1*-c.2528G > A encodes an unstable Pex1 mutant protein that has residual activity and is associated with a milder clinical phenotype without developmental defects, rather a progressive disorder due to ongoing impairment of peroxisomes [118, 119]. In addition, heterozygosity for Pex1-G843D results in an intermediate phenotype, and homozygosity for this allele is associated with a mildest phenotype of ZSD [120, 121]. The mouse equivalent model of ZSD caused by human Pex1-G843D allele reflects the hypomorphic *PEX* alleles that are commonly found in ZSD patients on the intermediate and milder end of the Zellweger spectrum. Mice homozygous for the Pex1-G844D allele, which is the murine ortholog of the human Pex1-G843D mutation, represented growth retardation and fatty livers with cholestasis [57]. The homozygous G844D mutation in mice allowed survival into adulthood, whereas the first 20 postnatal days were critical for survival with a 20% survival rate (Table 10.1). This is in contrast to other *Pex*-inactivated mice, generated by deleting different *Pex* genes, including *Pex2* [42], *Pex5* [40], *Pex13* [46], and *Pex14* [48], which represented the severe end of the ZSD spectrum and showed severe early-lethal phenotypes. Lethality of Pex1-G844D mice was likely due to a combination of cholestatic liver problems, probably as a consequence of a defective BA biosynthesis [58]. Surviving mice showed similar hepatic problems as reported for mild ZSD patients, including hepatomegaly, bile duct proliferation, liver fibrosis, and mitochondrial alterations [58]. Moreover, Pex1-G844D mice developed a retinopathy similar to that observed in human patients, associated with a loss of cone photoreceptors [57]. In particular, the liver and retina were affected in the Pex1-G844D mouse, whereas other tissues were relatively spared. The treatment of fibroblasts from the patients with Pex1-G843D and Pex1-G844D MEF with chemical chaperones resulted in the recovery of peroxisomal matrix protein import [57, 122]. Therefore, the treatment with chemical chaperones can be a potential candidate for the

therapy for the patients with Pex1-G843D mutation.

10.6 Conditional *Pex*-KO Mice

10.6.1 Tissue-Specific Inactivation of *Pex* Gene in Mice

The mice each with inactivated respective *Pex* genes in the whole body show a short life span. A majority of the mutant mice die within several days, and a small number of the pups survive over a week, which result in the difficulty in pathological analysis of ZS model mice. It is also possible that any combined effects of dysfunctions in multiple organs, such as the brain, liver, and kidney, cause the severe phenotype of ZS model mice. For the generation of mice with tissue-specific or cell type-specific KO of *Pex* genes, *Pex5* [123] and *Pex13* [124] genes floxed by *loxP* sites, have been established.

10.6.1.1 CNS-Specific *Pex*-KO Mouse (*Nes-Pex5*$^{-/-}$ and *Pex13* BM)

The mice with CNS-specific dysfunction of peroxisomes were generated by mating floxed mice, *Pex5*$^{flox/flox}$ and *Pex13*$^{flox/flox}$, with the mice carrying *Nestin-Cre* transgene, termed *Nes-Pex5*$^{-/-}$ and *Pex13* brain mutant (BM) mice, respectively [125, 126]. The mutation of *Pex* genes in the CNS resulted in the dysfunction of peroxisomes in neurons, oligodendrocytes, and astrocytes, giving rise to abnormal development and aberrant brain morphology (Table 10.2) [125, 126], as observed in *Pex*-null mice [40, 42, 44, 46].

The majority of CNS-specific *Pex*-KO mice exhibited growth retardation during the pre-weaning period and died by approximately 5–6 weeks of age, with a peak in the postweaning period [74, 126]. Behavior assessments of the mutant mice revealed neuromotor defects such as ataxia and dyskinesia, suggesting the impairment of the cerebellum. Learning and memory impairments and the defect of exploratory transitions were also indicated [74, 126].

During embryonic development, *Nes-Pex5*$^{-/-}$ mouse showed the migration delay of cortical neurons as assessed by BrdU incorporation assay, while less severe than *Pex5*-null mouse [125]. At P5, accumulation of cortical neurons in IZ was not detected [125]. These milder phenotypes of the cerebral cortex in *Nes-Pex5*$^{-/-}$ mouse suggested that the neuronal migration is mediated by interorgan communication. Especially, the importance of hepatic peroxisome function was strongly suggested by the phenotypes in *LR:Pex5*$^{-/-}$ mouse [41] and liver-specific *Pex5*$^{-/-}$ mouse (see below section) [125]. *Pex13* BM mouse also showed the neuronal migration defect accompanied with impaired neurogenesis [127].

Both *Nes-Pex5*$^{-/-}$ and *Pex13* BM mice showed the defect of cerebellar development, while foliation pattern of cerebellum was slightly different [125, 126]. At P5 of *Nes-Pex5*$^{-/-}$ mouse, the cerebella were slightly reduced in size and had a moderate foliation defect, but, at 3 weeks, *Nes-Pex5*$^{-/-}$ mice formed regular foliation pattern of the cerebellum [125]. Taken together, *Nes-Pex5*$^{-/-}$ mouse showed a developmental delay of cerebellar foliation. On the other hand, *Pex13* BM mouse exhibited the persistent defect of cerebellar formation [126]. At P5, there was no difference in the cerebellar formation between control and mutant mice. But at P10, the lack or shallower fissures of the cerebellum in *Pex13* BM mouse emerged and persisted beyond P20. There was no obvious explanation for the discrepancy of the developmental impairment of the cerebellar foliation between *Nes-Pex5*$^{-/-}$ and *Pex13* BM mice. Excision efficiency of the target gene mediated by Cre recombinase expression driven by nestin promoter is a plausible explanation for this discrepancy. Similarly to the phenotypes in *Pex2*-null mouse [44], granule cell migration from EGL to IGL was impaired in both CNS-specific *Pex*-KO mice [125, 126]. Abnormal differentiation of Purkinje cells was common phenotype of *Pex*-null mice [44, 48] and CNS-specific *Pex*-KO mice. In agreement with the findings in *Pex2*$^{-/-}$ mice, no major heterotopias of Purkinje cells were observed in the cerebellum of either *Nes-Pex5*$^{-/-}$ or *Pex13* BM mice.

Table 10.2 Phenotypes of conditional Pex knockout mice

		Phenotypes				
Mouse genotype	Pex-depleted tissue or cell type	Cortex	Corpus callosum	Cerebellum	Others	References
Nes-Pex-5$^{-/-}$	CNS	Neuronal migration defect Demyelination Mild microgliosis Mild astrocytosis	Dysmyelination Axonal swelling Microgliosis Astrogliosis	Abnormal foliation Migration defect of granule cells Abnormal Purkinje cell dendrite Dysmyelination	Lipid accumulation in the spinal cord Axonal damage in hippocampus	[74, 75, 125]
Pex13 BM	CNS	Neuronal migration defect Defect of neurogenesis Astrocytosis Microgliosis		Abnormal foliation Migration defect of granule cells Abnormal Purkinje cell dendrite Dysmyelination	Decrease of serotonergic neurons	[126–128]
Alfp-Pex5$^{-/-}$	Liver	Neuronal migration defect		Abnormal foliation Migration defect of granule cells Abnormal Purkinje cell dendrite Dysmyelination	Life span: ~8 d (80%) ~16 d (20%) Rarely survive 1 mo	[125]
Cnp-Pex5$^{-/-}$	Oligodendrocyte	Normal	Demyelination Neuroinflammation	Normal	Hind limb ataxia	[129–131]
GFAP-Pex5$^{-/-}$	Astrocyte	Normal		Elevation of catalase Lipid accumulation		[130]
NEX-Pex5$^{-/-}$	Projection neuron	Normal	Normal	Normal		[130]
CMV-Tx-Pex5$^{-/-}$	Whole body (tamoxifen-inducible)		Microgliosis	Axonal impairment Demyelination Microgliosis	Ataxia Microgliosis in the brain stem	[75]

Myelin abnormalities were observed in the cerebellum and corpus callosum of *Nes-Pex5*$^{-/-}$ mouse [75]. Together with cerebellar demyelination, axonal damage and swelling emerged. Reactive gliosis describes the rapid response to neuronal injury of astrocytes and microglia [132]. Excess proliferation of astrocytes, astrogliosis, were observed at P20 in the cerebellum of *Pex13* BM mouse [126] and all the brain region of a 3-month-old *Nes-Pex5*$^{-/-}$ mouse [74]. Microglia abundance was increased in the cerebellum at P20 of *Pex13* BM mouse [126] and in all the brain regions of a *Nes-Pex5*$^{-/-}$ mouse but was most pronounced in the white matter [74]. Demyelination in the brain stem of the *Nes-Pex5*$^{-/-}$ mouse was accompanied by microgliosis and astrogliosis [75].

In the midbrain of the *Pex13* BM mouse, the expression level of *tph2* encoding the enzyme involved in the synthesis of serotonin [133] and TPH2-positive serotonergic cells were decreased [128]. Neuroinflammation including astrogliosis and microgliosis were also activated in the midbrain region. Serotonergic system is involved in the brain development and motor control. Dysfunction of this system would account in part for the dysmorphogenesis of *Pex13* BM mouse.

10.6.1.2 Liver-Specific *Pex5*$^{-/-}$ Mouse (*Alfp-Pex5*$^{-/-}$)

Liver-specific *Pex5*$^{-/-}$ mouse was generated by mating *Pex5*$^{flox/flox}$ mice [123] with the mice expressing Cre recombinase in hepatocytes under the control of albumin promoter and α-fetoprotein enhancers (*Alfp*) [134, 135], and termed *Alfp-Pex5*$^{-/-}$ mouse [125, 136]. Liver-specific reconstitution of peroxisomes in *Pex5*$^{-/-}$ mouse (*LR:Pex5*$^{-/-}$) induces the improvement of neuronal migration in the cerebral cortex [41], suggesting that the integrity of hepatic peroxisomes affects the neuronal development. *Alfp-Pex5*$^{-/-}$ mouse showed severe developmental delay and growth retardation, with hypotonia (Table 10.2) [125]. About 80% of *Alfp-Pex5*$^{-/-}$ pups died within the first 8 days, and the remainder died between P9 and P16. Interestingly, the defect of hepatic peroxisomes exacerbated morphogenesis of CNS as observed in *Pex*-null mice. Neuronal migration in the cerebral cortex was severely affected in *Alfp-Pex5*$^{-/-}$ mouse. The mutant mice showed the impairment of cerebellar development such as the defect of foliation, migration delay of granule cells, and malformation of Purkinje cells. Therefore, the functional peroxisomes in the liver are most likely essential for the development of the cerebral cortex and cerebellum.

10.6.2 Cell Type-Specific Inactivation of *Pex5* Gene

In CNS-specific *Pex*-KO mice, peroxisome biogenesis is impaired in the three types of neuronal cells, neurons, oligodendrocytes, and astrocytes. To investigate which types of peroxisome biogenesis-defective cells are responsible for the defect of CNS development in the patient with ZS, cell type-specific *Pex5*$^{-/-}$ mice were generated (Table 10.2) [129, 130].

10.6.2.1 Oligodendrocyte- and Schwann Cell-Selective *Pex5*$^{-/-}$ Mouse (*Cnp-Pex5*$^{-/-}$)

Oligodendrocytes and Schwann cells are involved in the formation of myelin for axonal insulation and rapid impulse propagation in CNS and PNS, respectively. The patients with ZS show the demyelination throughout the brain [11, 12]. To generate the oligodendrocyte- and Schwann cell-selective *Pex5*$^{-/-}$ mouse, the *Pex5*$^{flox/flox}$ mice were mated with mice expressing Cre recombinase in oligodendrocytes and Schwann cells under the control of 2′,3-′-cyclic nucleotide phosphodiesterase gene (*Cnp*) promoter [137, 138], termed *Cnp-Pex5*$^{-/-}$ mice [129]. Growth retardation and dysmyelination in CNS were not observed during *Cnp-Pex5*$^{-/-}$ mice development. Up to the age of 2 months, myelin sheaths were morphologically normal. At 3 months, hind limb ataxia emerged with demyelination at the corpus callosum of *Cnp-Pex5*$^{-/-}$ mice. Disease progression with extensive neurodegeneration was most obvious at 9 months, and finally the complete loss of myelinated axon was observed at the most severe clinical stage. No mutant mouse was alive past 13 months.

Astrogliosis and microgliosis were restricted in the white matter at the early stage of neurodegeneration and spread to all the brain area at latter stage. The expression of proinflammatory cytokines and invasion of immune cells into the lesioned brain areas were elevated in *Cnp-Pex5*$^{-/-}$ mice [129].

In PNS of *Cnp-Pex5*$^{-/-}$ mice, peroxisomal protein import was impaired in Schwann cells, whereas myelination was normal at the sciatic nerves [131, 139]. Peroxisomal dysfunction in Schwann cells gave rise to the accumulation of VLCFA-containing ganglioside in lysosomes, thereby resulting in the impairment of the anchorage of juxtaparanodal K_v1-channels required for the regulation of fiber excitability [139].

These results suggested that peroxisomes in oligodendrocytes and Schwann cells are required for maintaining the structural and functional integrity of myelinated fibers, but not for the myelination process in CNS and PNS.

10.6.2.2 Astrocyte-Specific *Pex5*$^{-/-}$ Mouse (*GFAP-Pex5*$^{-/-}$)

Astrocytes support the neuronal activities, including guidance of neurons during development, removal of excessive glutamate from the synaptic cleft, and protection of neurons from oxidative stress. Peroxisome deficiency causes the astrogliosis in the white matter of the patients with ZS [13] and CNS-specific *Pex*-KO mice [75, 126, 128]. *GFAP-Cre* mice [140] were mated with the *Pex5*$^{flox/flox}$ mice, generating *GFAP-Pex5*$^{-/-}$ mice [130]. Biochemical defects including accumulation of VLCFA and ether lipid depletion were detected in *GFAP-Pex5*$^{-/-}$ mice. However, the mutant mice showed no significant macroscopic abnormalities during their life span longer than 24 months. Astrogliosis and excess proliferation of astrocytes were also absent throughout the brain of the *GFAP-Pex5*$^{-/-}$ mice. Mild morphological abnormalities such as elevation of catalase and lipid accumulation in the molecular layer of the cerebellum were observed. Therefore, peroxisome deficiency in astrocytes is not responsible for the astrogliosis in peroxisome-deficient mouse brain, but rather demyelination seems a causative event as observed in *Cnp-Pex5*$^{-/-}$ mouse [129].

10.6.2.3 Projection Neuron-Specific *Pex5*$^{-/-}$ Mouse (*NEX-Pex5*$^{-/-}$)

Disturbance of cortical laminar structures is a characteristic feature of ZS [11, 13, 64]. To delete the peroxisome in projection neurons of cerebral cortex, the mice expressing *NEX* promoter-driven Cre recombinase (*NEX-Cre*) [141] were mated with the *Pex5*$^{flox/flox}$ mice, thereby generating *NEX-Pex5*$^{-/-}$ mice [130]. Although the defect of peroxisome biogenesis in projection neurons was detected in the primary culture, neuronal migration defect in *NEX-Pex5*$^{-/-}$ mice was not observed. Since *NEX* promoter activity is only measurable in postmitotic neurons that migrate from the VZ to cortical plate [141], it is plausible that projection neurons reached the cortical plate before complete impairment of peroxisome functions.

10.6.3 Tamoxifen-Inducible *Pex5*$^{-/-}$ Mouse (*CMV-Tx-Pex5*$^{-/-}$)

To investigate the function of peroxisome in adult mouse, tamoxifen-inducible *Pex*-KO mouse was generated using CMV-Cre-ER mouse, in which the expression of Cre recombinase fused to a mutated estrogen receptor ligand-binding domain was driven by the ubiquitously active CMV promoter [75]. CMV-Cre-ER mouse was mated with *Pex5*$^{flox/flox}$ mouse, generating *CMV-Tx-Pex5*$^{-/-}$ mouse. The administration of tamoxifen led to inactivation of *Pex5* at a level less than 20% wild-type *Pex5* mRNA. At 5 or 8 months after tamoxifen administration, *CMV-Tx-Pex5*$^{-/-}$ mouse showed motor problems, demyelination, axonal impairment, and microgliosis (Table 10.2). This finding suggests that peroxisomal integrity is essential for not only development but also maintenance of CNS.

10.6.4 Other Conditional *Pex*-KO Mice

Using the *Cre-loxP* system, *Pex* genes have been inactivated in various tissues: adipocyte-specific *Pex5*-KO (*AKO-Pex5*$^{-/-}$) [142] and *Pex16*-KO (*Pex16*-AKO) [143] mice; the mouse with

inactivation of *Pex5* in PNS and adrenal medulla, *Wnt1-Pex5*$^{-/-}$ mice [144]; nonselective aP2 promoter-dependent Cre expression, *aP2-Pex5*$^{-/-}$ mouse [144]; germ line-specific *Pex13*-KO (*Stra8-Pex13*$^{-/-}$) mouse [145]; and β-cell-specific inactivation of *Pex5*, *Rip-Pex5*$^{-/-}$ mouse [146]. Although CNS abnormality was not described in these conditional KO mice, novel roles of peroxisomes were addressed, including the starvation-induced lipolysis mediated by peroxisome-lipid droplets interactions in *AKO-Pex5*$^{-/-}$ mouse adipocytes [142], the impairments of adipose thermogenesis caused by impediment of cold-induced mitochondrial fission in *Pex16*-AKO mouse [143], and the disturbance of glucose tolerance and enhancement of apoptosis induced by peroxisome deficiency in β-cell of *Rip-Pex5*$^{-/-}$ mouse [146]. Thus, conditional *Pex*-KO mice are highly useful for elucidating peroxisomal functions in the regulation of other organelles homeostasis and cellular functions in various tissues.

10.6.5 Summary of Conditional *Pex*-KO Mice

As described above, although CNS-specific *Pex5*$^{-/-}$ mice show an abnormal brain development [74, 125, 126]; the mice with neural cell type-specific inactivation of *Pex5* normally grow to adulthood [129, 130]. Normal development in these mice has been suggested to be owing to the shuttling of peroxisomal metabolites and supportive effects between different cell types of the brain [130]. Therefore, investigation of the interaction between neural cells might serve as a potential clue to delineate the pathogenic mechanisms underlying the abnormal development of neural cells.

10.7 Molecular Mechanisms Underlying Pathogenesis of Neurodegeneration in PBDs

To elucidate pathogenic mechanisms underlying the neurodegenerative symptoms, a number of ZS model and tissue-specific model mice have been generated. However, molecular mechanisms underlying the pathogenesis remain enigmatic.

Recently, using *Pex14*$^{\Delta C/\Delta C}$ mouse, we reported that dysregulated brain-derived neurotrophic factor (BDNF)-tropomyosin-related kinase B (TrkB) pathway causes the impaired dendritic development of Purkinje cells in the cerebellum [48]. The elevated level of BDNF together with the enhanced expression of TrkB-T1, a dominant-negative isoform of the BDNF receptor, gave rise to the inactivation of extracellular signal-regulated kinase (ERK) and AKT signaling. During cerebellar development, the BDNF-TrkB signaling pathway plays pivotal roles in dendrite formation of Purkinje cells [147–149] and in granule cell migration [150]. Heterotopia of Purkinje cells is a characteristic feature of the patients with milder ZSDs, including NALD and IRD [151–153]. Therefore, the BDNF-TrkB signaling pathway in the cerebellum is most likely susceptible to the impaired peroxisomal metabolism in ZSDs, including ZS, NALD, and IRD.

The impairment of myelination is commonly observed in ZS [11, 12], NALD [151], and RCDP [154, 155], but not in IRD [152, 153]. Indeed, dysmyelinations were apparent in the cerebellum of *Nes-Pex5*$^{-/-}$ mouse [74, 75]. On the other hand, *Cnp-Pex5*$^{-/-}$ mouse [129] and *Pex7:Acbd1* DKO mouse [51] showed normal development of myelin in CNS until 2 or 3 months after birth, in contrast to the progressively enhanced demyelination in aged mice. Since dysmyelinated axon is observed in plasmalogen-deficient (*Dhapat*$^{-/-}$) mouse, involvement of plasmalogens in formation and maintenance of myelin is suggested [156, 157]. The defect of plasmalogen synthesis leads to the elevation of squalene monooxygenase (SQLE) expression, resulting in the suppression of cholesterol biosynthesis [158]. In *Pex14*$^{\Delta C/\Delta C}$ mouse cerebellum, the marked reduction of plasmalogens and elevation of SQLE are evident, and thus the cholesterol biosynthesis is likely inhibited [73]. The decrease of cholesterol leads to the reduction of 24(S)-hydroxycholesterol (24OHC), a natural ligand for liver X receptors (LXR) α and β. Indeed, transcriptional activities of LXRs are reduced, i.e., mRNA level of myelin-enriched proteins,

Mbp, and proteolipid protein 1 are apparently depleted in the cerebellum of *Pex14*$^{\Delta C/\Delta C}$ mouse [73]. These findings partly indicate a physiological role of plasmalogens in the cerebellar development via the metabolite-mediated transcriptional regulation.

Impairment of myelination in PNS was observed in mice deficient in *Pex7* or *Dhapat*, thereby suggesting the involvement of plasmalogens in Schwann cell-mediated myelination [50]. Schwann cells in *Pex7*$^{-/-}$ mouse showed deactivation of AKT signaling, leading to an overt activation of glycogen synthase kinase 3 β (GSK3β). Active GSK3β inhibits the differentiation and myelination of Schwann cells. Notably, treatment with GSK3β inhibitors restored the defects of myelination of Schwann cells, effectively bypassing the plasmalogen deficiency [50]. These findings indicate the physiological role of plasmalogens in the differentiation and myelination of Schwann cells via signal transduction. However, a recent study with *Cnp-Pex5*$^{-/-}$ mice did not show such abnormalities in myelination [139].

10.8 Other Animal Models of Peroxisome Biogenesis Deficiency

In addition to the mouse model of PBD, other peroxisome biogenesis-defective animal models including nematode (*Caenorhabditis elegans*) and fruit fly (*Drosophila melanogaster*) have been established [159]. In *C. elegans*, silencing of *prx-5* (*Pex5*) led to the arrest of the initiation of normal postembryonic cell divisions and normal cell migrations of neuronal cells during the L1 stage [160]. In contrast to the early larval developmental impairment caused by *Pex5*-depletion, silencing of *Pex* genes such as *Pex1*, *Pex5*, and *Pex13* at later stages extended life span [161, 162], suggesting that peroxisomal biogenesis is essential for the nematode development, but of less importance in the adult stage.

In *Drosophila*, mutation or silencing of *Pex* genes including *Pex1*, *Pex5*, and *Pex19* showed larval lethality with abnormal development of CNS and PNS neurons and disorganization of glia cells [163–165].

Taken together, the peroxisomal functions are essential for normal neural development in nematode, fruit fly, mouse, and humans. Peroxisome-mediated neuronal development is very likely conserved in the animal kingdom. Therefore, the studies using peroxisome-deficient models, such as nematode and fruit fly, would also clarify the molecular mechanisms underlying pathogenesis of PBDs.

10.9 Conclusions and Perspectives

To address pathogenic mechanisms underlying PBDs, several mice with inactivation of *Pex* genes in a whole body have been generated. Neurodegenerative phenotypes of *Pex*-KO mice are very similar to those of the patients with PBDs, manifesting severe hypotonia, neuronal migration defect in the cerebral cortex, impairment of Purkinje cells arborization, and migration delay of the cerebellar granule cells. However, a few phenotypic differences are distinct between *Pex*-KO mice and PBDs, such as sparse heterotopia of Purkinje cells and mild dysplasia of inferior olivary nuclei. Addressing these issues will lead to a way to delineate the onset mechanism of PBDs.

Tissue-specific and cell-type specific conditional *Pex*-KO mice have also been generated using *Cre-loxP* system. Neurodegenerative impairments in CNS-specific *Pex*-KO mice are less severe than those in *Pex*-KO mice. Noteworthily, liver-specific *Pex*-KO mice represent the neurological impairments similar to *Pex*-KO mice, suggesting that peroxisomes in the brain and liver are coordinately engaged in the neuronal development. On the other hand, cell type-specific *Pex*-KO mice show no developmental abnormality in CNS, hence implying the somewhat limitation in the studies of neural development using *Cre-loxP* system. Therefore, more detailed studies are required to unveil the molecular mechanisms underlying PBDs.

Although a number of genetically modified mice have been generated, there is a few findings

with respect to the pathogenic mechanisms of PBDs. Plasmalogen-dependent myelinations in CNS and PNS are partly unraveled [50, 73]. In contrast, neuronal migration defects are observed in the cerebral cortical neurons and cerebellar granule cells, while almost no molecular insight into these processes has been reported. The mechanisms of aberrant expression of *Bdnf* and *TrkB-T1* during cerebellar development [48] also need to be defined in more detail. As suggested in cell type-specific *Pex*-KO mice, there is a difficulty in the *in vivo* analysis: the shuttling of peroxisomal metabolites and supportive effects among different cell types of the brain could not envisage the phenotypes notable in each cell type. Primary culture of Purkinje cells from $Pex14^{\Delta C/\Delta C}$ mouse revealed that the elevated BDNF induced the aberrant dendrites development [48]. Thus, the primary culture of neural cells likely has a potential way to elucidate the pathogenic mechanisms of PBDs, under the exclusion of the effect from impaired liver functions *in vivo*.

Several different types of therapeutic approaches for PBDs have been investigated. Oral administration of bile acids (BAs) to $Pex2^{-/-}$ mice prolongs the postnatal survival and partly improves the arborization of Purkinje cells, despite the exacerbated degree of hepatic steatosis [77]. Essentially the same results are observed in ZSD patients treated with BA administration [166, 167]. $LR{:}Pex5^{-/-}$ mice show that peroxisome biogenesis is restored in the liver and the neuronal migration in cortex is significantly improved [41]. Demaret et al. [168] reported that liver transplantation for a patient manifesting mild ZSD improved the neurodevelopmental status. Given that the outcomes from these approaches are compatible with the findings between *Pex*-KO mice and patients with ZS, the *Pex*-KO mice are highly useful for developing the therapies for PBDs.

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11 The *Drosophila melanogaster* as Genetic Model System to Dissect the Mechanisms of Disease that Lead to Neurodegeneration in Adrenoleukodystrophy

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Abstract

Drosophila melanogaster is the most successful genetic model organism to study different human disease with a recent increased popularity to study neurological disorders. *Drosophila melanogaster* has a complex yet well-defined brain with defined anatomical regions with specific functions. The neuronal network in the adult brain has a structural organization highly similar to human neurons, but in a brain that is much more amenable for complex analyses. The availability of sophisticated genetic tools to study neurons permits to examine neuronal functions at the single cell level in the whole brain by confocal imaging, which does not require sections. Thus, *Drosophila* has been used to successfully study many neurological disorders such as Parkinson's disease and has been recently adopted to understand the complex networks leading to neurological disorders with metabolic origins such as Leigh disease and X-linked adrenoleukodystrophy (X-ALD).

In this review, we will describe the genetic tools available to study neuronal structures and functions and also illustrate some limitations of the system. Finally, we will report the experimental efforts that in the past 10 years have established *Drosophila melanogaster* as an excellent model organism to study neurodegenerative disorders focusing on X-ALD.

Keywords

Drosophila melanogaster · X-linked adrenoleukodystrophy · Peroxisomes · ABCD1 transporter · Acyl-CoA synthase · Very-long-chain fatty acids · β-oxidation · Optogenetics

11.1 Introduction

The fruit fly, *Drosophila melanogaster*, has been an excellent genetic model system to dissect the basic mechanisms of heritability and for the study of neuroscience for the past 100 years. Recently, the fruit fly has been adopted as a prime model to elucidate the genetic and cellular mechanisms that lead to the development of human neurologic disorders [1]. The first work that reported the use of the humble fruit fly to model neurodegenerative disorders can be dated back to 1971 [2] when Herman and co-workers used the fly to study the changes in brain morphologies that are

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observed during aging. Their work was also important to establish the parallel between the human and insect brain functional and structural organizations. Since this early study, *Drosophila melanogaster* has been used to successfully model different human neurological disorders such as Huntington's disease [3], Alzheimer's disease [4], and Parkinson's disease [5]. The growing popularity of *Drosophila* as a model organism for human neurological disorders is based on the unparalleled versatility and speed in genetic manipulation of the fruit fly model, the extensive genomic conservation between flies and humans, and the low redundancy of the fly genome [6]. Moreover, the fly has a nervous system that, despite apparent structural divergences with the human brain, shows anatomical similarity and recapitulates a wide range of cellular signaling networks relevant to human diseases that can be easily studied both at the cellular and at the organismal level [7].

Emerging evidence from genome-wide association studies (GWAS) widely used to understand the genomic basis of human neurologic disorders revealed the need to adopt complementary experimental strategies to confirm the functional impact of allelic variants of the genomic loci linked to a particular disease; to elucidate the roles for new identified or poorly studied genes in the nervous system; and to probe network interactions within the identified involved regulatory pathways. To date, studies carried out using the *Drosophila* model system for human neurological diseases have demonstrated the ability to fulfill these experimental aims and have contributed to the mechanistic understanding of some of these complex genetic disorders [8].

Debilitating neurodegenerative conditions with metabolic origins affect millions of individuals worldwide [9–11]. The causes leading to neurometabolic disorders are extremely complex to dissect, and there are neither cure nor therapeutic interventions that can notably improve the patient's life. Thus, there is a pressing need to elucidate the molecular mechanisms of disease for these pathologies with the hope to identify potential therapeutic targets. To date, the study of metabolic neurodegenerative diseases in mammalian models has brought little success, most likely because the majority of models carry a single gene mutation in the implicated pathways. As a result, these models often underestimate the extensive redundancies within intertwined metabolic gene networks [12]. Thus, there is a need to establish novel genetic model systems with a low genomic redundancy and larger genetic amenability. Due to its limited genetic redundancies, the *Drosophila melanogaster* model system has been proved extensively useful for the study of severe neurodegenerative disorders with a metabolic origin such as the Zellweger syndrome [13] and adrenoleukodystrophy [14–17].

Here we will report how the fruit fly model system has recently helped to dissect the mechanisms of diseases of severe human neurological disorders. We will illustrate the main analogies between the *Drosophila* and human brain anatomy, and we will report the arsenal of genetic tools that can be used in *Drosophila* to study the signaling implicated in the aforementioned disorders. Finally, we will describe the advancement that studies in the *Drosophila* model system have brought to the understanding of adrenoleukodystrophy (ALD), a rare and oftentimes fatal progressive neurodegenerative disease caused by dysfunctions in fatty acid (FA) metabolism, and the potential implications of the *Drosophila* studies in the understanding of ALD. We will then examine points of debate on the validity of the *Drosophila* studies for our understanding of the factors that determine the onset of ALD in humans.

11.2 Drosophila as a Model for Neurological Disorders

Neurodegenerative disorders are an increasing burden in an ageing society [18]. As individuals grow old, regulatory mechanism of the inflammatory response and its resolution are increasingly likely to fail. The result is a constant state of low-grade inflammation that triggers neurodegeneration, a state referred to as "inflammaging." The failure of immune response

regulation is associated with common neurological disorders like Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) [19]. Similar events take place in aging flies [20], and their fast reproduction cycle, short life span, and complex yet accessible brain have led to seminal studies characterizing the mechanisms of age-related neurodegenerative disorders.

The impact of immune response regulation failure in the aging brain became evident in a recent study showing that the NF-κB-dependent expression of antimicrobial peptides (AMPs) in the head and brain of *Drosophila* increases with age and causes progressive neurodegeneration and a decline in locomotion abilities [21]. AMPs are an integral part of the innate immune system in flies. The tissue that typically expresses systemically acting AMPs is the fat body, which is orthologous to the mammalian adipose tissue and liver [22]. The head also contains fat body tissue, but the authors show that even the brain itself expresses AMPs. Mutants with constitutive NF-κB signaling had high levels of brain AMP expression, leading to shortened life span, neurodegeneration, and locomotor defects, while reduction of NF-κB-dependent AMP expression prolonged the life span of the animals and improved health at old age [21].

Drosophila can be employed as a model for neurodegenerative diseases in two ways: by the identification of fly genes homologous to genes directly associated with neurodegeneration in humans and by the ectopic expression of the human disease gene. Examples for the first approach are the Pink1 and parkin mutants, models for PD. PD is characterized by the loss of dopaminergic neuron function due to α-synuclein deposits. Pink1 is the homolog of the mammalian PINK1 gene [23] and encodes for a mitochondrial serine/threonine kinase. It acts upstream of parkin, an E3 ubiquitin ligase, in the control of mitochondrial dynamics [24]. Their mutation leads to degeneration of dopaminergic neurons, which is characteristic for PD [25–28]. Recent work using Pink1 mutants as PD models show that the Pink1 protein mediates calcium exchange at contact sites between the endoplasmic reticulum (ER) and mitochondria and that this is critical for the function of dopaminergic neurons [29]. PD is characterized by a broad range of non-motor deficits, such as deficits in learning and memory and sleep irregularities. These are recapitulated in Pink1 and parkin mutant flies [30]. The membrane contact sites between the ER and mitochondria that are mediated by Pink1 are also important for PD-associated non-motor deficits involving a circadian rhythm: in Pink1 and parkin mutants, excessive formation of ER-mitochondria contact sites in neuropeptidergic neurons leads to depletion of phosphatidylserine in the ER, which disturbs the sleep-wake pattern. The circadian rhythm in PD mutants could be restored by administration of phosphatidylserine, which highlights the importance of *Drosophila* research in the identification of novel therapeutic targets [31].

The second approach for which the *Drosophila* model can be employed is by the ectopic expression of human genes in the fly and is an approach widely used to model AD. One advantage of this strategy is that the disease-mediating gene can be expressed in a nonessential organ like the eye [32], where neurodegeneration can be studied without affecting the health of the animal. Microtubule-associated Tau protein is one of the key players in AD, because its hyperphosphorylation leads to the formation of neurofibrillary tangles. While mutants for the *Drosophila* Tau protein do not show any adverse effects [33], ectopic expression of hyperphosphorylated Tau prevents synaptic transmission. In the brains of AD patients, Tau binds to presynaptic vesicles, and work in *Drosophila* has established that the interaction is mediated by synaptogyrin-3 and that reduction of this protein prevents Tau from binding to presynaptic vesicles and restores neurotransmitter release [34]. Human genes encoding for the Aβ42 protein with mutations known for causing AD have been successfully studied in *Drosophila*. Their neuronal expression leads to Aβ accumulation and neurodegeneration. Similar to mammals, this triggers an immune response: Aβ plaques are engulfed by phagocytes by means of the receptor Draper, a process that activates JNK signaling [35].

Other examples for modeling of neurodegenerative diseases by expression of human genes

with disease-linked mutation include ALS and PD. In the ALS model, the expression of human superoxide dismutase 1 (SOD1) in motor-neurons leads to neuronal defects and a stress response in the surrounding glia [36]. In the PD model, the pan-neuronal expression of the human α-synuclein leads to changes in actin skeleton dynamics that impair mitochondrial function, ultimately causing neurodegeneration [37]. Several exciting new directions in PD research come from a study showing that the transcriptional output of α-synuclein expression shows large differences depending on whether it is expressed in glia or neurons [38]. Expression of α-synuclein in glia results in α-synuclein aggregation and cell death of dopaminergic neurons and other neurons, but not of glial cells. Glial expression leads to an upregulation of the majority of all differentially expressed genes, with proteolysis and cell surface receptor signaling among the top overrepresented biological processes. By contrast, neuronal expression leads to downregulation of 98% of the differentially expressed genes. Lipid metabolism is one of the upregulated biological processes, and differentially expressed genes in animals with ectopic expression of α-synuclein in both glia and neurons include fatty acid elongases and several peroxisome-located lipid metabolic enzymes [38]. This is particularly intriguing since a decline in peroxisomal function and the resulting alterations in lipid metabolism are associated with neurodegeneration in general, but their relevance for PD is hardly understood thus far.

Taken together, *Drosophila* has helped to elucidate novel principles in the disease mechanisms of neurodegenerative disorders. Its value as a model system for human neurodegenerative disorders becomes evident by its rather complex central nervous system, which has roughly 135,000 neurons, and its organization into defined brain regions that resembles the mammalian brain [39]. Efforts to map the whole brains to the resolution of single synapses are ongoing for several species. The CNS of *Caenorhabditis elegans*, which contains only 302 neurons, has been mapped already in 1986 [40]. The FlyEM Project aims at defining the complete connectome with all synapses involved of the fly central and ventral nervous system, and several specific regions such as the mushroom body, the learning and memory center, are already published [41].

The different brain regions are susceptible to different neurological disorders, e.g., the dopaminergic neurons in the substantia nigra pars compacta are affected in PD, and neurons in the hippocampus and entorhinal cortex are affected in AD [42]. Whether human neurodegenerative diseases can be modeled in *Drosophila* with spatial resolution, thus reflecting the susceptibility of the specific brain regions to different disorders is not fully elucidated yet, but a similar specificity seems likely. The insect CNS can be categorized into defined, non-overlapping regions and subregions, such as the optic lobe comprising lamina and medulla, or the central complex comprising the central body, which can be further subdivided into fan-shaped body and ellipsoid body [43]. The structure and organization of the fly brain resemble the human brain to an extent that makes a common evolutionary origin likely; additionally, gene expression patterns during brain development are conserved between the phyla. Brain structures with similar organization and function in flies and humans include higher brain centers required for complex behaviors and memory formation: the human basal ganglia and the fly's central complex. The conservation extends to subordinate brain structures, such as the fan-shaped and ellipsoid body in the central complex that resemble the striatum and pallidum, respectively [44].

The optic lobe is often used for neurodegeneration assays: it is adjacent to the retina, and the structure of lamina and retina can be analyzed for dissipation and wholes as a readout for neurodegeneration [45].

A complex and intensively studied fly brain region is the mushroom body, the center for olfaction, learning, and memory. Mushroom body neurons, the Kenyon cells, are subdivided into three groups according to the region their axons project to the α and β lobe, the α' and β' lobe, or the γ lobe. The three subsets of neurons have different functions in memory processing [46, 47]. *Drosophila* dopaminergic neurons form

distinct clusters, and a specific subset innervating the β' lobe of the mushroom body is responsible for progressive motor function decline upon expression of α-synuclein, similar to dopaminergic neurons in the human substantia nigra pars compacta [48, 49].

11.3 Drosophila Tools to Study Neurobiology and the Cause of Neurodegenerative Diseases

The *Drosophila melanogaster* is particularly suited to address certain questions pertaining to neurodegenerative disorders. Although evolutionarily separated, flies and humans share basic molecular mechanisms. The sequencing of the fly and human genomes revealed remarkable genes and pathways conservation between the fly and human [50]. Moreover, in the context of neurodegenerative diseases, the fact that around 85% of disease-associated human genes have a fly homolog [51] makes research with this organism relevant. There is a reasonable similarity between the central nervous systems of flies and humans, for example, the mushroom body in the protocerebrum is the higher brain center required for learning and memory in the fly and has often been compared to the human cerebellum (see Sect. 11.2). The reduced genome complexity allows easier interpretation of loss-of-function studies which in combination with the great variety of established genetic tools, and a fully sequenced genome [52], makes a useful model organism to study the etiology of human neurodegenerative diseases [7].

The fruit fly is inexpensive and easy to handle, and multiple strains have been developed over the years that are available to the community and can be obtained from stock centers with public access. Finally, large-scale screening approaches are easy to perform [53], and over the years screens have allowed to identify many genes in flies and elucidate their functions which later were confirmed to be conserved in humans (such as oncogene functions [54], homeotic gene developmental cascades [55], circadian rhythms [56], and innate immune responses [57, 58]). Indeed, gene manipulation is fast and relatively simple. Over the years, the fly community has developed an astonishing number of mutant and transgenic fly lines, and all fly lines are maintained alive in stock centers and available to the scientific community. The Bloomington Stock Center at Indiana University maintains a comprehensive collection of mutant, RNAi, misexpression [32, 59, 60] and sgRNA expressing flies for CRISPR mutagenesis as well as other stocks [60]. Additional stock centers, such as the Harvard Transgenic RNAi Project (TRiP) [61] and the Vienna Research Center (VDRC) [62], have generated genome-wide RNAi collections. Precise control over gene expression can be easily achieved using large collections of time-specific and cell type-specific drivers, as well as compound- or temperature-activated driver fly lines. FlyBase, the website of genes and genomes, curates published phenotypes, gene expression, and genetic and physical interactions [45] allowing researchers access to high-quality information (Fig. 11.1). Taken together, these advantages illustrate the reasons for which *Drosophila* has greatly contributed to biological research in general and to neuroscience research in particular.

The approach of recreating a simplified version of human neurological disease started by expressing the human mutant gene for the polyglutamine disease in the fly, and this genetic approach has now been used to model aspects of many neurological and neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, fragile X syndrome, and spinocerebellar ataxias [4, 5, 63, 64]. The results coming from these studies combined with findings from human patient pathology, human population genetics, and other biological understanding have provided mechanistic insight into the molecular pathways of disease.

Using the binary UAS/Gal4 expression system, gene silencing or misexpression of endogenous genes or homologous human genes in different neurons and at the desired developmental time can be easily achieved [65]. This permits the analysis of the detrimental effects of gene inactivation or overexpression of mutant gene

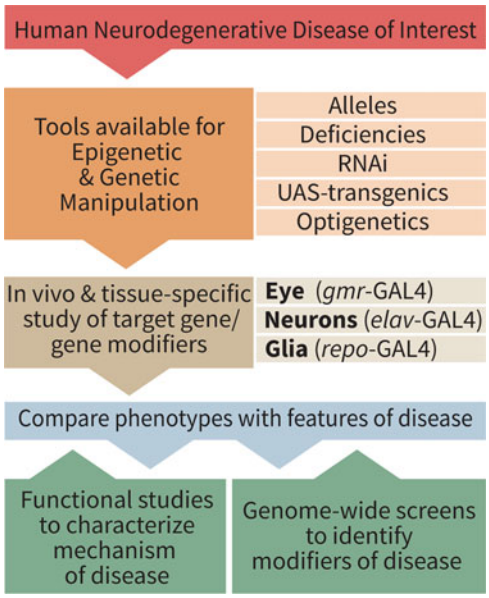

Fig. 11.1 Diagram of the rational to study *Drosophila* model for neurological diseases. Genes that are linked to disease based on genetic studies or pathological examination of the human neural tissue usually serve as a starting point. The second step is to identify between the available tools the most appropriate one to model the disease of interest. Many strategies for genetic manipulation exist in the fly, allowing tissue-specific and age-specific expression of transgenes for both up- and downregulation of gene expression. Examples for loss-of-function studies include hypomorphic or null alleles, deficiency lines in which a stretch of DNA that includes all or part of the gene of interest or a region of the genome covering many genes has been deleted, and shRNA and dsRNA transgenes to knockdown gene expression. For upregulation, a commonly used strategy is the expression of a transgene under the control of the upstream activating sequence (UAS). There is a rich collection of the GAL4 transcriptional activators placed under a cell type-specific promoter. Many of these Gal4 are available for very select neuron expression patterns. Upon establishment of a model, the effects of the disease gene are examined and compared to known manifestations and features of the human disease. Extracted and modify from [7]

variants in cells of the nervous system. To this aim, there are over 7000 GAL4 lines in the adult nervous system of *Drosophila melanogaster* with specific expression patterns [66, 67]. While many lines show expression in a small fraction of neurons in the adult brain, only ~1 in 1000 appears to drive expression in a single cell type. To achieve greater specificity, there is the split-GAL4 intersectional method [68] where individual spatial or time-specific enhancers drive the expression of either GAL4's DNA-binding domain or an activation domain joined to a leucine zipper-dimerization domain. When expressed individually, each half is insufficient to activate transcription of the upstream activating sequence (UAS) reporter. When both the activation domain and the DNA binding domain are present in the same cell, they bind to make a functional transcription factor that can activate the UAS DNA sequence and trigger transcription of a reporter gene [65].

The Gal4/UAS system has been widely used to perform modifier screen of a defined neurophenotype caused by mutation or overexpression of a disease-linked gene (Fig. 11.2). In a very easy and popular modifier screens used to study neurogenes, flies in the F1 generation where the gene of interest is knocked-down or overexpressed in the optical neurons display a rough eye phenotype (REP) [32]. The F1 flies with REP are crossed with flies with either loss of function mutations or misexpression of endogenous genes under UAS control and screened for obvious changes in REP appearance. This approach is very effective because the external investigation of the REP is very easy and the degree of photoreceptor loss as an indicator for neurotoxicity can be quickly evaluated (Fig. 11.2 a and b). This type of screen allows the screening of large collections of potential genetic interactors of the gene of interest in vivo (Fig. 11.2 b–e) [72]. In addition to the REP, there are multiple other readout systems to assess neurodegeneration and neuronal dysfunction in *Drosophila* mutant. Some of these readout systems address parameters of fly behavior like locomotion [73] and longevity (Fig. 11.2 e and f). Moreover, histological analysis is frequently used to address neuronal cell death (e.g., vacuolization of fly brains upon neuron loss) (Fig. 11.2 c, d, g) [15]. In the context of neurodegenerative diseases, these assays are more significant than the REP; however, REP can be used in large-scale screen because it is the easiest to score and then candidate genes can be further studied using more appropriate assays.

In conjunction with thousands of promoter elements that are widely available to drive the

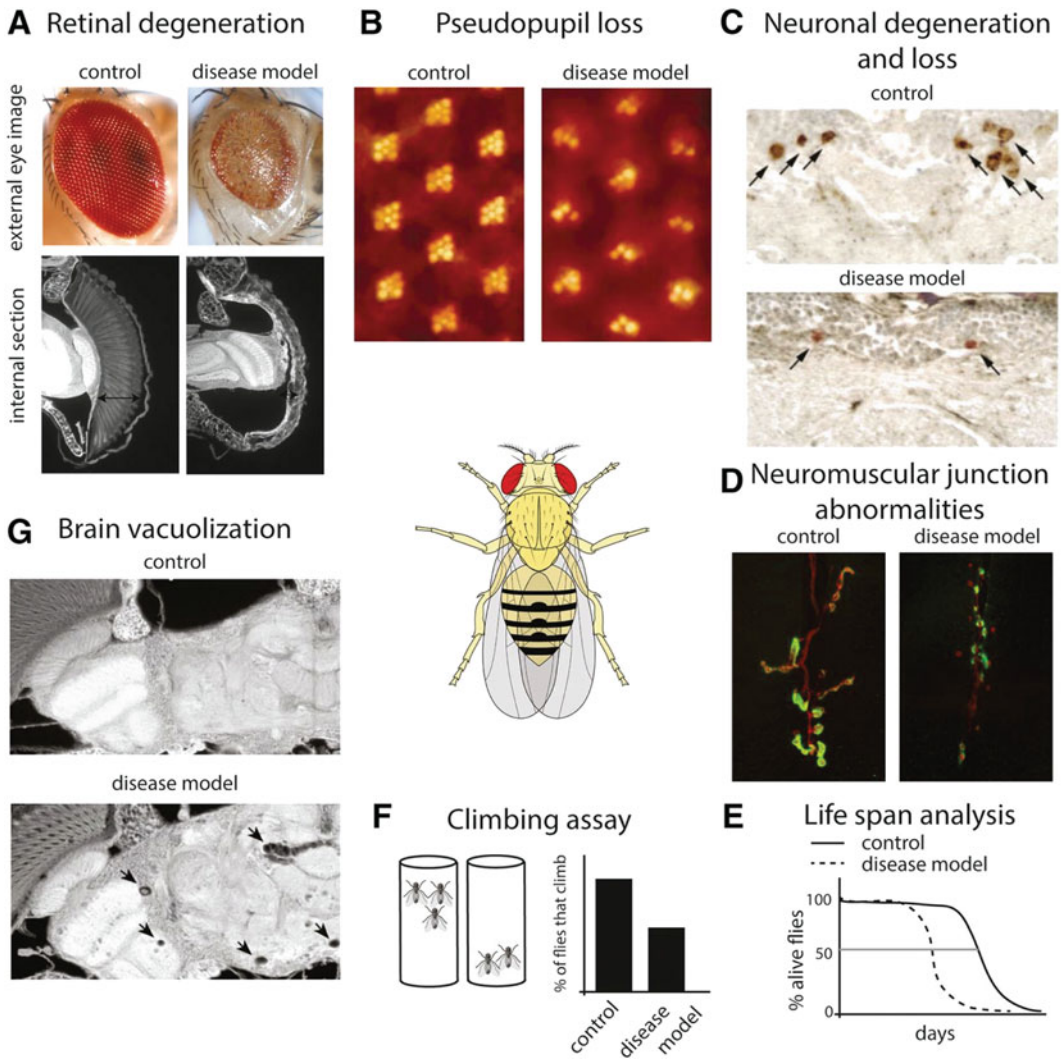

Fig. 11.2 Examples of assays to study neural degeneration in *Drosophila melanogaster*. (**a**) Using an eye-specific GAL4 driver, both the disease gene and candidate modifiers can be expressed in the eye. As the eye is a non-vital tissue, the effects of highly toxic genes can be assessed in adult flies without concerns of lethality. The external eye (top panel) offers a rapid readout. Compared with the normal eye, the degenerative eye can show disruption of ommatidial structure, reduced size, and loss of pigmentation. Internal sections (bottom panel) allow examination of the retinal tissue. Arrows indicated collapsed retina. (**b**) The pseudopupil assay allows a quantitative measure of degeneration in adult flies by assessing the structure of the photoreceptor cells by counting the number of intact rhabdomeres (the specialized organelles of the photoreceptor cells). Each ommatidium is composed of eight photoreceptor cells. Using back illumination of the head, seven rhabdomeres of each ommatidium can be visualized (left panel) and their structural deterioration over time due to degeneration can be monitored (right panel). (**c**) Labeling of specific neuronal cell types, either by using selective antibodies (such as antityrosine hydroxylase to label dopaminergic neurons) or by expression of markers using the *GAL4-UAS* system can allow one to assess the integrity of neurons in the brain [69]. (**d**) Expression of disease genes in motor neurons, followed by immunostaining for presynaptic (red) and postsynaptic (green) neuromuscular junction (NMJ) markers can reveal NMJ abnormalities such as reduced bouton numbers and increase in "ghost boutons" that lack opposing postsynaptic structure [70]. (**e**) Life span analysis of flies expressing disease-related genes. Transgene expression can be limited to the nervous system, to specific neuronal cell types, or to the adult stage to bypass developmental effects by using the range of driver lines available. (**f**) The negative geotaxis climbing assay of *Drosophila* can be used to examine motor deficits with age. Flies are tapped to the bottom of an empty vial and the number of flies that can climb above

Gal4/UAS system, there are two other binary systems, LexA/LexAop and QF/QUAS, that can be used to express pre- and postsynaptic markers/gene variant in distinct sets of neurons but also in combination with Gal4/UAS to direct expression to highly precise subpopulations of neurons [74].

Functional imaging and optogenetic techniques are available in *Drosophila* and have been employed to identify which neurons are activated to drive a specific behavior or to respond to a sensory stimulus and to define neurons whose activation/silencing causes changes in behavior, respectively [75]. The two techniques are highly complementary and can help to map neurons necessary for given behaviors.

Genetically encoded calcium indicators (GECIs) [76] and genetically encoded voltage indicators (GEVIs) [77], which allow us to measure activity in the specific neurons in which the sensors are expressed, can be used to detect which neurons are activated in a specific area of the brain during a specific process and how this activation is affected in mutant fly model of a neurological disorder [75]. For instance, by expressing a GECI (such as GCaMP6) in many neurons, applying the stimulus, and fast-scanning a focused area of the brain, we can identify the responding region of the brain to the stimulus. More detailed or rapid scans of a smaller volume (region of interest) can be performed to pinpoint the specific subset of activated neurons.

GRASP (GFP reconstitution across synaptic partners) is a genetic method used to identify cell contacts and synapses in living animals during a specific behavior or under specific stimuli [78]. GRASP labels synapses based on the proximity of the pre- and postsynaptic plasma membranes. GRASP is based on the reconstitution of two fragments of the split GFP across the synapses of interacting neurons. Each of the two nonfluorescent split GFP fragments is added to carrier transmembrane proteins that are in the two cells forming the synapsis. The two fragments of the split GFP assemble into a fluorescent form only when the membranes are sufficiently close to permit carrier proteins to interact [79]. GFP signal is recorded by live confocal microscopy.

Optogenetics uses genetically targeted expression of light-activated proteins, particularly ion channels and pumps, to alter neural activity. Currently, preferred neuronal activators include ChR2, a green light (470 nm)-sensitive nonselective cation channel derived from the green alga *Chlamydomonas reinhardtii* [80], and CsChrimson, a red light (590 nm)-activated channel from the red alga *C. subdivisa* [81]. In *Drosophila*, there are a variety of microscopic methods for delivering excitation light and collecting emitted photons during optogenetic studies. Confocal microscopes (scanning or spinning disk) deliver excitation illumination broadly and collect it from planes of interest. Two-photon microscopy is preferred in this approach when studying adult flies since it can penetrate the cuticle better [75].

In summary, *Drosophila melanogaster* is an established model organism to study human neurological diseases. The high degree of conservation in molecular pathways between flies and humans together with the rich arsenal of tools developed to study neuronal identity and function has led to the discovery of novel mechanisms of disease.

11.4 Adrenoleukodystrophy

ALD is a rare and progressive neurodegenerative disease that mainly affects children. The disease can be oftentimes fatal and presents with clinically heterogeneous features [82]. The most common form of the disease is X-linked adrenoleukodystrophy (X-ALD), which has an

Fig. 11.2 (continued) a certain height is recorded. (**g**) Brain degeneration in *Drosophila* is often accompanied by the formation of vacuoles as shown here upon expression of a CAG-repeat RNA [71]. This figure is a reprint from [7]

estimated incidence of 1:17,000 in all ethnic groups and shows incomplete penetrance and variable expressivity. ALD can be classified based on its severity: the adrenomyelopathy (AMN); Addison's disease; and—the most common and most devastating form—cerebral ALD. Cerebral ALD is diagnosed in boys between the ages of 4 and 10 that present progressive disability that usually lead to death within 4 to 8 years [82].

X-linked cerebral ALD affects ~40% of all affected individuals. Demyelination in the central nervous system (CNS) constitutes the clinical feature of ALD that eventually lead to a permanent disability and death. The most established genetic cause of X-ALD was identified in the 1990s: a mutation in the *ABCD1* gene on the X chromosome [83]; thus females are carriers of ALD but most of the time present no symptoms, while all the males are affected with the disease. The *ABCD1*gene encodes for a peroxisomal membrane half-ATP-binding cassette ABC transporters. The typical structure of an ABC transporter is composed of two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). There are several different arrangements of the TMD and NBD domains found among the human ABC proteins [84]. The two NBD domains bind and hydrolyze ATP, which provides the driving force for transport. The two TMDs participate in substrate recognition and translocation across the membrane. The adrenoleukodystrophy protein (ALDP) is a half-transporter that must dimerize with another half-transporter to form a full-transporter [84].

ABCD1 localizes at the peroxisomal membrane and mediates the influx of very-long-chain fatty acids (VLCFA, 22 or more carbon) acyl-CoA esters into the peroxisome where they will be catabolized via peroxisomal β-oxidation (Fig. 11.3) [85]. Indeed, the accumulation of VLCFA in the adrenal glands, brain, and plasma of all the affected patients is a hallmark of ALD, although VLCFA accumulation and disease intensity do not always correlate in patients or animal models of the disease (Fig. 11.3). For instance, in X-linked human ALD [86, 87] and in three knockout mice of the ABC transporter gene, there was no correlation between the amount of VLCFAs and the severity of pathology [88]. These observations suggest that excess in VLCFAs and the pathology may not have a direct causal relationship, but may be separate ramifications of another defect. H. W. Moser et al. [88] suggested that autosomal modifier genes may play a role in the pathogenesis of ALD. Interestingly, in a *Drosophila* genetic model for X-ALD, it was observed that affected homozygous females show the neurodegenerative defects but do not present VLCFA accumulation [15]. The differences between males and females are consistent with the idea that other unknown modifiers might affect the physiopathology for the disease. Thus, the use of *Drosophila* models should facilitate genetic analysis to clarify this problem by identification of suppressor and enhancer genes (see sections below).

Mutations in another gene that encode for the acyl-CoA synthase (ACS), an enzyme that functions immediately upstream of the ABCD1 transporter in the peroxisomal FA β-oxidation process, have been more recently linked to the ALD pathology [89, 90]. *ACS* encodes for members of an enzyme family that catalyze the second step of the fatty acid esterification process (Fig. 11.2). FAs cannot pass through membranes unless they are esterified. ACSs are responsible for the different FA esterification to acyl-CoA that can enter the plasma and organelle membranes. In invertebrates as well in vertebrates, there are different ACS genes [14, 89]. Knockouts in mammalian models of one or several genes that encode for the ACS proteins impede the ability to come to firm conclusions on the substrate specificity of the different ACSs. Problems are caused in part by the genetic redundancy of the members of this gene family. Indeed, the expression or activity of other ACS isoforms may increase to compensate for the absent ACS enzyme, and this is the reason for which for long time mutations in ACS genes were not found linked to ALD.

Recently it was determined that the human ACSL4 has an important role in neural development since mutations in *ACSL4* have been found in the form of X-linked mental retardation

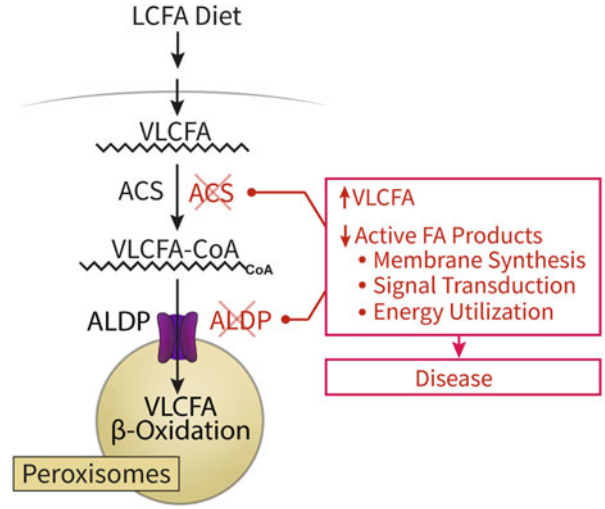

Fig. 11.3 Diagram of the very-long-chain fatty acid (VLCFA) degradation in peroxisomes. When transport to the peroxisome is blocked by mutation of either the acetyl-CoA synthetase (ACSL) or the transporter (ABCD1 also referred to as adrenoleukodystrophy protein, ALDP), which is shown here as a dimer (oval pair) resident in the peroxisomal membrane, VLCFAs accumulate inside the cell

[90]. This linkage to X-ALD is confirmed by studies in the *Drosophila* model system, of *dAcsl* mutants, where dAcsl is considered the "functionally homologous" to human ACSL4 (see next section). *dAcsl*-mutant flies have impaired synaptic vesicle transport and function and accumulate axonal aggregates that can be rescued by human ACSL4. It was therefore suggested that dAcsl regulates the retrograde transport of synaptic vesicles, possibly by increasing specific lipid-signaling molecules (Fig. 11.3) [91].

ACSL4 in humans seems to regulate eicosanoid synthesis by inhibiting their production. Since eicosanoids are pro-inflammatory lipids, and brain inflammation appears to be a feature of X-ALD patients, mutation in ACSL4 could induce the disease contributing to a high pro-inflammatory status of the brain [92].

Recently nonsense mutation for another member of the ACSL superfamily was found in a whole-exome sequencing of an X-ALD child. SLC27a6 encodes ACS belonging to the FATP (fatty acid transport protein) lineage of the ACS superfamily.

Further studies have revealed that mutations in *SLC27a6* leads to an incompletely penetrant leukodystrophy as observed for ABCD1, confirming that disease risk is enhanced by additional genetic and/or environmental factors. This discovery that the loss of SLC27a6 ACS function is associated with leukodystrophy together with the ACS loss-of-function studies in the fruit fly [14, 93] is very important given clinicians' frequent inability to recognize the heritable basis of leukodystrophy [94].

The power of the humble fruit fly has helped to identify a new candidate susceptibility gene for adrenoleukodystrophy, and further analyses in *Drosophila* offer great potentials to advance our understanding of the genetics of this devastating disorders.

11.5 *Drosophila* Models for ALD

The necessity of a genetically defined model for ALD becomes evident by the fact that VLCFA levels, which serve as a diagnostic marker for peroxisome-related disorders, do not correlate with the neurological conditions of the patients, and the differences in the neurodegenerative phenotype of patients with mutations in the ABCD1 gene [16]. Literature on *Drosophila* models for adrenoleukodystrophy is, however, scarce. Nevertheless, data obtained from the *Drosophila* ALD models *bubblegum* and *double bubble* have helped to show that VLCFA accumulation is rather a concomitant feature of ALD disease but not causative for neurodegeneration. Acyl-

CoA synthetases with substrate specificity for very-long-chain fatty acids (Acslvl) were originally suspected to be affected in ALD patients [95], before mutations in the ABCD gene were identified in the patients. Research on ALD in *Drosophila* has been based on mutants for *dAcsl* rather than ABCD transporters until recently [14, 15]. In 1999, Kyung-Tai Min and Seymour Benzer isolated the *bubblegum* mutant in a screen for neurodegeneration, and the affected gene was identified as a VLCFA acyl-CoA synthetase [15]. *Bubblegum* mutants display age-dependent degeneration of the optic lobe, and the resulting holes resemble the name-giving bubbles. The authors observed an increase in VLCFA in male flies, but not in female flies, despite the fact that both sexes showed the same neurodegenerative phenotype. Analogous to Lorenzo's oil treatment, the authors fed *bubblegum* mutants with glyceryl trioleate oil. In animals treated with the oil from early larval stages to adulthood, VLCFA were reduced to normal levels, life span prolonged, and neurodegeneration ameliorated.

The corresponding human ortholog was described in 2000 by Steinberg and colleagues [17]. Like its *Drosophila* counterpart, human *bubblegum* is capable of activating very-long-chain fatty acids [17]. There are two human homologs of *bubblegum*, termed *ACSBg1* and *ACSBg2*, which differ in their expression pattern: ACSBg1 is relevant in the brain and steroidogenic tissues, while ACSBg2 is active in the testis and brain stem [89]. Whether human bubblegum is relevant for ALD is unclear [96–98].

Building on the work of Min and Benzer, Sivachenko and colleagues proposed an improved *Drosophila* model for ALD in 2016 [14]. The phenotype of the original bubblegum mutant is not fully penetrant, and apart from the degenerative phenotype of the optic lobe, *bubblegum* mutants display only subtly compromised health and life span. Sivachenko and colleagues report that *Drosophila* has seven genes encoding for ACSL enzymes and that *bubblegum* (*bgm*) has a close homolog that resides next to it in the genome. They termed this gene *double bubble* (*dbb*) and further report that *bgm* and *dbb* have overlapping substrate specificity; thus, ACSL enzymes can be redundant in their function, which explains the mild phenotype of the *bubblegum* mutant. Double mutants for the two genes show severe degeneration of the retina and the lamina, and their lifespan is shortened by 10%.

With these publications, *Drosophila* ALD models were still represented by mutants for genes distinct from the disease-causing mutations in humans, in genes for ACSL enzymes rather than ABCD transporters. In 2018, Gordon and colleagues further identified the gene *CG2316* as the ABCD1 homolog. ABCD1 is expressed in the adult head, and ubiquitous and neuron-specific RNAi-mediated knockdown of the gene results in neurodegeneration [16]. The authors also found that the phenotype of the *bgm-dbb* double mutant is exacerbated under stress, showing a gene-environment interaction that could possibly explain the varying severity in ALD patients. Due to the uncertain contribution of VLCFA to disease progression, Gordon et al. hypothesized that it is rather the lack of products of VLCFA-CoA that influence the phenotype. They fed a medium-chain fatty acid-enriched diet to *bgm* and *dbb* mutants and found that it ameliorates the neurodegenerative phenotype. Strikingly, a similar treatment has been used in fly models for peroxisomal biogenesis disorders: *Peroxin 19* mutants display elevated VLCFA but reduced MCFA levels. When supplemented with medium-chain fatty acids, their neurodegeneration and early lethality were rescued, but VLCFA levels were unchanged [99].

11.6 Conclusions and Remarks

Drosophila melanogaster has emerged to be the best genetic model system to identify genes linked to human neurological disorders and to define the mechanisms of diseases. The fruit fly's low genomic redundancy together with its strong genetics and the growing development of optogenetic tools and neuron-specific labeling techniques to be used in in vivo studies has growing potential to quickly advance our understanding of devastating neurological disorders and to be used for the development of more effective

therapies. *Drosophila* has been employed to model a large number of neurological disorders, and studies carried out in the fruit fly have elucidated genetic and cellular signaling affected in the specific diseases and/or have helped to identify new genes linked to the diseases. Thereby, the fly has contributed to improve early diagnostic in complex human disorders. One example here is represented by the study of the often fatal neurodevelopmental disease, X-ALD. The literature presented here leaves the question open whether *Drosophila* mutants are valid model for X-ALD. The consequences of the modeled enzyme dysfunction are similar since they take part in the same metabolic defect, fatty acids metabolic pathway, but several studies on the human *bubblegum* came to the conclusion that it has no direct relevance for ALD [96–98, 100]. Wiesinger and colleagues showed that not only free VLCFA but also VLCFA-CoA esters accumulate in the cells of ALD patients and that this is a direct effect of ABCD1 deficiency [101]. However, Sivachenko et al. describe an X-ALD patient with an additional mutation in a close homolog of *bubblegum*, the very-long-chain ACS SLC27a6 [14], which raises the possibility that the role of acyl-CoA synthetase in ALD may be underestimated and highlighting the necessity of a fast, efficient, and genetically tractable model organism like *Drosophila* for further studies on this topic.

Moreover, it has firmly been established that several peroxisomal metabolic pathways require the participation of one or more ACSs and one of the peroxisome-associate ACS seem to have a role in inhibiting pro-inflammatory lipids, thus connecting the biochemical defects to the inflammatory dysfunction to the neurodegeneration defects all observed in X-ALD patients. Thus, the identification of the linkage between mutations in the ACS superfamily member and X-ALD using the *Drosophila* model might have unraveled a previously unknown mechanism of disease. Clarification of these complex issues as well as the identification of new genes involved in X-ALD pathogenesis is warranted to explain the different expressivity and penetrance of the X-ALD associate mutations.

Strategic collaboration between neurologists, human geneticists, and the *Drosophila* researchers holds great promise to accelerate progress in this and other neurological diseases in the post-genomic era.

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12 Human Peroxisomal 3-Ketoacyl-CoA Thiolase: Tissue Expression and Metabolic Regulation

Human Peroxisomal Thiolase

Norbert Latruffe

Abstract

This paper reports that the human peroxisomal 3-ketoacyl-CoA thiolase expression shows three transcripts: Tr1 (1705 bp), Tr2 (1375 bp) and Tr3 (1782 bp). Their highest expression is observed in the human liver and at a lesser extent in hepatic-derived HepG2 cells. The intestine and blood and endothelial cells show lower expression. The lowest expression is found in adipocytes. The transcript Tr3 appears to be the most abundant. So far, no data have been published regarding the regulation of the human peroxisomal thiolase. After cloning a fragment of the 5′ region involved in the regulation of the human thiolase gene, the effects of different treatments have been studied on the thiolase expression in the hepatoma HepG2 human cell line. Biocomputing analysis indicates that (i) a GRE (glucocorticoid response element) is located at −650 bp upstream of the transcription initiation site; (ii) a C/EBPα (CCAAT/enhancer-binding protein) binding site is located at − 1000 bp upstream of the transcription initiation site – and (iii) there is no putative PPRE (peroxisome proliferator-activated receptor response element). In the human HepG2 cells, thiolase expression is upregulated by glucose and downregulated by insulin and sterols, while dexamethasone and fatty acids have no effect. The ciprofibrate, a peroxisome proliferator, leads only to a weak stimulation of the mRNA expression as compared to thiolase B expression in the rat liver.

Keywords

Human peroxisome · Thiolase · Tissue expression · Regulation · Fatty acids · PPAR agonists · Sterols · Dexamethasone · Glucose · Insulin · Transcription factors

Abbreviations

C/EBPα	CCAAT/enhancer-binding protein
Chol/25-OHC	cholesterol/25-hydroxycholesterol
GRE	glucocorticoid response element
PEX proteins	peroxins
PPAR	peroxisome proliferators-activated receptor
PPRE	peroxisome proliferator-activated receptor response element
PTS2	peroxisomal targeting sequence type 2

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TAT	tyrosine aminotransferase
TH	3-ketoacyl-CoA thiolase
Tr1,2,3	thiolase transcripts 1,2 or 3
SCPx	sterol carrier protein
VLCFA	very-long-chain fatty acid
X-ALD	X-linked adrenoleukodystrophy

12.1 Thiolase: State of the Art

Thiolase activities are found in mammals, plants, bacteria and yeasts. They belong to two classes. Class I corresponds to the enzymes with a 3-ketoacyl-CoA thiolase activity (EC 2.3.1.16), while class II represents the enzymes with an acetoacetyl-CoA thiolase activity (EC2.3.1.9). In the presence of coenzyme A, thiolase catalyses the last step of the fatty acid β-oxidation, which cleaves the 3-ketoacyl-CoA, releasing then an acetyl-CoA molecule and a fatty acyl-CoA shortened by two carbon atoms (Fig. 12.1). In humans, there is one straight length peroxisomal thiolase: the 3-ketoacyl-CoA thiolase (TH). In rodents, three enzymes harbour 3-ketoacyl-CoA thiolase activity: thiolases A and B that are specific toward VLCFAs, as well as SCPx (sterol carrier protein), a second peroxisomal thiolase that specifically oxidizes the branched chain fatty acids, as the pristanic acid, the intermediate of bile acids and dicarboxylic fatty acids as well. Rodent thiolases A and B have been previously cloned by Hijikata et al. (1990) [1], and the corresponding cDNA by Chevillard et al. (2004) [2]. Tsukamoto et al. (1994) [3] reported that rat peroxisomal 3-ketoacyl-CoA thiolase contains a signal peptide at the amino terminus of the precursor, which is involved in the enzyme import into peroxisomes via a PTS2 (peroxisomal targeting sequence type 2), as shown by Otera et al. (1998) [4]. Thiolase B gene expression strongly depends on the activation by the peroxisome proliferators-activated receptor PPARα [5] (and contains several PPRE according to Chamouton et al. (2010, 2012) [6, 7]. Thiolase B null mice have been generated by Chevillard et al. (2004) [8]. Fidaleo et al. (2010) [9] and Nicolas-Frances et al. (2014) [10] showed that these thiolase B KO mice exhibit several metabolic disturbances of cholesterol and glucose pathways. Tsukamoto et al. (1994) [3] reported that rat peroxisomal 3-ketoacyl-CoA thiolase contains a signal peptide at the amino terminus of the precursor, which is involved in the enzyme import into peroxisomes via a PTS2 (peroxisomal targeting sequence type 2), as shown by Otera et al. (1998) [4].

Numerous organelle genetic deficiencies have been reported in several human diseases with various physiopathological consequences; this includes mitochondria, peroxisome, lysosome and endoplasmic reticulum deficiencies. According to the recent review of Vamecq et al. (2014) [11], the first human peroxisomal disease recognized to result from a deficiency of peroxisomes was the cerebrohepatorenal syndrome of Zellweger, a peroxisomal biogenesis disorder in which there are abnormal peroxisomes from different sizes and shapes. Children having such a disease present major hepatomegaly and very enlarged fontanelle and often die during the first months after birth. Human peroxisomal genetic disorders were discovered due to the establishment of around a dozen of complementation groups which allowed to the identification of several peroxins (PEX proteins), involved in the formation of the peroxisomal envelope /membrane, in the import of newly synthesized proteins into the matrix of the organelle and in the division of the organelle. Peroxisomal diseases are now classified as peroxisomal biogenesis disorders (Zellweger syndrome) and as peroxisomal protein defects affecting a single or few enzyme functions; these latters include neonatal adrenoleukodystrophy, infantile Refsum disease and rhizomelic chondrodysplasia punctata type 1. Inborn errors leading to a single peroxisomal transporter deficiency or a single peroxisomal enzyme deficiency may affect peroxisomal β-oxidation (X-linked adrenoleukodystrophy (X-ALD), acyl-CoA oxidase1 deficiency (pseudo-neonatal adrenoleukodystrophy/ACOX1 deficiency), D-bifunctional protein/MFP2 deficiency (pseudo-Zellweger syndrome), peroxisomal 2-methylacyl-CoA racemase deficiency, fatty acid α-oxidation (Refsum disease), ether phospholipid biosynthesis (rhizomelic

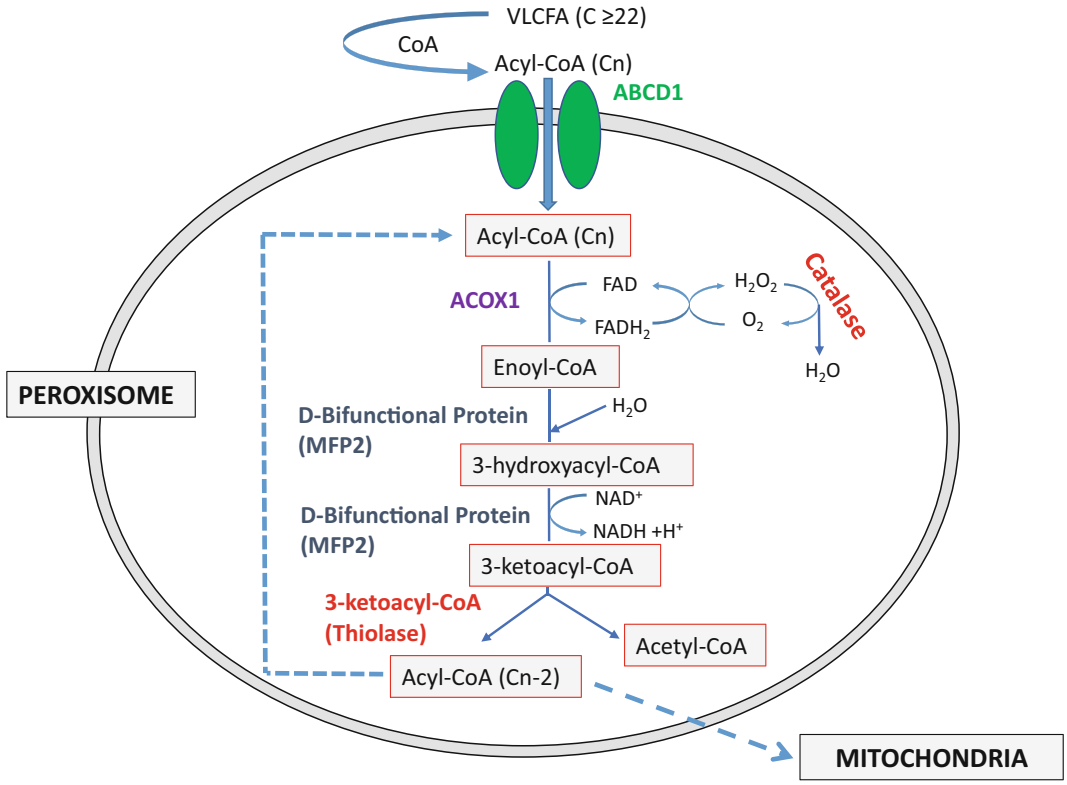

Fig. 12.1 Schematic representation of a peroxisome illustrating the peroxisomal β-oxidation and the contribution of thiolase. Very-long-chain fatty acids (VLCFA; C ≥ 22) are activated by CoA and give acyl-CoA which are taken in charge by ABCD1 to enter inside the peroxisome. The peroxisomal β-oxidation which successively involved the enzymes ACOX1, MFP2 and thiolase allows to reduce the length of VLCFA from two carbons at each cycle of β-oxidation and gives (acyl-CoA n-2) and acetyl-CoA. The final product of peroxisomal β-oxidation is octanoyl-CoA which is transported from the peroxisome to the cytosol via carnitine O-octanoyltransferase for further oxidation in the mitochondria in acetyl-CoA**.** The present figure has been realized by Ms. Aline Yammine (PhD student; University Bourgogne Franche-Comté & Lebanese University) at the Bio-peroxIL laboratory EA7270/Inserm and at the Bioactive Molecules Research Laboratory, Doctoral School of Sciences and Technologies, Lebanon

chondrodysplasia punctata type 2), dihydroxyacetone phosphate acyltransferase deficiency and rhizomelic chondrodysplasia punctata type 3 (alkyl-dihydroxyacetone phosphate synthase deficiency), alanine-glyoxylate aminotransferase (hyperoxaluria type 1, glutaryl-CoA oxidase (glutaric aciduria type 3), peroxisomal mevalonate kinase/hyper-IgD syndrome, catalase deficiency (acatalasemia), peroxisomal TRIM37 protein (Mulibrey nanism) and sterol carrier protein 2 (SCPx). Many of these abnormalities lead to neurodegenerative disorders.

Concerning thiolase, a possible innate defect has been claimed for a certain time by Schram et al. (1987) [12] in a case of peroxisomal thiolase deficiency of the human 3-oxoacyl-coenzyme A, but Ferdinandusse et al. (2002) [13] reviewed the peroxisomal 3-ketoacyl-CoA thiolase and identified the real defect in the D-bifunctional protein/MFP2.

The human 3-ketoacyl-CoA thiolase is encoded by a single gene called ACAA1 located on chromosome 3 (p22–13) [14]. This gene has a length of 9 kbp like, contains 12 exons and 11 introns as the rat thiolase genes. It shows

three transcripts: Tr1 (1705 bp), Tr2 (1375 bp) and Tr3 (1782 bp). This promoter organization is closer to that of the rat thiolase A gene. The coding sequence of the human gene enables the synthesis of a precursor of 424 amino acids, showing 86% identities with rat thiolase protein sequence. The protein can also be addressed by a PTS2 signal; the mature form after cleavage contains 398 amino acids, as in rat and mouse. To our knowledge, no data have been published regarding the regulation of human peroxisomal thiolase by peroxisome proliferators and therefore the presence of a possible PPRE in the gene sequence.

12.2 Estimation of the Expression of Human Thiolase in Tissue-Derived Cells and Exploration of Its Metabolic Regulation

After cloning a fragment of the upstream 5′ region involved in the regulation of the human thiolase gene expression, we studied the effect of different treatments on the thiolase expression in the human HepG2 hepatoma cell line.

By biocomputing analysis, we revealed the presence of putative binding sites of numerous transcription factors including:

- GR (glucocorticoid receptor) which recognizes a GRE located at −650 bp upstream of the transcription initiation site. GR exhibits two distinct binding domains: a ligand-binding domain and a DNA-binding domain. GR acts as a dimer; binds to GGTACAnnnTGTTCT sequence, called GRE; and plays a pivotal role in sugar metabolism and inflammation.
- C/EBPα (CCAAT/enhancer-binding protein) binding site is located at −1000 bp upstream of the transcription initiation site. C/EBP contains a DNA-binding domain and acts like a dimer. C/EBP binds to A/G TTGCG C/T AA C/T response element of its target genes and plays a role in adipogenesis and in hepatic regeneration.
- Binding sites of other identified transcription factors (USF, Sp1, etc.).

12.3 Material and Methods

- Cultures of HepG2 and other human cell lines and treatments of cultured cells with either fatty acids, cholesterol or derivatives as well as with dexamethasone, glucose and insulin were done according to Chevillard et al. (2004) [2].
- RNA extraction followed by RT-qPCR, for estimation of the different putative transcripts (Tr1, Tr2 and Tr3) and by northern blotting with a thiolase probe as compared with ribosomal phosphoprotein (36B4) probe as standard according to Chevillard et al. (2004) [6].
- Cloning of the 740 and 1200 pb fragments upstream of the transcription site (+1) of the regulatory 5'region of the thiolase gene, the so-called TH740 and TH1200 constructs. Evaluation of transcription regulatory properties by transient transfections on HepG2 hepatic-derived cell line (Hansmannel et al., 2003) [14].

12.4 Results

- *Tissue expression of the different human thiolase transcripts*
  The bioinformatic analysis revealed three putative transcripts from the human thiolase gene, Tr1, Tr2 and Tr3, respectively, of 1705 bp, 1375 bp and 1782 bp length. Tr1 and Tr3 only could be revealed in the tested cell lines. The highest expression is seen in the human liver followed by the hepatoma-derived HepG2 cell line. The intestine and blood and endothelial cells show lower expression. The lowest expression is seen in adipocytes. The transcript Tr3 appears to be the most abundant.
- *Influence of the metabolic state on the expression of the human thiolase transcripts in HepG2 cells*

Table 12.1 Influence of metabolic parameters on the expression of peroxisomal human thiolase in HepG2 cells

Pharmaco-metabolic conditions	Effect on thiolase transcription
Fatty acids [a]	No effect
Ciprofibrate [b]	x 2
Dexamethasone [c]	No significant effect
Glucose [d]	x 1.9 (Tr3) [g]
Insulin [e]	x 0.8
Sterols (Chol/25-OHC) [f]	x 0.65 (x 0.85 *)
CEBP alpha	x 3.4 to 4.5
CEBP beta	No stimulation

Experimental conditions
[a]50–300 μM, 48 h
[b]500 μM, 48 h
[c]1 μM, 48 h
[d]4.5 g/L, 48 h
[e]5 μg/mL, 48 h
[f]30/3 μg/mL (* with 10/1 μg/mL), 48 h
[g]Thiolase transcript 3 (Tr3)
Chol = cholesterol; *25-OHC = 25-hydroxycholesterol*

The data obtained are summarized in Table 12.1.

- Northern blotting analysis of the thiolase transcripts expression did not show any effect of fatty acid treatment at different concentrations (50–300 μM) on HepG2 cells, whatever the fatty acid carbon length or unsaturation (18:0, 18:1, 18:2, 18:3, 20:4, 20:5 and 22:6).
- The implication of PPARα pathway in the thiolase expression has been studied by Nicolas-Frances et al. (2000) [15] in HepG2 cells under treatment with ciprofibrate, a PPARα agonist. Northern blotting analysis showed a twofold induction in HepG2 cells of the thiolase mRNA expression after 48 h treatment with 500 μM ciprofibrate. This corresponds to a weak induction as compared to those observed in rat Fao cells (x 13) reported by Hansmannel et al. (2003) [14].
- The effect of dexamethasone at 1 μM on the thiolase transcript level has been evaluated by northern blotting. Dexamethasone did not show any significant effect on thiolase mRNA level in the presence of glucose 4.5 g/L despite the presence of a well-conserved GRE. However, control of the treatment efficacy by dexamethasone (1 μM) has been made by following the transcriptional activation of the *tyrosine aminotransferase* (TAT) gene: TAT expression is stimulated by twofold, indicating that the treatment by dexamethasone was efficient. The lack of GR-dependent transcription activation was studied by transient gene reporter transfection in HepG2 cells. There is no stimulation of the transcriptional activation of the thiolase 740 bp promoter fragment by GR in the presence or the absence of dexamethasone (1 μM versus vehicle).
- The study of glucose effect on the thiolase mRNA expression by northern blotting and by RT-qPCR showed that the transcript Tr3 level was increased in the presence of glucose 4.5 g/L (1.9-fold) as compared to low glucose medium (1 g/L). By contrast, no variation was observed for the Tr1 thiolase transcript level.
- There was a slight repression of thiolase expression in HepG2 in the presence of insulin at 5 μg/mL (factor x 0.8), whatever the glucose concentration (1 or 4.5 g/L).
- The sterol effect has been studied in HepG2 on the thiolase expression by northern blotting analysis. The data obtained revealed a dose-dependence repression of thiolase

expression by sterols treatment: factor of 0.85 and 0.65 with concentration ratio of cholesterol/25-hydroxycholesterol of 10/1 and 30/3, respectively.

- *Study of human C/EBPα by transient transfection in hepatic human HepG2 cells*
  We observed a stimulation of transcriptional activation of the 740 bp and 1200 bp fragments containing CEBPα putative binding site of the thiolase gene promoter. The stimulation is lower for the 740 bp fragment than for the 1200 bp fragment. Data, which are not reported here, showed also a similar stimulation in the human CaCo2 intestinal cells. For control, no stimulation of the transcription, but rather a repression (factor 0.7) was seen in the presence of C/EBPβ, whatever the fragment length (740 or 1200 bp).

12.5 Discussion

This work reports the isolation and the cloning of the 5′ regulatory region fragments of the human thiolase. The lack of thiolase expression stimulation by peroxisome proliferator (ciprofibrate) is in accordance with the absence of a PPRE. The tissue specificity of the human thiolase expression is the highest in liver cells.

The treatment of cells with fatty acids does not trigger any induction of thiolase expression in contrast with peroxisomal ACOX1 (acyl-CoA oxidase 1). This can reveal the lack of regulation of the last step of long-chain fatty acyl-CoA peroxisomal oxidation.

GR activation by dexamethasone does not see any significant effect on thiolase gene expression. Stimulation of thiolase mRNA expression by glucose is coherent with the repression seen with insulin. On the other hand, sterol-dependent repression could be linked with the availability of the acetoacetyl-CoA for cholesterol synthesis since thiolase breaks down acetoacetyl-CoA on the last cycle of fatty acyl-CoA shortening. On the other hand, the implication of C/EBPα in the regulation of the thiolase gene expression has been shown in both hepatic HepG2 and intestinal Caco-2 cell lines. The binding site to C/EBP response element appears specific to C/EBPα since no additional stimulation was seen in the presence of C/EBPβ. Interestingly the encoded protein is a key regulator of adipogenesis and of the lipid accumulation in these cells, as well as in the hepatic metabolism of glucose and lipids.

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Part V

Treatments of Peroxisomal Diseases

Biological Functions of Plasmalogens 13

Md Shamim Hossain, Shiro Mawatari, and Takehiko Fujino

Abstract

Plasmalogens (Pls) are one kind of phospholipids enriched in the brain and other organs. These lipids were thought to be involved in the membrane bilayer formation and anti-oxidant function. However, extensive studies revealed that Pls exhibit various beneficial biological activities including prevention of neuroinflammation, improvement of cognitive function, and inhibition of neuronal cell death. The biological activities of Pls were associated with the changes in cellular signaling and gene expression. Membrane-bound GPCRs were identified as possible receptors of Pls, suggesting that Pls might function as ligands or hormones. Aging, stress, and inflammatory stimuli reduced the Pls contents in cells, and addition of Pls inhibited inflammatory processes, which could suggest that reduction of Pls might be one of the risk factors for the diseases associated with inflammation. Oral ingestion of Pls showed promising health benefits among Alzheimer's disease (AD) patients, suggesting that Pls might have therapeutic potential in other neurodegenerative diseases.

Keywords

Plasmalogens · Neuroinflammation · Neurodegenerative diseases · Aging · Signaling mechanism

13.1 What Are Plasmalogens (Pls)?

Pls are one kind of ether phospholipids found in mammals, invertebrate organisms, and in anaerobic bacteria. Pls are the major phospholipids in mammals, because they constitute 20% of total phospholipids. Pls are plasmenyl type ether phospholipids having vinyl ether bond at their sn-1 position, different from other plasmanyl phospholipids having only the ether bond at sn-1 (Fig. 13.1). The ether phospholipid and plasmanyl are often called as alkyl phospholipids and plasmenyl as alkenyl phospholipids. The expressions of ether phospholipids including Pls are ubiquitous. Ether phospholipids are important structural components of cell membranes in mammals. It has been known that in mammals the fatty acid at the sn-1 position can be derived from the three kinds of fatty alcohols (16:0, 18:0, and 18:1). The sn-2 position is mainly occupied by polyunsaturated fatty acids (PUFA), but monounsaturated fatty acid, such as oleic acid

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(18:1), is also common. The head group of Pls is mainly of two kinds, ethanolamine and choline, but the other head group is also present including serine-Pls found in the human retina. Ethanolamine Pls (Pls-Etn) are highly enriched in the brain and nervous tissues. Choline Pls (Pls-Cho) are enriched in other peripherals organs including the heart, kidney, and intestinal tissues. It is believed that Pls-Etn can form rigid and condensed lipid bilayer compared to Pls-Cho, which can form relatively flexible lipid bilayer. Because of the smaller head group, the Pls-Etn and serine-Pls are believed to be enriched in the inner leaflet of lipid bilayer, whereas the Pls-Cho are present in the outer leaflet. The scientists, Feulgen and Volt, first described Pls in their studies of tissue section where they reported that these special lipids are present inside of the cells [1]. Pls are ubiquitous and enriched in the cell membranes including the plasma membrane, mitochondrial membrane, and nuclear membrane of the mammalian cells [1, 2]. Pls are also found to be enriched in lipid raft domains of cell membrane, suggesting that they might regulate cellular signaling events. Besides their enrichment in cell membrane, Pls are found to be secreted from the cells including glial and neurons. The normal concentration of Pls in the human blood is about 5 mg/dl. It is still unknown whether secreted Pls exist as free lipids or lipoproteins. By localizing in the inner leaflet of the plasma membrane, Pls-Etn might bind with membrane proteins to modulate their intracellular signaling. Pls-Etn were shown to activate cellular signaling, but it remained unknown whether Pls-Cho could induce cellular signaling. The Pls-Etn-mediated cellular signaling events will be discussed in the latter part of this chapter. Like other phospholipids, Pls can be degraded to various other components which could induce several cellular events. Among the secondary products, the most common are lyso-Pls, lyso-PAF (platelet-activating factor), PAF, and alkyl LPA (Fig. 13.2). Pls in the cell membrane can be degraded by reactive phospholipase A2 (PLA2), resulting in the formation of lyso-Pls and free fatty acids including arachidonic acid (AA) and DHA (Fig. 13.2). It has been known that AA shows various beneficial effects in brain protective functions in the cells. The activation of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) is also shown to be triggered by AA [3]. The lyso-Pls are mainly enriched in the cell membrane and known to be functionally important.

13.2 Extraction and Purification of Pls

Although it has been known that mammalian tissues contained enriched amount of Pls, it was not known how to extract these intact Pls, aiming at studying the biological significances of these lipids in health. Mawatari et al. succeeded in separating purified Pls from various cells and tissues by high-performance liquid chromatographic (HPLC) method [4], which enabled them to examine biological activities of Pls. On the basic concept of acid hydrolysis of Pls, which could yield lysophospholipid and a fatty aldehyde, they analyzed the purity of extracted Pls from the sources. To purify Pls by the single chromatographic (HPLC) run, they first collected the total chloroform lipid extracts. The tissue samples were subjected to lipid extraction using the method of Bligh and Dyer [5]. To put it simply, the tissues were homogenized in 3 ml of methanol-chloroform (2:1) mixture followed by addition of 1 ml chloroform. After addition of 2 ml of 0.88% KCl solution followed by a brief centrifugation, the lower chloroform lipid portion was collected and dried under a stream of nitrogen gas. It is to be noted that all the solvents used for lipid extraction contained 1.2 mM butylhydroxytoluene (BHT). These lipid extracts were then subjected to the single chromatographic run by the HPLC system aimed at getting purified Pls [4]. To our knowledge, it is the first report of successful extraction of intact Pls from animal tissues. Both the ethanolamine Pls (Pls-Etn) and the choline Pls (Pls-Cho) could be separated by the HPLC technique described by the Fujino's group [4]. Mawatari et al. also identified novel approaches to extract and purify plasmalogens by hydrolyzing diacylphospholipids with

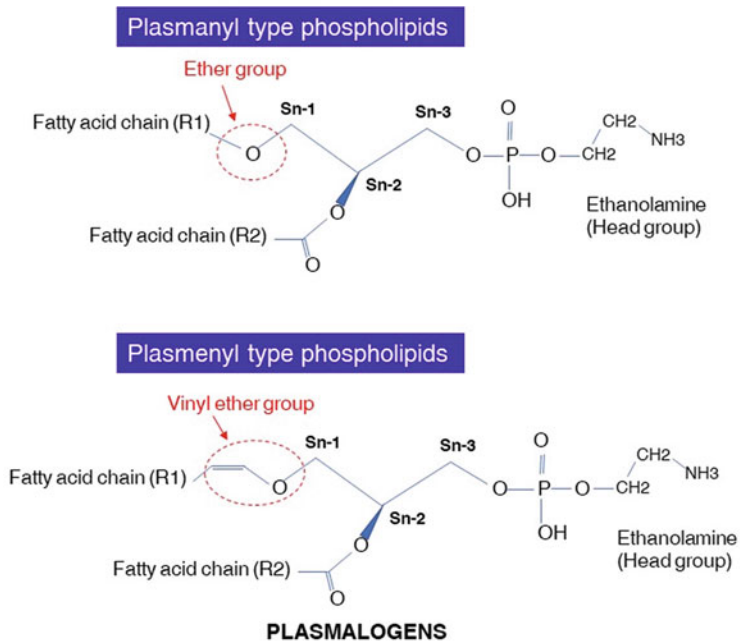

Fig. 13.1 Schematic diagram. General structures of plasmanyl- and plasmenyl- phohspholipids. Plasmalogens (Pls) are plasmenyl type phospholipids. Pls contain vinyl ether group at sn-1 position compared to the ether group of plasmanyl type phospholipids. Fatty acids at sn1 position of Pls are mainly palmitic acid (16:0), stearic acid (18:0), and oleic acid (18:1). The fatty acids at sn-2 may contain polyunsaturated (PUFA) and monounsaturated fatty acids. The head groups might be ethanolamine, choline, serine, etc

phospholipase A_1 (PLA_1) [6]. In this method, PLA_1 treatments degraded diacylphospholipids including the phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in the crude plasmalogens mixture which was obtained from the hexane/acetone solvent extraction of total lipids. The PLA_1 treatments yielded a large amount of lysophospholipids and other substances which were removed from the plasmalogens enriched with hexane portion by making the Na_2SO_4 partition in the hexane/2-propanol (3:2) solvent layers [6]. This process yielded 92% pure Pls which were then subjected to hexane/acetone (1:1) solvent extraction, leading to the highly pure Pls (97% of phospholipids). To purify further, the HPLC separation was conducted. Here, we describe the relative contents of Pls obtained from the chicken breast meat and scallop (Table 13.1). The scallop-derived Pls were enriched in EPA- and DHA-containing Pls compared with the chicken breast meat-derived Pls which were found to be enriched in arachidonic acid- and DHA-containing Pls (Table 13.1). Both the chicken- and scallop-derived plasmalogens were found to be effective in inhibiting neuroinflammation, and there were no comparative studies. Additional experiments are needed to examine the differences in their biological activities to address whether scallop-Pls, which are enriched in EPA-containing Pls, have better function than the chicken breast meat-derived Pls.

13.3 Antiapoptotic Function of Plasmalogens

During the development process of early mammalian life, various extracellular factors remained highly active to support the growth of neuroblast and immature neuronal cells. Interestingly, PUFA-containing Pls are found to be enriched in the mouse brain during the developmental

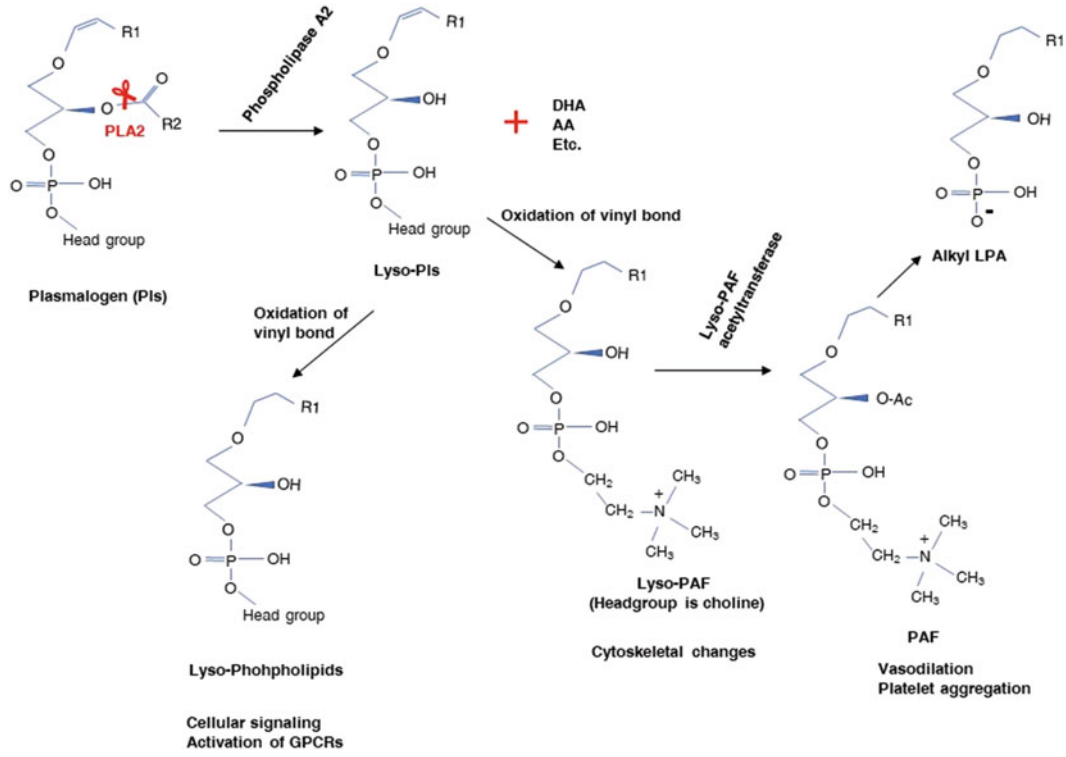

Fig. 13.2 Schematic diagram. Plasmalogens can undergo enzymatic reaction and oxidation reaction to form various bioactive substances

stages compared to the brain of older mice. The embryonic and young mouse brains are enriched with PUFA-containing Pls more than the MUFA-containing Pls such as oleic acid Pls. When we treated neuronal cells with purified Pls enriched with PUFA, we observed the attenuation of neuronal apoptosis caused by the nutrient deprivation [7]. This evidence could suggest that PUFA-Pls are crucially important for immature neurons for their survival during embryonic development and young stage of life. Neuronal apoptosis is accompanied with activation of caspase proteins. There are two major kinds of apoptosis events such as intrinsic and extrinsic apoptosis pathways. Extrinsic pathway of apoptosis is caused by membrane-bound death receptors including fas-associated death domain (FADD) and associated with the activation of caspase-8- protein. Intrinsic pathway is mediated by activation of caspase-9 protein and is associated with the failure of mitochondrial membrane integrity. A dysfunction of the mitochondrial membrane integrity is triggered by increased expression of proapoptotic Bax protein compared to the antiapoptotic factor Bcl2 which could lead to the release of cytochrome-C from the mitochondria to induce activation of caspase-9. Besides these two major pathways of apoptosis, there is another apoptosis pathway involved with the stress signaling of the endoplasmic reticulum (ER), which leads to the activation of caspase-12. These activations of caspases can further activate Caspase-3 protein which could induce apoptosis by inducing the DNA fragmentation. Our experimental evidence showed that nutrient deprivation induced th activation of caspase-9 and Pls treatments inhibited this effectively (Fig. 13.3) [7]. In the adult brain, neuronal cell death can occur by a sudden deficiency of nutrients, especially during the ischemic condition such as

Table 13.1 Relative composition of purified Pls from scallop and chicken breast meat

Types of fatty acids-containing Pls-Etn	Pls from scallop (% of Pls-Etn)	Pls from chicken breast meat (% of Pls-Etn)
Eicosapentaenoic acid (EPA) (20:5)	23.9%	–
Docosahexaenoic acid (DHA) (22:6)	38.9%	18.6%
Arachodonic acid (20:4)	9%	24.9%
α-Linolenic acid (18:3)	1%	7.2%
Linoleic acid (18:2)	1.2%	4.1%
Oleic acid (18:1)	2.6%	26.3%
Stearic acid (18:0)	2%	2.2%
Palmitic acid (16:0)	5.2%	3.6%
Others	16.2%	13.1%

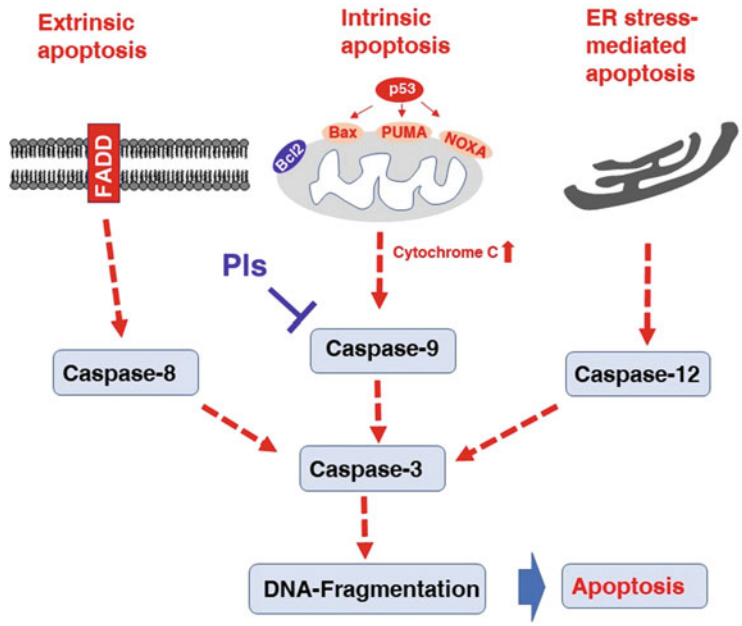

Fig. 13.3 Pls inhibit neuronal cell death by attenuating activation of Caspase-9. Neuronal cells undergo apoptosis by intrinsic pathway by nutrient deprivation. The nutrient deprivation did not activate extrinsic and endoplasmic reticulum (ER) stress-mediated apoptosis pathways. The Pls treatments inhibited intrinsic apoptosis pathways associated with inhibition of caspase-9, caspase-3 and DNA fragmentation. The intrinsic pathway is caused by mitochondria-mediated release of cytochrome-C, which is associated with the abnormal expression pattern of various mitochondrial membrane proteins including Bax, PUMA and NOXA. The expression of these proteins are known to be regulated by apoptosis inducer protein p53. (Sources: Hossain et al., *Plos One,* 2013)

following brain stroke. The stroke-induced neuronal cell death is often associated with the activation of Caspase-9, an intrinsic pathway [8]. It has been shown that Pls inhibit apoptosis of neuronal-like cells (Neuro2A) originated from adult mice, further suggesting that Pls might prevent neuronal cell death in the adult brain following stroke. Future studies are necessary to address this issue to explore a novel therapeutic strategy to reduce the brain injury following stroke.

13.4 Plasmalogens and Neuroinflammation

13.4.1 Reduction of Pls-Caused Neuroinflammation

Neuroinflammation is associated with neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD). The reduction of Pls has been found in the postmortem brains of patients with AD. However, it was not known whether brain Pls could be reduced at the early stage of AD, especially when the neuroinflammation could be seen without massive accumulation of Aβ proteins. It has been known that neuroinflammation occurs at the early stage of AD including the people with MCI (mild cognitive impairment) [9]. Professor Fujino's group found that the Pls are reduced in serum and the red blood cells (RBC) in patients with MCI compared to the control healthy individuals (manuscript in preparation). This evidence could indicate that Pls are reduced at the early stages of AD, which could be one of the risk factors for neurodegenerative diseases. To understand what would happen when brain Pls are reduced, we knocked down the Pls-synthesizing enzyme GNPAT in the mouse brain. Surprisingly, we observed that the GNPAT knockdown-mediated reduction of brain Pls increased the activation of glial cells associated with upregulation of pro-inflammatory cytokines [10]. These findings suggest that the reduction of brain Pls is one of the causes of neuroinflammation, and it might induce the progression of AD. Various studies showed that neuroinflammation caused by LPS injection in mice could increase the accumulation of Aβ proteins and phosphorylation of tau proteins (p-Tau) in the brain. Therefore, it is likely that the reduction of brain Pls could lead the progression of AD by enhancing neuroinflammation-mediated accumulation of toxic substances in the brain such as Aβ and p-Tau. Neurofibrillary tangles (NFTs), marked by excessive accumulation of p-Tau proteins, are found in the progressive stages of AD but not in patients with MCI, suggesting that prolonged neuroinflammation could result in the formation of NFTs. Therefore, inhibition of neuroinflammation at the early stages of AD could prevent the disease progression and prolong the life span.

13.4.2 Inflammation Can Reduce Pls

13.4.2.1 Pls Biosynthesis

The schematic diagram (Fig. 13.4) shows the enzymes involved in Pls biosynthesis pathway in cells [11, 12]. Two known peroxisome-bound enzymes involved in Pls synthesis are glyceronephosphate O-acyltransferase (GNPAT) and alkyl glyceronephosphate synthase (AGPS). The fatty acyl-CoA reductase 1 (FAR1), present in the peroxisomal membrane, catalytically converts lipogenesis-derived acyl-CoA to fatty alcohols necessary for Pls synthesis in the cells. It is known that the catalytic N-terminal domain of FAR1 faces outside of peroxisome membrane and the C-terminal is inside of peroxisome. In peroxisome, the intermediate product of glycolytic pathway, dihydroxyacetone phosphate (DHAP), and fatty alcohols undergo enzymatic reactions by GNPAT and AGPS to produce 1-0-alkyl-DHAP. The peroxisome-derived product, 1-0-alkyl-DHAP, can then undergo various enzymatic reactions in the endoplasmic reticulum (ER) to form Pls. Depending on the cell types, the head group of Pls can be ethanolamine, choline, or serine. In the brain it is mostly ethanolamine, whereas choline-type Pls are enriched in the heart tissue and the serine-type Pls are enriched in the retina. Although GNPAT and AGPS catalyze two different reactions of Pls synthesis, they can physically interact with each other for their activation [13], suggesting that the reduction of either protein, GNPAT, or AGPS can cause a reduction of Pls biosynthesis. This can be suggested further by the findings that mutation of either of these genes resulted in the reduction of Pls [14].

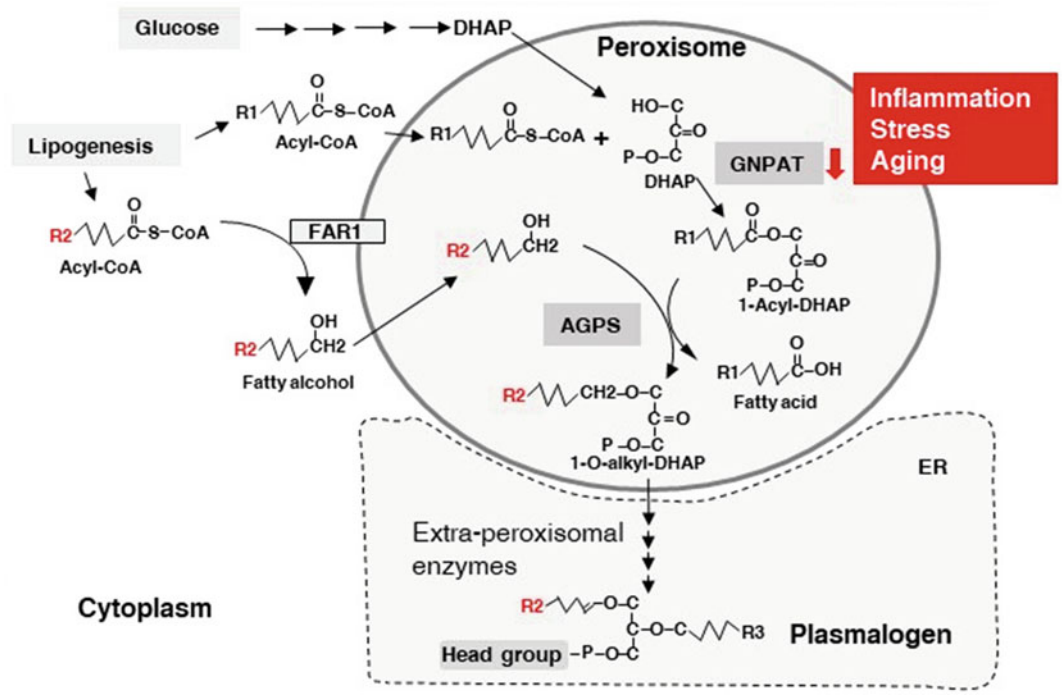

Fig. 13.4 Schematic diagram shows the plasmalogen biosynthesis pathway in cells. Pls were reduced by inflammation, stress and aging associated with the reduction of Pls synthesizing enzyme GNPAT. *DHAP* Dihydroxyacetone phosphate, *FAR1* Fatty acyl-CoA reductase 1, *GNPAT* Glyceronephosphate O-acyltransferase, *AGPS* Alkyl glyceronephosphate synthase, *ER* Endoplasmic reticulum, *R1,R2* Fatty acid chains, *Head group* Ethanolamine, choline, serine, etc

13.4.2.2 Inflammation in Various Diseases and Reduction of Plasmalogens

Inflammation is an event to fight against infection or pathogenic substances that are harmful to our health. Although inflammation is one kind of defensive events of our body, chronic inflammation could cause various disorders. The brain inflammation, marked with the increased number of activated glial cells, is often found among neurodegenerative diseases including AD [15]. It has been known that Pls are reduced among the patients with AD and PD (Table 13.2). Inflammation is also found to be associated with some cancers including colon cancer, where chronic bowel inflammation is one of the main pathogenic factors. It is unknown whether the reduction of Pls could be associated with cancers including colon cancer. However, it is likely that a reduction of Pls could accelerate inflammation of macrophages underlying the colon tissues, which might cause the formation of colon cancer. Inflammation of macrophages is known to be associated with the ulcerative colitis and in inflammatory bowel syndrome. It has not been known whether Pls are reduced by ulcerative colitis or by inflammatory bowel syndrome. However, we detected the reduction of Pls contents in colon tissues of DSS-mediated Inflammatory bowel disease (IBD) model mice (manuscript in preparation), which could suggest that Pls might be reduced in colon tissues during inflammation by the similar mechanism found in glial cells. We previously found the inflammation reduced Pls in microglial cells which are one kind of macrophages present in the central nervous system [10]. The adipose tissue inflammation, marked by the increased number of activated macrophages within adipose tissue, is reported in patients with diabetes. The activated

Table 13.2 Reduction of plasmalogens in various diseases and pathological conditions

Diseases/pathological conditions	Reduction of plasmalogen	References
Rhizomelic chondrodysplasia punctate (RCDP)	Reduction of PlsEtn in serum	(18)
Alzheimer's disease (AD)	Reduction of blood and brain PlsEtn	(14,19,20)
Parkinson's disease (PD)	Reduction of PlsEtn in lipid rafts of the brain tissue	(21,30)
Chronic heart disease (CHD)	Reduction of PlsEtn in plasma and erythrocytes	DOI:https://doi.org/10.9734/CA/2018/44602
Down syndrome	Reduction in PlsEtn in frontal cortex and cerebellum	(22)
Schizophrenia	Reduced PlsEtn and PlsChoin plasma	(23,24)
Autism	Reduced PlsEtn in plasma and in erythrocytes	(25)
Diabetes	Reduction of Pls in serum and red blood cells (RBC)	Endocrine Abstracts (2019) 63 P572I DOI: 10.1530/endoabs.63.P572
Multiple sclerosis (MS)	Reduction of PlsEtn in cerebral cortex	(26,27)
	Reduction of DHA-containing PlsEtn in serum	
Ischemia/lschemic stroke	Reduction of Pls in spinal cord ischemia in rabbits	(28,29)
	Reduction of PlsEtn in the rat model of ischemic stroke	
Neuroinflammation and stress	Reduction of PlsEtn in the mice brain	(10)
	Reduction of PlsEtn in murine glial cells by treatments with LPS and IL-1β	

macrophages release cytokines which could reduce insulin sensitivity of surrounding adipocyte cells resulting in the type 2 diabetes (T2DM). In type 1 diabetes, the chronic inflammation increased reactive T cells, inflammatory cytokines, and monocytic cells that could destroy the insulin-producing pancreatic β cells [16]. Therefore, inflammation of pancreatic tissue is believed to be one of the causes of diabetes. Increased pro-inflammatory cytokines, including TNF-α and IL-1β, were also reported among diabetic patients [17], suggesting further that inflammation is closely linked to metabolic disorders. It has been reported that Pls are reduced in RBC cell membrane and in blood plasma among the diabetic individuals when compared with healthy individuals (*Endocrine Abstracts (2019) 63 P572 | DOI:* https://doi.org/10.1530/endoabs.63.P572). The reduction of Pls in various diseases is shown in Table 13.2 [10, 14, 18–30]. Although Pls are found to be reduced in serum of diabetes patients, it remained unknown whether Pls are also reduced in the activated macrophages within the adipose tissue. Further studies will be necessary to address this issue to suggest that the reduction of Pls in macrophages is the pathogenic marker for diabetes. Atherosclerosis is the main cause of cardiovascular diseases and is caused by inflammation. In atherosclerosis, cytokines released from activated macrophages in the blood vessels can cause the inflammation resulting in the formation of plaques by foam cells. The foam cells are converted from the activated macrophages in the atherosclerosis plaques. Clinical studies showed that Pls are reduced among patients with chronic heart diseases (CHD) compared to the control age-matched healthy individuals (Cardiology

and Angiology: An International Journal, DOI: https://doi.org/10.9734/CA/2018/44602)
(Table 13.2). The reduction of Pls was found in the blood plasma and cell membrane of the RBC among the patients with CHD, suggesting a possibility that reduction of Pls might be linked with the activation of the macrophages and formation of atherosclerosis plaques. Inflammation is also reported to be associated with the severity of chronic fatigue syndrome (CFS) [31]. CFS is linked to anxiety- and depression-like symptoms. In animal experiments, systemic inflammation by injection with LPS often causes behavioral changes related to major depression and fatigue [32]. Inflammation-mediated increase of cytokines in the brain and peripheral organs might be directly linked with pathogenesis of depression-like behaviors. During the systemic inflammation by LPS injection in mice, we observed a reduction of brain Pls associated with neuroinflammation. This evidence could suggest that reduction of brain Pls could induce CFS and depression-like behaviors. It has been reported that there is an elevated level of pro-inflammatory cytokines in blood samples of major depression patients [33]. Although it remained unknown whether Pls are reduced in patients with major depression- or anxiety-related disorders, it could be likely that oral ingestion of Pls could reduce systemic inflammation in these patients and improve their health condition. It remained mostly unknown how the peripheral cytokines could affect brain function to elicit depression-like behaviors. Future studies will be necessary to address this issue. Oral ingestion of Pls improved cognitive function in AD patients [34], suggesting that a therapeutic approach by Pls supplement might be beneficial in other diseases marked with the Pls reduction.

13.4.3 Mechanism of Pls Reduction

Reduction of Pls is observed in many disease conditions, but the mechanism remained mostly elusive. There are two most possible causes of Pls reduction such as (1) genetic causes and (2) enzymatic degradation.

13.4.3.1 Reduction of Pls by Gene Mutations

It has been well known that mutation of the genes, encoding Pls biosynthetic enzymes, is associated with the reduction of Pls. One of the common examples is the mutations of GNPAT gene in RCDP patients [35]. Interestingly, the GNPAT knockout mice showed RCDP-like phenotypes with the reduction of blood Pls, suggesting that inhibiting GNPAT can induce the disease progression. Mutation in AGPS gene was also reported in RCDP patients associated with the reduction of Pls [35]. Mutation in the FAR1 gene has also been reported in RCDP patients [36]. Genetic mutations of these genes in other diseases associated with Pls deficiency are poorly studied. Besides the genetic mutation, downregulation of these genes could also reduce Pls.

13.4.3.2 Reduction of Pls by the Enzymatic Degradation

The Pls, like other glycerophospholipids, could be degraded by various enzymes including the phospholipase A2 (PLA2) [37]. PLA2 catalyzes the degradation of phospholipids at sn-2 position, and activation of this enzyme was found in AD [38]. Therefore, it could be possible that an increased activity of PLA2 may be one of the causes of Pls reduction in the brain. There are two kinds of PLA2: cytosolic and extracellular. PLA2 activity could produce free fatty acids and the lyso-Pls from Pls. The lyso-Pls might also have Pls-like activity because they contain the vinyl ether bond at the sn-1 position. Further studies are necessary to address whether lyso-Pls and free fatty acid could have biological function like Pls.

13.4.3.3 Reduction of Pls in the Brain by Inflammation, Stress, and Aging

It has already been argued before that inflammation is associated with many diseases and some of them were also characterized with the reduction of Pls. This evidence suggested that there might be some common mechanisms of Pls reduction in cells, especially in macrophages. Glial cells are

one kind of macrophages present in the central nervous system, and it has been found that inflammation reduced Pls in these cells. Activation of Glial cells, characterized by the increased number in reactive glial cells, are one of the markers of neuroinflammation in the brain. Resting glial cells can transform to reactive glial cells by the treatments of bacterial toxin called lipopolysaccharide (LPS), which has been used as the model of neuroinflammation. Glial cells could induce expression of pro-inflammatory cytokines including TNF-α and IL-1β by the LPS treatments. The intraperitoneal injection (i.p.) of LPS can cause peripheral and brain inflammation marked with the increased number of reactive glial cells in the brain. In these model mice of neuroinflammation, we found a significant reduction of Pls in the brain. The LPS treatments also reduced cellular Pls in murine glial cells including microglia and astrocytes. To examine the mechanism how Pls are reduced by neuroinflammation, we studied genetics of GNPAT promoter (Fig. 13.5). Bioinformatics studies showed that GNPAT promoter has two c-Myc binding sites near the transcriptional start site. By chromatin immunoprecipitation (ChIP) studies, we found that inflammatory signals increased recruitment of c-Myc transcription factor onto the *GNPAT* transcription start site (Fig. 13.5). The c-Myc is a transcription factor which has various beneficial roles in cells including DNA replication during cell division. However, excess activation or amplification of c-Myc gene is often associated with bad prognosis of cancers. The c-Myc can transcriptionally activate or suppress various gene expressions, which depend on the co-factors that bind with c-Myc. The c-Myc-mediated transcriptional suppression of target gene is known to be mediated by binding with Miz1 protein. The c-Myc and Miz1 protein complex could displace p300 co-activator from the promoter region of target genes, resulting in the transcriptional inhibition from the nearby gene promoter. It is known that the recruitment of c-Myc protein onto the *GNPAT* promoter was prerequisite for downregulation of transcription from *GNPAT* promoter. Therefore, inflammation could reduce *GNPAT* expression by increasing recruitment of c-Myc proteins onto the *GNPAT* promoter. This is the genetic mechanism of Pls reduction in glial cells, and similar phenomena were also found in the murine brain by neuroinflammation, stress, and aging. The chronic restrain stress in mice also reduced Pls in the murine brain. Pls were also found to be reduced in the brain of old mice. Interestingly, increased expression of c-Myc and reduction of *GNPAT* were observed in AD model mice and in the postmortem AD brain tissues [10], which suggests that c-Myc-mediated downregulation of *GNPAT* could be one of the common causes of Pls reduction by neuroinflammation, stress, and aging. In our study, the reduction of Pls by LPS treatments was observed among glial cells, but not in neuronal cells, which could be because neuronal cells have very low expression of TLR4 proteins. Interestingly, we observed the upregulation of c-Myc protein also in neuronal cells in by LPS-mediated neuroinflammation [10]. This could be explained by the fact that during neuroinflammation various cytokines can be released from the glial cells which could induce activation of NF-kB in neuronal cells to induce c-Myc protein, which could reduce Pls synthesis in neuronal cells. Therefore, we could propose that neuroinflammation might reduce Pls in the brain not only in glial cells but also in neuronal cells probably by the increased activity of c-Myc protein.

The c-Myc could be increased by NF-kB, because of the presence of NF-kB binding consensus sequences onto the c-Myc promoter (Fig. 13.5). Therefore, during inflammation processes, the NF-kB is recruited onto the c-Myc promoter and induces its transcription, resulting in upregulation of c-Myc gene expression. Expression of c-Myc gene is increased by aging and is believed to be a risk factor for cancer progression among older people. The increased expression of c-Myc among cancer patients is often associated with amplification of c-Myc genomic region. In neurodegenerative brains, the c-Myc expression is also found to be increased but is not associated with the amplification of the genome. This increased expression of c-Myc could be due to the increased level of inflammatory events in the brain by aging

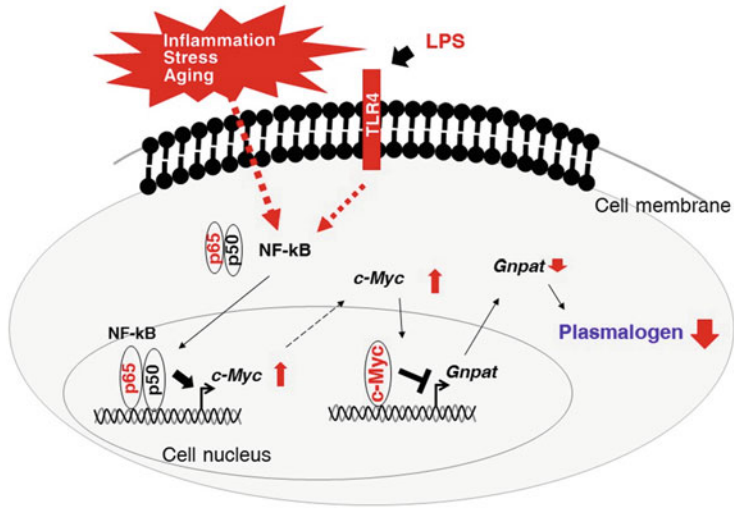

Fig. 13.5 Schematic diagram showing reduction of Pls in glial cells by inflammation. Inflammation, stress and aging enhanced NF*k*B (heteromeric complex of p65 and p50) activity to reduce Pls. LPS is the bacterial toxin that activates TLR4 to induce inflammation, a model for neuroinflammation. Activation of NF-kB increased transcription from c-Myc promoter, resulting in an increase in expression of c-Myc protein in cells. The c-Myc transcription factor recruitment onto the *Gnpat* promoter reduced the transcription, resulting in downregulation of *Gnpat* expression. Downregulation of *Gnpat* by inflammation could reduce plasmalogen synthesis in glial cells. (Source: Hossain et al., *J. Neuroscience,* 2017)

processes. The c-Myc expression is increased in astrocytes of the brain tissues of patients with AD and Parkinson's disease (PD) [39]. Myc haploinsufficient mice ($Myc^{-/+}$) live longer than the wild-type mice, suggesting that reduction of Myc protein could enhance life span [40]. Therefore, the increase of c-Myc in older people could accelerate aging process and could reduce life span, which could be due to the reduction of Pls contents. We can suggest that the increased level of Pls and reduced level of c-Myc protein could reduce the risk of cancer and increase life span (Fig. 13.6). It is still unknown whether Pls can reduce c-Myc expression and inhibit cancer progression. More studies will be necessary to address this issue. It will be very important to know the contents of Pls in the c-Myc-amplified tumors to examine the role of Pls in tumor growth and regression. Chronic inflammation is often associated with cancers such as colitis, pancreatitis, and hepatitis which are linked to colon, pancreatic, and liver cancers, respectively. The Pls could be reduced by those chronic inflammation processes, resulting in the increase of risk of having cancers. Therefore, any therapeutic approach to increase Pls in the affected tissues might have promising anti-inflammation and anticancer activities. Additional studies will be necessary to address these issues.

13.4.4 Pls Inhibit Neuroinflammation

It has been known that inflammation signals reduce cellular Pls by downregulating the Pls-synthesizing enzyme called GNPAT via activation of NF-*k*B and c-Myc. Therefore, the next question which remained unknown was whether Pls could prevent neuroinflammation. Interestingly, i.p. injection of purified ethanolamine Pls (Pls-Etn) effectively inhibited neuroinflammation in the murine brain [41]. The i.p. injection of Pls-Etn attenuated the downregulation of Pls contents during neuroinflammation caused by LPS. These findings suggest that peripheral injection of Pls could prevent the reduction of Pls

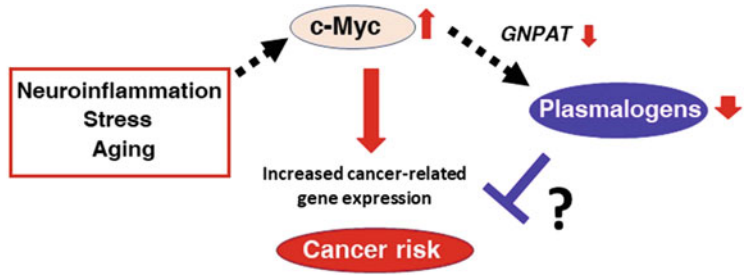

Fig. 13.6 Schematic diagram shows the c-Myc-mediated reduction of Pls might increase the risk of cancer. Neuroinflammation, stress and aging increased c-Myc. The c-Myc could increase cancer risk by inducing proto-oncogene expression. c-Myc could also reduce Pls by downregulating GNPAT expression. Reduction of Pls by c-Myc might accelerate cancer progression, suggesting that Pls might have inhibitory effects on cancer progression

during brain inflammation. Therefore, it is possible that Pls intake can prevent brain inflammation. Here, the brain inflammation means the activation of glial cells in the murine brain by i.p. injection of LPS. In addition to the i.p. injection of Pls, the oral intake of Pls could also prevent neuroinflammation and reduce accumulation of Aβ in the brain cells including neurons and astrocytes [42]. It is still unknown how the peripherally injected Pls prevented neuroinflammation and attenuated the reduction of Pls in the brain. Further studies are necessary to address this issue. Direct application of Pls to the cultured microglial cells prevented the LPS-mediated inflammatory signals in microglial cells and reduced expression of pro-inflammatory cytokines including IL-1β, TNF-α, and MCP-1 [41–44]. This evidence suggests that Pls could have direct effects on the glial cells to protect from inflammation. To examine the mechanism how Pls could inhibit neuroinflammation, we investigated the inflammatory processes in microglial cells by in vitro experiments. It has been known that endocytosis of TLR4 is one of the key events to induce inflammatory signal in microglial cells. We found that the inhibition of TLR4 endocytosis by dynasore, a GTPase inhibitor, in microglial cells effectively inhibited LPS-mediated inflammatory signal [43]. In addition, inhibition of caspase-3 also prevented the LPS-induced inflammatory signal. These results suggest that in microglial cells, LPS-mediated inflammatory signal is effectively controlled by two events: the first is the endocytosis of TLR4 and the second is the activation of caspase-3. Activation of caspase-3 is found to be associated with induction of NF-kB-mediated transcriptional regulation of pro-inflammatory genes (Fig. 13.7). Treatments of microglial cells by purified Pls extracted from a scallop, which are rich in the DHA-containing Pls, inhibited endocytosis of TLR4 (Fig. 13.7) [43]. The endocytosis process of TLR4 is influenced by various factors. It is known that LPS treatments could enhance endocytosis of TLR4 by increasing its recruitment with the adaptor proteins such as CD14 and MD2. LPS treatments enhance the formation of the complex, TLR4-CD14-MD2, which is necessary for the endocytosis of TLR4 to induce inflammatory signal in glial cells. Pro-inflammatory cytokines are not always bad for the health, because they also have defensive roles against infection and other minor inflammation. However, nitric oxide (NO) is a signal molecule which is well known to be involved in the pathogenesis of inflammation-related diseases. The increased level of NO is produced by overactivity of nitric oxide synthase-2 (NOS2) gene in cells. Overproduction of NOS2 could induce NO which is able to maintain the inflammatory signals in various diseases including rheumatoid arthritis and lung cancers. Macrophage-inducible NO production could initiate tumor cell growth such as in squamous cell carcinoma (SCC)

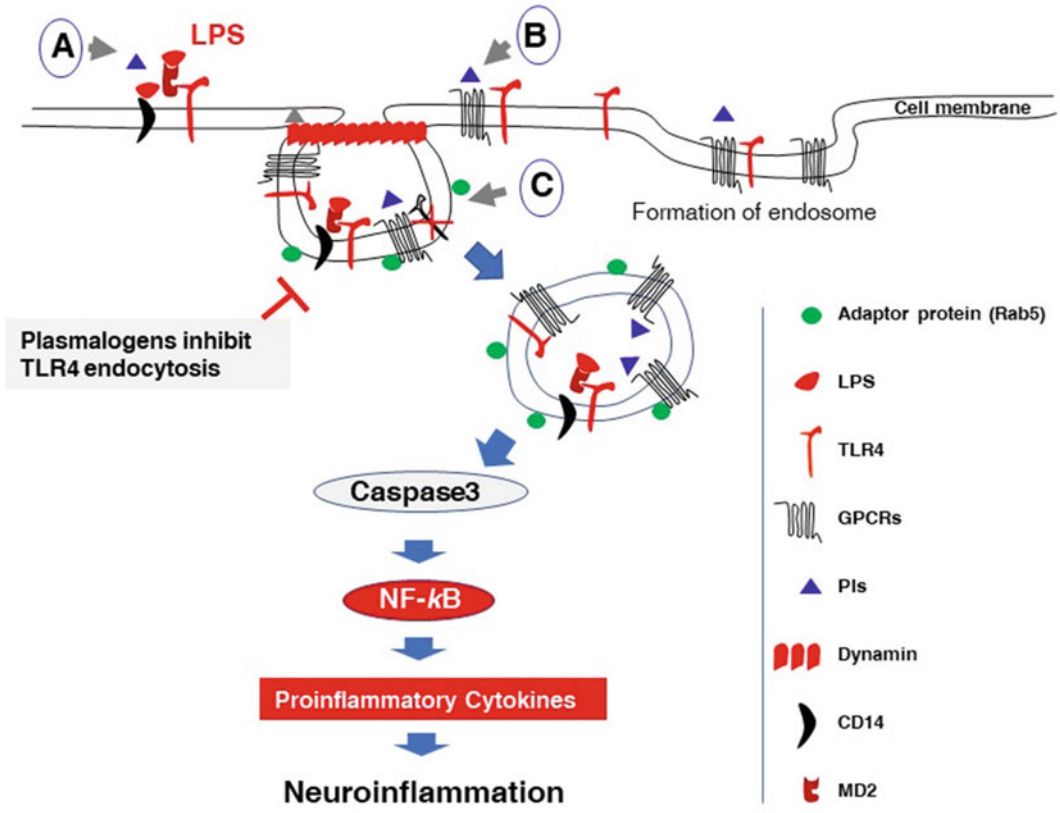

Fig. 13.7 Schematic diagram of Endocytosis of TLR4 and the inhibitory mechanism of plasmalogens. (**a**) Pls might function as ligands for CD14 or MD2 to block the LPS effects on TLR4 activation. (**b**) Pls-mediated activation of the GPCRs (possible ligands for Pls) might inhibit recruitment of CD14 and MD2 to TLR4. (**c**) Pls-mediated activation of the GPCRs might reduce TLR4 recruitment to endosomal vesicles. (Source: Fatma A. et al., Mol. Neurobiol., 2018)

[45]. Elevated NO in serum could be a marker for systemic inflammation. Macrophages are the prime sources of NO, and we examined whether Pls could inhibit NO production during the inflammation. We used the brain macrophage-like cells, microglia, and found that treatments with scallop-Pls inhibited NO production and also inhibited the NOS2 gene expression during the LPS-mediated inflammation (Fig. 13.8) [44]. It has been known that there are two LPS-induced inflammatory signals, MyD88-dependent and MyD88-independent, which can induce NOS2 expression in macrophage-like cells (Fig. 13.8). The scallop-Pls, which are high in PUFA-containing Pls, inhibited both the pathways to attenuate upregulation of NOS2 and NO. The LPS-mediated inflammation also reduced the Pls synthesis by downregulation of two key enzymes of Pls synthesis, GNPAT and AGPS (Fig. 13.8). Pls inhibited not only the NF-kB but also the p38MAPK activity in glial cells [44]. The c-Fos/c-Jun transcriptional activity was involved in upregulation of NOS2 expression in microglial cells, and this was inhibited by DHA-Pls but not by oleic acid Pls [44], suggesting that PUFA-containing Pls are effective in inhibiting inflammation signals in microglial cells. It is still unknown whether the DHA-containing Pls-Etn, which showed the inflammation inhibitory effects in the microglial

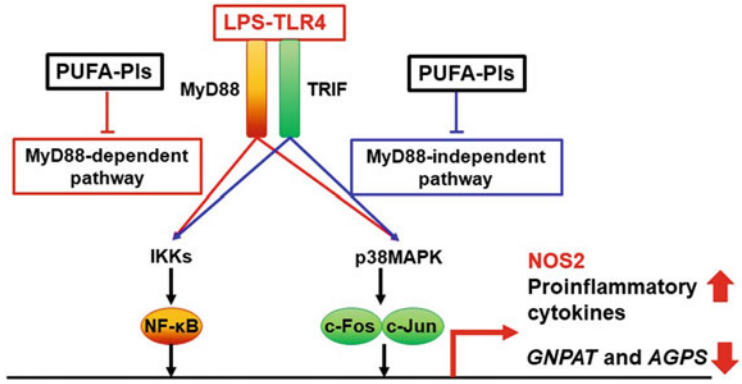

Fig. 13.8 PUFA-Pls inhibit LPS-mediated inflammatory processes and *NOS2* expression in microglial cells. PUFA-Pls could inhibit both MyD88-dependent and independent pathways of inflammatory signals in microglial cells. Activation of NF-kB and p38MAPK were inhibited by Pls. LPS-mediated inflammatory signals induced NOS2 expression and at the same time reduced the Pls synthesizing enzymes GNPAT and AGPS. (Source: Youssef et al., Neuroscience, 2019)

cells, is the main Pls component to show anti-inflammatory activities. Additional experiments will be necessary to address this issue by using the other PUFA-containing Pls inducing EPA and arachidonic acids.

13.5 Plasmalogens and Cognitive Function

Pls have long been known to be reduced among AD patients especially in the brain and serum, but it remained unknown whether Pls had any influences on cognitive function. In experimental mice, and in human subjects, the deficiency of cognitive function has been linked with brain inflammation. Although the ethanolamine Pls were found to be reduced in the postmortem brains of AD, it is likely that the downregulation of Pls can occur at the early stage of disease progression especially before the excessive accumulation of amyloid beta in the brain. This can be suggested by the findings that Pls-Etn are reduced in serum among the patients with MCI. MCI is the early stage of AD and the excessive accumulation of Aβ is not seen in these brains.

13.5.1 Plasmalogens Enhance Memory

In the clinical trial, oral ingestion of Pls showed beneficial effects to improve memory [34]. The oral ingestion of Pls for 6 months improved cognitive function among patients with AD and MCI. In this clinical study, the scallop-derived Pls which are high with DHA- and EPA-containing Pls were used. We still do not know whether the Pls, extracted from chicken, which are high with arachidonic acid-containing Pls, are also effective to enhance cognitive function of patients with AD. In Morris water maze memory test, it has been found that oral ingestion of scallop-Pls could improve learning and memory (manuscript in preparation). Therefore, oral intake of Pls has cognitive improvement function in mice and in human. These findings are very interesting especially in the aspect of therapeutic potential to improve cognitive function. The clinical studies were carried out among the patients with cognitive impairments. Therefore, it is still unknown whether oral intake of Pls could prevent the onset of AD. Additional experiments will be necessary to address this issue. Pls were found to be reduced at the early stage of AD and among the aged mice, which suggests that a reduction of Pls might initiate the progression of AD. Therefore, it

could be likely that oral ingestion of Pls might prevent the onset of AD. This hypothesis could be supported by our recent findings that daily intake of Pls prevented the onset of AD-like pathologies such as accumulation of Aβ in the brain of triple transgenic AD (3xTg-AD) model mice (manuscript in preparation). It is still not known how the oral intake of Pls prevented the accumulation of Aβ proteins in the brain of AD mice. Additional studies could reveal the mechanism how the orally ingested Pls could prevent the onset of AD. We previously found that brain inflammation reduced learning and memory among experimental mice and the oral intake of Pls attenuated the learning and memory processes associated with attenuation of glial activation in the brain [42]. The changes in the brain to boost cognitive function among patients with AD who underwent clinical studies of oral intake of Pls remained largely unknown. To examine the memory-related changes in the brain by Pls diet, we performed mice experiment.

13.5.2 Plasmalogens Induce Memory-Related Gene Expression in Neuronal Cells

It remained unknown how the oral intake of Pls improved cognitive function among the AD patients. To examine the possible memory-boosting role of plasmalogens, we performed mice study and in vitro studies with cultured neuronal cells. Oral ingestion of scallop-Pls for 2 months improved learning and memory tasks in Morris water maze test. This memory is called hippocampal-dependent spatial memory. Spatial memory is also found to be reduced in patients with AD and even in older population. The brain-derived neurotrophic factor (BDNF) is a well-known neuropeptide that plays a crucial role in maintaining spatial memory in mice and in human. The reduction of hippocampal BDNF might reduce spatial learning and memory. BDNF could play one of the key roles in memory process because of their ability to regulate various events related to memory such as increasing the dendritic spines and enhancing the synaptic transmission (Fig. 13.9). Interestingly, the oral ingestion of Pls increased expression of BDNF in the hippocampus of adult mice and associated with the improvement of learning and memory tasks. These findings could suggest a possible mechanism of Pls-mediated improvement of cognitive function in patients with AD. BDNF could be secreted from neuronal cells and astrocytes in the brain. The Pls treatments in cultured neuronal cells induced BDNF expression and associated with the increased phosphorylation of AKT, ERK, and the transcriptional factor cAMP response element-binding (CREB) proteins (Fig. 13.9). Bioinformatic studies revealed that there are various CREB bindings sites onto the BDNF promoter and the Pls treatments increased the recruitment of p-CREB protein onto those binding sites, resulting in upregulation of *BDNF* transcription (Fig. 13.9). We previously found that Pls treatments increased phosphorylation of ERK and AKT in neuronal cells via the membrane-bound GPCR proteins [7, 46]. Activation of ERK and AKT could increase phosphorylation of CREB and could enhance their localization into the nucleus, resulting in the recruitments onto the BDNF promoter. The increased BDNF in extracellular fluids could increase translocation of the kinase receptor TrkB (receptor for BDNF) into lipid rafts (Fig. 13.9). The recruitment of TrkB or recruitment of GPCR in the lipid rafts could induce cellular signaling to enhance gene expression related to synaptic function and memory processes (Fig. 13.9). The Pls-mediated memory signaling is positively regulated by BDNF, and it is also known that BDNF stimulation could also induce Pls in neuronal cells. Therefore, Pls could have a very vital role in the memory processes of humans via their potentials to regulate BDNF in the hippocampus. Additional experiments are necessary to address this in the future.

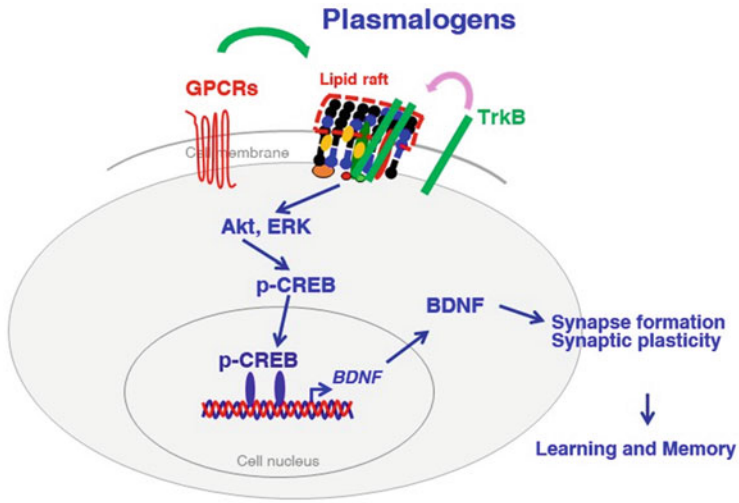

Fig. 13.9 Schematic diagram showing the possible mechanism of Pls-mediated memory signaling in neuronal cells. Lipid raft localization of TrkB and GPCRs (possible receptors for Pls) could induce activation of AKT and ERK proteins. AKT and ERK could induce phosphorylation of CREB transcription factor which could be recruited onto the *BDNF* promoter to induce its transcription. Pls-mediated BDNF induction in neuronal cells could play one of the major roles in improving cognitive function by increasing dendritic spines and synaptic plasticity

13.5.3 Reduction of Brain Pls Inhibits Learning and Memory Performance

It has been known that oral intake of Pls improved cognitive function among AD patients and in mice. However, it was mostly unknown whether a reduction of brain Pls itself could reduce cognitive function. The hippocampus is the key region of the brain controlling spatial memory. Spatial memory can be analyzed in mice by Morris water maze (MWM) tests. The MWM tests are one kind of navigation tasks which is often used to check hippocampal-mediated learning and memory process. We found a significant reduction of learning and memory tasks in mice when Pls concentration was reduced in the hippocampus by knockdown of Pls-synthesizing enzyme GNPAT (manuscript in submission). This finding suggests that reduction of brain Pls has negative effects on cognitive function in mice and probably also in humans. In mice, Pls reduction in the hippocampus caused the downregulation of *BDNF* gene associated with reduced expression of phosphorylated AKT and ERK proteins (Fig. 13.9). The Pls reduction in neuronal cells reduced recruitment of CREB transcriptional factor onto the *BDNF* promoter (manuscript in submission). In addition, the reduction of brain Pls showed a reduction of dendritic spines of the hippocampal neurons. Direct application of Pls in cultured neurons increased the dendritic spines, suggesting that Pls could induce BDNF expression to regulate dendritic function in maintaining memory processes in the hippocampus. Therefore, a reduction of hippocampal Pls content might impair cognitive function because of the reduction of BDNF. It is still unknown whether BDNF is reduced in the hippocampus of the AD patient's brain especially when Pls contents were decreased.

13.5.4 Plasmalogens Might Inhibit Systemic Inflammation to Enhance Cognitive Function

Besides the direct effect of Pls in the brain cells, it might also be possible that orally ingested Pls

could inhibit systemic inflammation to reduce neuroinflammation in the brain. Oral ingestion of Pls might inhibit the production of peripheral pro-inflammatory cytokines. In the gut, pathogenic bacteria can produce LPS that might induce systemic inflammation in older people. This systemic inflammation could have negative effects in cognitive function. In our previous study, we found that LPS injection reduced learning and memory in mice associated with the increased neuroinflammation in the brain. The oral ingestion of Pls inhibited this neuroinflammation in those mice, suggesting that Pls could prevent inflammatory signals to improve cognitive function [42]. Therefore, it is also likely that Pls-mediated improvement of cognitive function among the AD patients might be associated with the reduction of serum pro-inflammatory cytokines. Additional studies will be necessary to address whether Pls drinking could inhibit systemic inflammation or inflammation in gut macrophages. It has been known that systemic inflammation is increased among older people compared to young adult, suggesting that the anti-inflammatory role of Pls might be involved in part to improve the cognition.

13.5.5 Reduction of Brain Pls Is One of the Key Pathogenic Factors in Neurodegenerative Diseases

We recently identified that glial cells could secrete ethanolamine Pls (Pls-Etn) in extracellular medium, suggesting that microglia and astrocytes might also secrete Pls in the brain which could act on neuronal cells to modulate the brain function including cognitive function (Fig. 13.10). We previously argued that Pls synthesis in the glial cells could be reduced by aging, stress, and inflammation. The reduction of Pls in the brain might be one of the key pathogenic factors for neurodegenerative diseases associated with neuroinflammation. There are about 85 billion glial cells in the human brain, and they occupy about half the volume of the brain and spinal cord. The enrichment of glial cells in the brain gives rise to a possible hypothesis that a constant supply of Pls by glial cells could have tremendous effects on the neurons (Fig. 13.10). Pls are shown to have various beneficial effects related to neuronal function, which strongly suggests that a reduction of these lipids might have worse effects in the brain which could enhance the chances to have neurodegenerative diseases. These lipids are also reduced in neurodegenerative diseases including AD and PD. We also found that reactive glial cells in the AD brain have increased c-Myc expression that could reduce Pls synthesis by downregulating GNPAT. It is still unknown how Pls are reduced in the patient's brain with PD, but it could be due to neuroinflammation. Apoptosis or the shrinkages of healthy neurons is associated with neurodegenerative diseases. Because of antiapoptotic role, Pls in the adult brain could prevent neuronal cell death, suggesting that a reduction of brain Pls might lead to the neuronal cell death. Therefore, the reduction of Pls could be one of the major causes for neuronal damages associated with neurodegenerative diseases. A possible therapeutic approach to recover normal content of Pls in the neurodegenerative brains could have tremendous beneficial effects in attenuating the disease progression.

13.6 Plasmalogens and Aging

Many diseases including AD, diabetes, and immune dysfunction are related to aging because they appear to be common among older people compared to young individuals. Here, we discuss some aging-related phenotypes observed in *C. elegans* associated with the deficiency of Pls. It has been known that ethanolamine Pls are also enriched in *C. elegans*.

13.6.1 Deficiency of Pls Reduces Life Span

Interestingly, *C. elegans* has most of the Pls-synthesizing enzymes including *acl-7* (human analog of GNPAT), *fard-1* (human analog of FAR1), and *ads-1* (human analog of

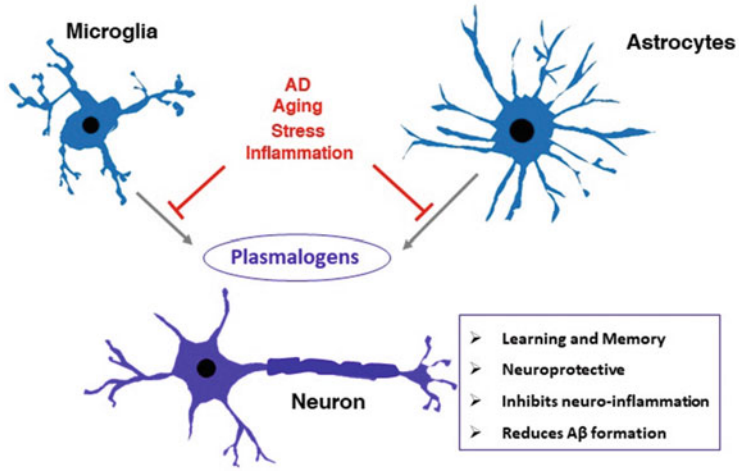

Fig. 13.10 Schematic diagram of biological effects of Plasmalogen in brain. Astrocytes and microglial cells could release plasmalogens in brain aiming to protect neuronal cells from various damages including neuroinflammation. Pls enhance learning and memory processes of brain. Pls reduce formation of toxic amyloid beta proteins in brain. Reduction of Pls in glial cells can be caused by aging, stress, and inflammation. This reduction is also found in patient's brain with Alzheimer's disease (AD)

AGPS). All these mutant (acl-7, ads-1 and fard-1) *C. elegans* showed a reduction of life span (manuscript in preparation). The contents of Pls-Etn were found to be reduced in these mutant *C. elegans* comparted to the wild-type *C. elegans* (N2). Primary evidence showed that reduction of Pls in these mutants was associated with the reduction of sir-2.1 gene (manuscript in preparation). In mouse cell line, Pls treatments induced protein expression of several analogs of sirtuins including Sirt1 (manuscript in preparation). These findings could suggest that Pls might enhance life span by regulating protein expression of sirtuins. Additional experiments will be necessary to address this.

13.6.2 Deficiency of Pls Shows Metabolic Syndrome-like Phenotype

The Pls-deficient *C. elegans* mutants (acl-7, ads-1, and fard-1) showed an increase in fat deposition compared to wild-type *C. elegans* when treated with high concentration of glucose (manuscript in preparation). It has been known that older people are more susceptible to getting fat compared to young individual. The glucose ingestion-mediated fat deposition in *C. elegans* could be a model of obesity. The mechanism of obesity in *C. elegans* could be different from humans. However, the increase in fat deposition among the Pls-deficient *C. elegans* could suggest that reduction of Pls among humans could have worse effects among diabetes patients. It has already been known that Pls are reduced among patients with diabetes (discussed earlier), suggesting that the Pls reduction might accelerate fat deposition among the patients with metabolic syndromes. Additional studies will be necessary to address this issue.

13.6.3 Deficiency of Pls Reduces Immune Function to Fight Against Pathogenic Bacteria

The reduction of immune defense is common among aged people, and they usually get worse effects when infected with bacteria or virus compared to younger people. We observed the increase in mortality of Pls-deficient *C. elegans*

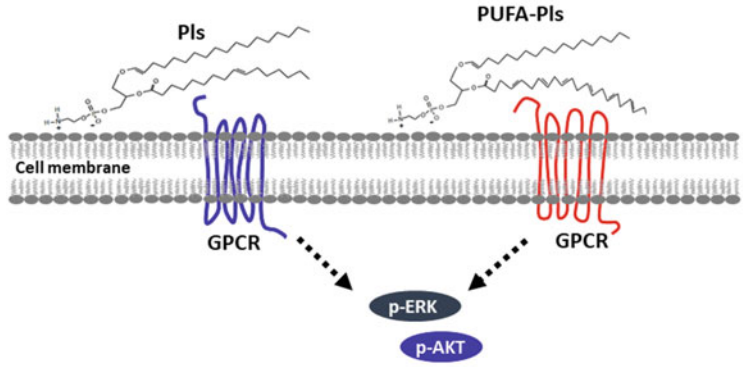

Fig. 13.11 Plasmalogens might function as hormones or ligands to elicit biological effects. Extracellular addition of Pls induces cellular signaling (phosphorylation of AKT and ERK) in a short period of time. Pls induce signaling events via the GPCRs (GPR1, GPR19, GPR21, GPR27, and GPR61). These GPCRs are found to be present not only in the brain but also found in gut epithelial cells, suggesting that Pls might have wider biological effects from brain to gut

mutants (acl-7, ads-1, and fard-1) compared to the wild type when subjected to infection with the pathogenic bacteria *Staphylococcus aureus* (manuscript in preparation). These findings suggest that Pls deficiency could reduce immune function to fight against the infection in *C. elegans*. It could be likely that reduction of Pls among older people might be linked to the reduction of their immune function to fight against bacterial infection or even viral infection. It is still unknown whether the Pls deficiency could be linked with the immune deficiency in human.

13.7 Mechanism of Action of Pls in Inducing Cellular Signaling

13.7.1 Plasmalogens Might Function as Ligands or Hormones to Elicit Biological Effects

Extracellular addition of Pls induces cellular signaling by GPCRs, which suggests that Pls might function as ligands or as hormones. We found that glial cells readily secret plasmalogens in extracellular space and the extracellular Pls can activate signaling molecules in neuronal cells, suggesting that Pls might function as paracrine hormones in the brain. In the periphery, Pls are detected in serum. Therefore, Pls could reach to various target organs and function as endocrine hormones to activate cells having the GPCR (possible receptor for Pls) expression. However, further studies will be necessary to address these issues. The oral ingestion of a very low dose of Pls (1 mg/day) has been shown to improve cognitive function in patients with AD, suggesting that Pls might function as potent bioactive compounds or as hormones. The in vitro studies showed that some membrane-bound GPCRs such as GPR1, GPR19, GPR21, GPR27, and GPR61 could regulate the Pls-mediated induction of cellular signaling. It is likely that Pls could modulate cellular function in any tissues of mammal if they expressed either of these receptors. These GPCRS are highly expressed in neuronal cells, suggesting that glial cell-mediated release of these bioactive phospholipids could stimulate the surrounding neurons by activating these GPCRs (Fig. 13.11). Interestingly, our recent evidence showed that GPR19 and GPR61 are expressed in the gut epithelial cells of mouse. Our study showed the presence of GPR19 and GPR61 in the gut epithelial cells, suggesting further that the low dose of Pls (1 mg/day) could elicit biological effects via activating these gut

GPCRs. In the clinical studies, 6-month oral ingestion of Pls at the low dose (1 mg/day) produced significant effect in improving cognitive function among AD patients. The blood plasma concentration of Pls is about 5 mg/dl, which raised a question on how a low dose of Pls, when ingested orally, could evoke physiological functions including the cognitive function. To answer this, we could suggest that the purified Pls, when ingested orally, could activate the GPCRs in the gut epithelial cells to induce biologically active substances which might affect the brain function either directly or indirectly. Pls are lipid soluble, and it is likely that they can be present in physiological system either as free or as lipoprotein. Lipoprotein formation of Pls has not been known, and it is still not known whether any soluble proteins could bind with free Pls. However, in physiological system like other lipids, Pls could exist as protein bond complexes called lipoproteins. However, when we treated the cells with purified Pls, which were free from any proteins, they could induce cellular signaling within 5 minutes. This evidence could suggest that free Pls are functionally active to induce cellular signaling via the membrane-bound GPCRs. However, the Pls could form lipoproteins inside the cells. The orally administered purified Pls used for the experiments and for the clinical trial were extracted from scallop and free of proteins, which suggests that the free-form of Pls are functionally active even when administered at a very low dose. This evidence could suggest that these special lipids could function as ligands or hormones to activate the GPCRs inside the gut epithelium or other tissues to elicit their biological activities. Pls have the polar hydrophilic group, ethanolamine, which could bind with polar groups on the cell membrane and could interact with surrounding GPCRs (Fig. 13.11). Additional experiments are necessary to address whether Pls could function as ligands or hormones to activate membrane-bound GPCR from outside of the cells.

13.7.2 Plasmalogens Might Change Membrane Dynamics to Induce Cellular Signaling

In addition to the hypothesis that Pls could function from outside of the cells as hormones or ligands, it could also be possible that these lipids can change the membrane dynamics. It has been known that phospholipids have a propensity to form liposome. The property to form liposome is the basis of their use as carriers for liposome-derived drug delivery systems. It is possible that Pls in extracellular space might form liposome because they could form hexagonal structure. Pls with longer tails could form bilayer of the liposome vesicles. These liposomes could integrate on the plasma membrane of live cells called fusion. The fusion of liposome is a well-known event in biological systems. This could change the membrane dynamics and could form lipid raft domains to induce the cellular signaling by recruiting the GPCRs or other membrane-bound receptors (Fig. 13.12). This hypothesis could be supported by the findings that Pls are highly enriched in lipid rafts of hippocampal tissues (manuscript in preparation). We noticed that glial cells can secret Pls-Etn in extracellular space, suggesting that these Pls could form liposome. In the brain, the glial cell-mediated secretion of Pls might lead to the formation of Pls-liposome. The Pls-liposome might fuse on the plasma membrane of the surrounding neurons to induce cellular signaling. It might also be possible that neuronal cells could release neurotransmitter by forming the Pls-liposome in the synaptic clefts. The glial cell-mediated formation of Pls-liposome could also enter in the synaptic cleft to modulate synaptic activity by integrating on axonal terminals or dendritic spines. Therefore, the existence of Pls-liposomes in the brain could have various biological effects probably by activating the membrane-bound GPCRs. Additional experiments will be necessary to address whether the GPCRs, which are the possible receptors of Pls, are present in neuronal synapses.

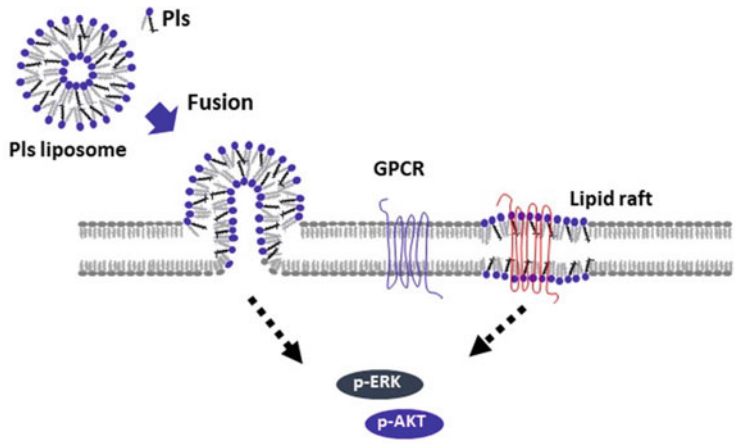

Fig. 13.12 Pls might induce cellular signaling by changing the membrane structure. Extracellular addition of Pls, which could exist as liposome, might integrate into cell membrane to induce cellular signaling by allowing activation of membrane protein(s) including GPCRs. Pls were found to be enriched in lipid raft and their enrichment might enhance localization of GPCRs in these compartments to induce cellular signaling

13.7.3 Other Possible Mode of Action of Plasmalogens

Like other phospholipids, Pls could be degraded to form several bioactive components including free fatty acids and lyso-Pls. These bioactive substances could also play a role to elicit physiological function. In addition to that, Pls could also maintain membrane structures in other intracellular organelles such as the mitochondrial and nuclear membrane. The membrane dynamics of these intracellular organelles could be strictly maintained by Pls, and a physiological reduction of Pls could have detrimental effects. Extracellular addition of Pls showed an increase in nuclear Pls, which suggests that because of their lipid solubility nature, Pls could penetrate the outer cell membrane to enter into the nucleus. The role of Pls in the nucleus is mostly unknown and mysterious. We found that Pls treatments increased transcriptional activity of the nuclear receptor, peroxisome proliferator-activated receptors (PPAR). However, we still do not know whether Pls can be integrated to nuclear membrane to modulate the PPAR activity or Pls could bind directly to PPARs transcription factor to increase their translocation onto genomic DNA. Further studies will be necessary to address these issues.

13.8 Conclusion and Future Direction

The deficiency of Pls in neurodegenerative diseases has been reported a long time ago, but until recently the function of Pls remained elusive. Recent studies showed that these lipids have various biological functions including their ability to inhibit neuroinflammation, increase neuronal survival, and enhance cognitive function. Direct application of these lipids to the cells showed promising effects in inhibiting inflammatory signals and in inducing activation of signaling molecules such as AKT and ERK. Pls also induced various gene expressions in neuronal cells related to memory including the neuropeptide BDNF. Surprisingly, Pls treatments for 5 minutes could increase phosphorylation of AKT and ERK proteins, suggesting that these lipids might function as hormones or ligands. Several GPCRs were also identified as possible receptors for these lipids. The Pls concentration of Pls in the human blood is about 5 mg/dl, but the oral ingestion of Pls at low dose (1 mg/day)

improved cognitive function in patients with AD, suggesting further that these lipids might work as hormones. We need further studies to elucidate the detailed mode of function of these lipids to explore novel therapeutics to prevent or even to cure various diseases which are associated with a reduction of Pls and systemic inflammation. To precisely understand the mode of function of Pls, we need to investigate the following: (1) What type of Pls is biologically active? (2) Do Pls work as hormone or ligands to activate membrane protein? (3) How did the extracellular Pls activate membrane receptors? (4) Do Pls activate nuclear receptor by a direct interaction? (5) Do Pls cross the blood-brain barrier? (6) Do the orally ingested Pls have any peripheral effects to enhance cognitive function? Therefore, a lot of studies will be necessary to understand the mode of function of Pls in our health. The outcomes of these studies could reveal novel therapeutic strategies to improve our health.

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14 Therapeutic Efficacy of Plasmalogens for Alzheimer's Disease, Mild Cognitive Impairment, and Parkinson's Disease in Conjunction with a New Hypothesis for the Etiology of Alzheimer's Disease

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Abstract

It has been reported in recent years that blood levels of plasmalogens (Pls) are decreased in various diseases. None of those reports, however, conducted any clinical trials to examine the effect of Pls on those diseases. This article describes our recent report on a therapeutic efficacy of orally administered Pls in mild cognitive impairment (MCI), mild to severe Alzheimer's disease (AD), and Parkinson's disease (PD). A 24-week, multicenter, randomized, double-blind, placebo-controlled trial was performed in patients with MCI ($n = 178$) and mild AD ($n = 98$). The study design for moderate AD ($n = 57$) and severe AD ($n = 18$) was 12-week open-labeled, and the design for patients with PD ($n = 10$) was 24-week open-labeled. They showed a significant improvement in cognitive function and other clinical symptoms with elevation of the blood Pls levels. No adverse events were reported. The baseline levels of plasma ethanolamine plasmalogen and erythrocyte ethanolamine plasmalogen in MCI, AD, and PD were significantly lower than those of normal aged. The degree of reduction in the blood Pls levels was in the order of MCI ≺ mild AD ≺ moderate AD ≺ severe AD ≺ PD. The findings suggest that the blood levels of Pls may be a beneficial biomarker for assessing AD severity. Based on these results, we have proposed a new hypothesis for the etiology of AD and other neuropsychiatric disorders.

Keywords

Plasmalogen · Scallop · Alzheimer's disease · Mild cognitive impairment · Parkinson's disease · Biomarker of severity

14.1 Background

A dramatic increase in the number of patients with cognitive impairment, especially Alzheimer's disease (AD), has emerged globally as an urgent issue to be addressed [1]. The measures taken up thus far are, however, not sufficient to control AD in the rapidly aging society, and effective therapeutic and preventive measures against AD remain to be established.

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A widely accepted hypothesis is that the pathological process of AD leading to cognitive deterioration is initiated by the accumulation of β-amyloid plaques and tau protein tangles in the brain, and pharmaceutical agents targeting the amyloid formation and clearance have been tested in the past decade [2]. However, clinical trials to demonstrate efficacy of these novel candidates have seen no success [2, 3].

Recently published research has suggested a potential efficacy of plasmalogens (Pls), a special class of glycerophospholipids, in the treatment of AD, as noted in some reviews [4–9]. The levels of Pls have been found to be decreased in the postmortem AD brain and in the blood of AD patients [10–18]. Pls are found in the cell membrane of many mammalian tissues, especially of the brain, heart, skeletal muscle, leukocytes, and sperm. Ethanolamine Pls (PlsPE) are abundant in the brain, while choline Pls (PlsPC) are abundantly found in the heart. Out of their various functions, special attention is given to the antioxidant and anti-neuroinflammatory properties that are linked to the chemical structure of Pls characterized by the vinyl ether bond at the sn-1 position of glycerol backbone. Other well-known properties include ion transport, membrane fusion, cholesterol efflux, and precursor of biologically active substances. All these properties are essential to maintain life [4–9].

We developed a simple method to extract large amounts of Pls from animals, by which the research on Pls treatment and AD was accelerated [19, 20]. Our studies using animal models demonstrated that Pls reduced the accumulation of β-amyloid and improved cognitive function by suppressing neuroinflammation [21–23]. Furthermore, we performed a 24-week placebo-controlled trial to examine the efficacy of orally administered Pls in patients with mild cognitive impairment (MCI) or mild AD [24, 25]. We also investigated the effect of treatment with Pls on cognitive function in patients with moderate-to-severe AD and Parkinson's disease (PD) in open-label studies [26, 27].

14.2 Efficacy in Patients with Mild Cognitive Impairment ($24 \leq$ MMSE-J ≤ 27) [24, 25]

14.2.1 Study Methods

A multicenter, randomized, double-blind, placebo-controlled trial was conducted to evaluate the efficacy of scallop-derived Pls in 25 hospitals or clinics in Japan from November 2014 to April 2016, targeting patients aged 60 to 85 years with the scores of 20–27 on the Mini-Mental State Examination-Japanese (MMSE-J) version. The participants who totaled 328 had no cerebrovascular dementia as confirmed by a CT scan or MRI, and they were given the clinical diagnosis of Alzheimer's disease or mild cognitive impairment. The enrolled participants were randomly assigned to receive either Pls 1.0 mg or matching placebo for 24 weeks. The processes of randomization, masking, and allocation concealment were strictly performed by the expert. Figure 14.1 shows the study profile.

The primary endpoint was change in the MMSE-J score. Its total score ranges from 0 to 30 and a lower score indicates poorer cognitive function. The secondary endpoints were Wechsler Memory Scale-Revised (WMS-R), Geriatric Depression Scale Short Version in Japanese (GDS-S-J), the levels of ethanolamine Pls (PlsPE) in plasma, and the relative concentration of PlsPE in erythrocyte membrane, shown as the percentage of Pls to the total phospholipids in erythrocyte membrane. Overall, 276 out of 328 participants completed the 24-week study period.

In this section, we describe the efficacy of Pls in patients with mild cognitive impairment (MCI) ($24 \leq$ MMSE-J ≤ 27). There were no statistically significant differences between the Pls-treated group ($n = 90$) and the placebo group ($n = 88$) on age and the baseline values for study outcomes as shown in Table 14.1.

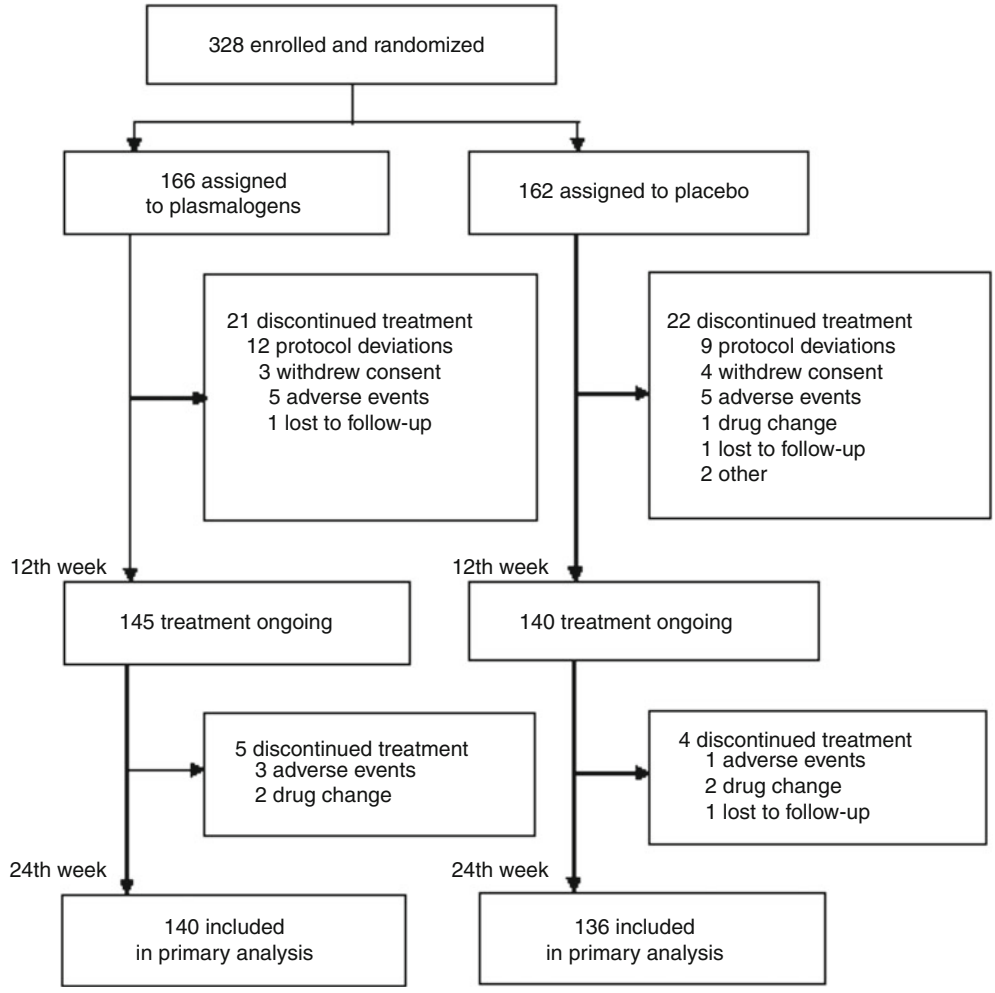

Fig. 14.1 Study profile of RCT (MCI and mild AD) [24]

14.2.2 Results

14.2.2.1 Change in Mini-Mental State Examination-Japanese and Wechsler Memory Scale-Revised

The MMSE-J total score increased at 24 weeks in both the Pls-treated group and the placebo group, although no significant between-group difference was found in the change of the MMSE-J total score. Out of the 11 domains of the MMSE-J, the orientation to place improved significantly in the Pls-treated group, but not in the placebo group (Table 14.2). The change in the domain score was significantly different between the two groups. With regard to the orientation to time, the Pls-treated group showed no notable baseline-to-endpoint change, while the placebo group showed a statistically significant decline at endpoint. However, the between-group difference in the change of the score for the orientation to time was not statistically significant (Fig. 14.2). Regarding the domains of calculation, registration, and other domains, there was no significant change in either group, nor was there any significant between-group difference at endpoint.

The WMS-R (0 min) and WMS-R (30 min) scores each showed a significant improvement in both groups, but with no statistically significant between-group difference.

Table 14.1 Baseline characteristics of RCT (MCI)[25]

Variable	Plasmalogen ($n = 90$)	Placebo($n = 88$)	P value[a]
Male, n (%)	37 (41.1%)	26 (29.5%)	0.12
Age in year	75.8 (6.1)	75.9 (5.5)	0.90
MMSE-J	25.6 (1.3)	25.6 (1.1)	0.99
1. Orientation to time	4.1 (1.0)	4.1 (1.0)	0.75
2. Orientation to place	4.3 (0.6)	4.4 (0.6)	0.13
3. Three-word registration	3.0 (0.2)	3.0 (0.3)	0.98
4. Attention and calculation	4.2 (1.0)	3.9 (1.1)	0.07
5. Three-word recall	1.4 (1.0)	1.5 (1.1)	0.56
6. Language (naming)	2.0 (0.2)	2.0 (0.1)	0.99
7. Language (repeating)	1.0 (0.1)	1.0 (0.1)	0.55
8. Language (three-step command)	2.8 (0.5)	2.8 (0.4)	0.66
9. Language (reading)	1.0 (0.0)	1.0 (0.0)	–
10. Language (writing)	1.0 (0.2)	1.0 (0.2)	0.98
11. Visual construction	1.0 (0.2)	1.0 (0.2)	0.72
WMS-R (0 min)	5.39 (3.97)	5.49 (4.32)	0.87
WMS-R (30 min)	3.66 (4.13)	3.90 (4.61)	0.71
Erythrocyte PlsPE (%)[b]	8.07 (1.09)	8.17 (1.00)	0.53
Plasma PlsPE (mg/dl)	3.92 (1.34)	4.03 (1.29)	0.56

Values are mean (SD) unless otherwise specified
MMSE-J = Mini-Mental State Examination-Japanese
PlsPE = ethanolamine plasmalogens
[a]Chi-square test for proportion and unpaired t-test for mean
[b]The number of the patients were 90 in the Pls-treated group and 87 in the placebo group

Table 14.2 Mean difference from baseline (MCI)

Variable	Plasmalogen ($n = 90$) mean (95%CI)		Placebo ($n = 88$) mean (95%CI)		P value[a]
MMSE-J	0.59	(0.13: 1.05)	0.39	(−0.18: 0.95)	0.58
1. Orientation to time	−0.01	(−0.31: 0.29)	−0.28	(−0.54: −0.02)	0.18
2. Orientation to place	0.36	(0.21: 0.50)	0.03	(−0.12: 0.19)	0.003
3. Three-word registration	0.02	(−0.03: 0.08)	0.05	(−0.01: 0.10)	0.55
4. Attention and calculation	0.10	(−0.15: 0.35)	0.24	(−0.06: 0.54)	0.48
5. Three-word recall	0.00	(−0.22: 0.22)	0.24	(−0.02: 0.50)	0.17
6. Language (naming)	0.02	(−0.02: 0.07)	0.02	(−0.01: 0.05)	0.99
7. Language (repeating)	0.01	(−0.01: 0.03)	0.02	(−0.01: 0.05)	0.55
8. Language (three-step command)	0.06	(−0.06: 0.17)	0.07	(−0.04: 0.18)	0.88
9. Language (reading)	0.00	(−: −)	0.00	(−: −)	–
10. Language (writing)	0.03	(0.00: 0.07)	0.02	(−0.02: 0.07)	0.72
11. Visual construction	0.00	(−0.04: 0.04)	−0.02	(−0.08: 0.03)	0.53
WMS-R (0 min)	1.51	(0.84: 2.18)	1.98	(1.28: 2.67)	0.34
WMS-R (30 min)	1.27	(0.60: 1.93)	1.67	(0.97: 2.37)	0.41
Erythrocyte PlsPE[b]	0.26	(0.07: 0.45)	0.32	(0.12: 0.53)	0.67
Plasma PlsPE	−0.47	(−0.70: −0.23)	−0.41	(−0.68: −0.13)	0.74

MMSE-J = Mini-Mental State Examination-Japanese
PlsPE = ethanolamine plasmalogens
[a]Unpaired t-test for mean
[b]The number of the patients were 90 in the Pls-treated group and 87 in the placebo group

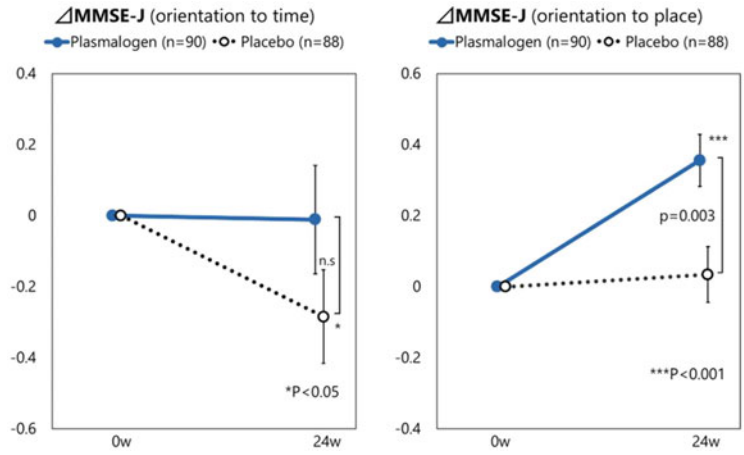

Fig. 14.2 Change in MMSE-J scores after Pls administration (MCI) [25] *MMSE-J* Mini-Mental State Examination-Japanese

14.2.2.2 Change in the Blood Level of Ethanolamine Plasmalogen

Both the Pls-treated group and the placebo group showed an improvement in the levels of erythrocyte PlsPE after Pls ingestion, but no significant difference was observed between the two groups. The levels of plasma PlsPE were decreased in both groups, producing no significant between-group difference (Table 14.2).

When compared to age-matched healthy controls, a slightly significant decrease was noted in baseline-erythrocyte PlsPE overall although there was no significant decrease in baseline-plasma PlsPE (Fig. 14.3).

14.2.3 Discussion

The MMSE-J total score improved statistically significantly in the Pls-treated group, while the change was not significantly different from the change in the placebo group. However, the analysis on each domain of the MMSE-J demonstrated that the orientation to place improved significantly and differentially in the Pls-treated group alone. This improvement of the orientation to place coincides with our preceding animal experiment, in which orally administered Pls improved learning and memory of mice using the Morris water maze test (manuscript in preparation). On the other hand, there was no significant between-group difference for the orientation to time. The finding is, however, not necessarily peculiar in view of the temporal occurrence of disorientation to time and place. Orientation to time is usually disturbed at first, and then orientation to place is lost during the progression form MCI to AD [28]. Hence, it is not a forced explanation that recovery of the late-disturbed orientation to place precedes that of orientation to time. Furthermore, the orientation to time in the placebo group significantly worsened after 24-week treatment in contrast to the Pls-treated group with no worsening. These findings indicate the possibility that Pls treatment may arrest the progression from MCI to AD.

14.3 Efficacy in Patients with Mild Alzheimer's Disease ($20 \leq$ MMSE-J ≤ 23) [24]

14.3.1 Study Methods

The study participants in this section were 98 patients with mild AD out of the 276 participants who completed the 24-week RCT described earlier in Sect. 14.2.1 (Fig. 14.1). There were no statistically significant differences between the Pls-treated group ($n = 50$) and the placebo group ($n = 48$) on age

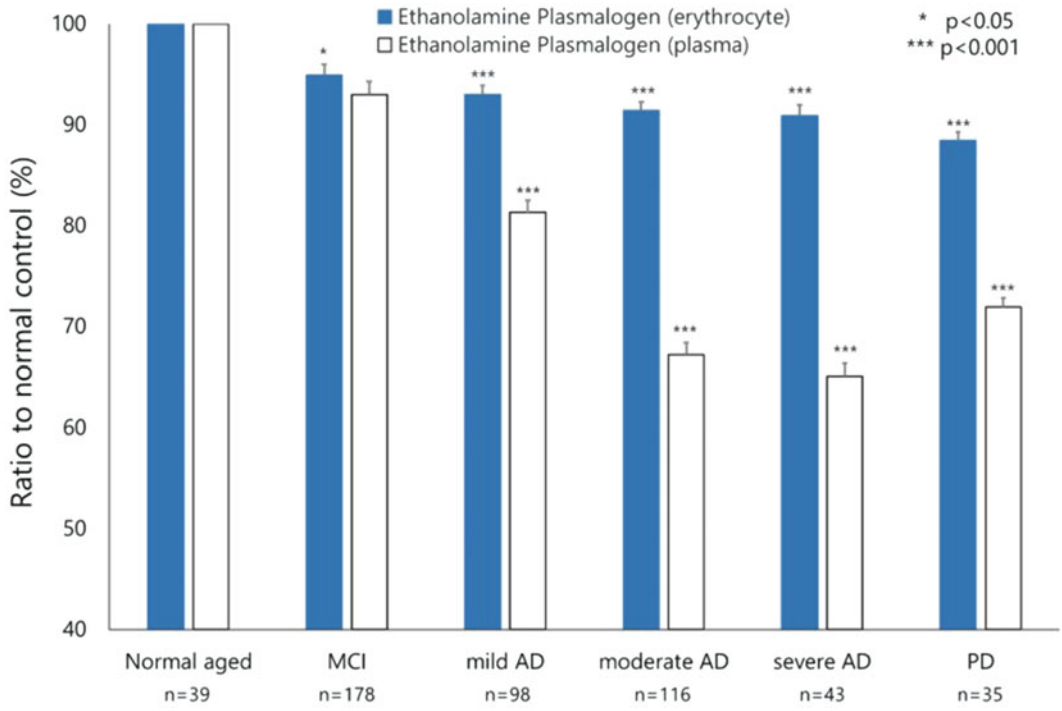

Fig. 14.3 Comparison with healthy controls in the blood levels of PlsPE *MCI* mild cognitive impairment, *AD* Alzheimer's disease, *PD* Parkinson's disease. The number of moderate AD, severe AD, and PD includes cases other than the study participants

and the baseline values for study outcomes as shown in Table 14.3.

14.3.2 Results

14.3.2.1 Change in Mini-Mental State Examination-Japanese and Wechsler Memory Scale-Revised

An intention-to-treat analysis yielded no significant difference between the Pls-treated and the placebo groups in the outcomes of cognitive function. The MMSE-J score showed a nearly significant improvement in the Pls-treated group versus no such improvement in the placebo group, producing no statistically significant between-group difference. The WMS-R (0 min) and WMS-R (30 min) scores each showed a significant improvement in the Pls-treated group with no significant between-group difference (Table 14.4).

We, furthermore, analyzed the WMS-R score by sex and age. Participants were divided into two groups based on the median value, 77 years or younger and 78 years or older. In the age group of 77 years or younger, WMS-R (30 min) increased statistically significantly in the Pls-treated group, showing a significant between-group difference. Focusing on the sex differences, the female of all ages in the Pls-treated group made a significant improvement in WMS-R (30 min) with a statistically significant between-group difference (Fig. 14.4).

14.3.2.2 Change in the Blood Level of Ethanolamine Plasmalogen

The levels of plasma PlsPE improved in the Pls-treated group after Pls ingestion in contrast to a decline in the placebo group, showing a statistically significant difference between the

Table 14.3 Baseline characteristics of RCT (mild AD)

Variable	Plasmalogen ($n = 50$)	Placebo ($n = 48$)	P value[a]
Male, n (%)	25 (50.0%)	18 (37.5%)	0.23
Age in year	77.3 (5.9)	77.6 (5.6)	0.82
MMSE-J	21.4 (1.1)	21.7 (1.0)	0.30
1. Orientation to time	2.5 (1.1)	2.7 (1.4)	0.42
2. Orientation to place	4.0 (0.8)	3.8 (0.7)	0.24
3. Three-word registration	2.9 (0.3)	3.0 (0.0)	0.05
4. Attention and calculation	2.8 (1.5)	2.8 (1.5)	0.87
5. Three-word recall	0.6 (0.9)	0.8 (1.0)	0.38
6. Language (naming)	2.0 (0.0)	2.0 (0.1)	0.31
7. Language (repeating)	1.0 (0.2)	1.0 (0.2)	0.97
8. Language (three-step command)	2.8 (0.4)	2.9 (0.4)	0.86
9. Language (reading)	1.0 (0.0)	1.0 (0.0)	–
10. Language (writing)	0.9 (0.3)	0.9 (0.3)	0.95
11. Visual construction	0.9 (0.4)	0.9 (0.3)	0.83
WMS-R (0 min)	2.36 (2.20)	2.21 (2.17)	0.74
WMS-R (30 min)	0.66 (1.85)	0.60 (1.04)	0.83
Erythrocyte PlsPE (%)	8.01 (0.97)	7.96 (0.88)	0.77
Plasma PlsPE (mg/dl)	3.23 (1.13)	3.71 (1.22)	0.05

Values are mean (SD) unless otherwise specified
MMSE-J = Mini-Mental State Examination-Japanese
[a]Chi-square test for proportion and unpaired t-test for mean

two groups. Regarding the levels of erythrocyte PlsPE, there was no significance in between-group difference (Table 14.4).

When compared to age-matched healthy controls, the baseline levels of erythrocyte PlsPE were markedly significantly decreased overall,

Table 14.4 Mean difference from baseline (mild AD)

Variable	Plasmalogen ($n = 50$) mean (95%CI)		Placebo ($n = 48$) mean (95%CI)		P value[a]
MMSE-J	0.06	(−0.76: 0.88)	0.19	(−0.59: 0.96)	0.82
1. Orientation to time	−0.18	(−0.65: 0.29)	−0.15	(−0.52: 0.23)	0.91
2. Orientation to place	−0.06	(−0.33: 0.21)	0.10	(−0.21: 0.42)	0.43
3. Three-word registration	0.02	(−0.07: 0.11)	−0.02	(−0.06: 0.02)	0.42
4. Attention and calculation	0.24	(−0.25: 0.73)	0.48	(0.02: 0.94)	0.48
5. Three-word recall	0.02	(−0.29: 0.33)	−0.19	(−0.45: 0.07)	0.30
6. Language (naming)	0.00	(−: −)	0.02	(−0.02: 0.06)	0.31
7. Language (repeating)	0.04	(−0.02: 0.10)	0.02	(−0.05: 0.09)	0.68
8. Language (three-step command)	−0.04	(−0.17: 0.09)	−0.10	(−0.25: 0.05)	0.51
9. Language (reading)	0.00	(−: −)	0.00	(−: −)	–
10. Language (writing)	0.02	(−0.07: 0.11)	0.04	(−0.04: 0.13)	0.73
11. Visual construction	0.00	(−0.13: 0.13)	−0.02	(−0.15: 0.11)	0.82
WMS-R (0 min)	1.24	(0.49: 1.99)	0.30	(−0.39: 0.99)	0.07
WMS-R (30 min)	0.78	(0.04: 1.52)	0.02	(−0.37: 0.41)	0.08
Erythrocyte PlsPE[b]	0.24	(−0.03: 0.52)	0.40	(0.11: 0.69)	0.42
Plasma PlsPE	0.16	(−0.07: 0.40)	−0.34	(−0.69: 0.00)	0.02

MMSE-J = Mini-Mental State Examination-Japanese
PlsPE = ethanolamine plasmalogens
[a]Unpaired t-test for mean
[b]The number of the patients were 50 in the Pls-treated group and 49 in the placebo group

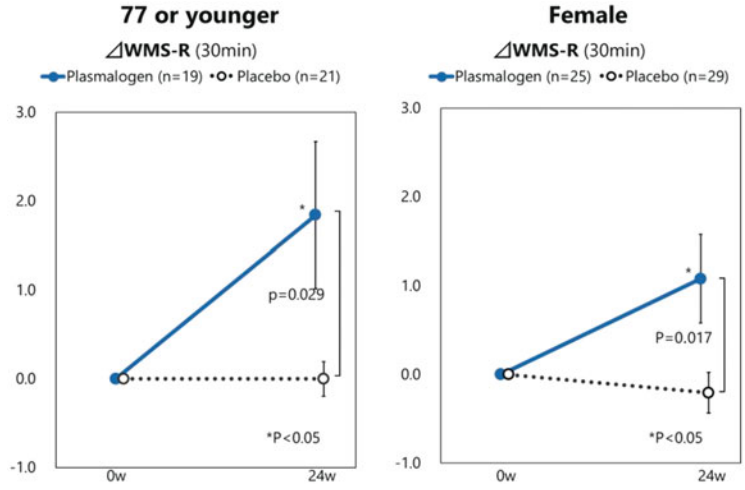

Fig. 14.4 WMS-R improvement in 77 years or younger participants and female participants (mild AD) *WMS-R* Wechsler Memory Scale-Revised

while those of plasma PlsPE were significantly lower than the controls (Fig. 14.3).

14.3.3 Discussion

No statistically significant difference was observed in the MMSE-J score between the Pls-treated and the placebo groups. However, the Pls-treated group showed a significant improvement in the WMS-R score, and the improvement seemed to be greater than that in the placebo group. Notably, there was a more remarkable improvement in WMS-R in the age group of 77 years or younger and in the female of all ages. These results indicate that oral administration of scallop-derived purified Pls might be effective in improving cognitive function of mild AD patients. This study did not confirm the efficacy of Pls ingestion in the age group of 78 years or older, or in the male of all ages. One reason for the lack of efficacy in these aged participants might be ascribed to age-related irreversible degenerative changes in the brain [29]. It is unknown why the efficacy was obvious in females but not in males.

The changes in the blood level of plasma Pls after treatment differed significantly between the Pls-treated and the placebo groups. Plasma PlsPE in the placebo group was significantly reduced after treatment, whereas that of the Pls-treated group remained unchanged. These findings may indicate that production of Pls in peroxisome was reduced concurrently with the progression of AD during the 24-week study period and that oral administration of Pls may contribute to maintain the production of Pls in peroxisome.

14.4 Efficacy in Patients with Moderate Alzheimer's Disease ($11 \leq$ MMSE ≤ 19) and Severe Alzheimer's Disease (MMSE ≤ 10) [26]

14.4.1 Study Methods

The efficacy of scallop-derived Pls was examined in an open-label study. A total of 103 participants aged 60 to 85 years were recruited from 23 hospitals or clinics in Japan from February 2015 to December 2015, consisting of moderate AD ($n = 57$) and severe-AD ($n = 18$). The baseline characteristics of completed participants are described in Table 14.5.

The enrolled participants received 1.0 mg of scallop-derived Pls for 12 weeks. Efficacy endpoints included change in the MMSE score, the levels of Pls in erythrocyte and plasma, and five-item neurofunctional evaluation rated by

Table 14.5 Baseline characteristics of open trial (moderate AD and severe AD)

Variable	Moderate AD ($n = 57$)	Severe AD ($n = 18$)	P value[a]
Male, n (%)	24 (42.1%)	8 (44.4%)	1.00
Age in year	77.2 (4.7)	74.9 (5.0)	0.09
MMSE	15.9 (2.5)	5.7 (2.4)	0.00
1. Orientation to time	1.2 (1.1)	0.1 (0.3)	0.00
2. Orientation to place	2.2 (1.4)	0.2 (0.4)	0.00
3. Three-word registration	2.9 (0.5)	1.6 (1.2)	0.00
4. Attention and calculation	1.2 (1.2)	0.1 (0.2)	0.00
5. Three-word recall	0.8 (1.0)	0.2 (0.5)	0.01
6. Language (naming)	1.9 (0.5)	0.9 (1.0)	0.00
7. Language (repeating)	0.8 (0.4)	0.0 (0.0)	0.00
8. Language (three-step command)	2.9 (0.4)	2.3 (1.0)	0.00
9. Language (reading)	0.9 (0.3)	0.3 (0.5)	0.00
10. Language (writing)	0.6 (0.5)	0.0 (0.0)	0.00
11. Visual construction	0.6 (0.5)	0.1 (0.2)	0.00
Erythrocyte PlsPE (%)	7.83 (0.91)	8.03 (1.20)	0.45
Plasma PlsPE (mg/dl)	3.05 (1.22)	3.15 (1.24)	0.76

Values are mean (SD) unless otherwise specified
MMSE = Mini-Mental State Examination
PlsPE = ethanolamine plasmalogen
[a]Chi-square test for proportion and unpaired t-test for mean

caregivers. The degree of improvement in the MMSE was classified as remarkable improvement (ΔMMSE≥4), improvement (ΔMMSE 2 to 3), no change (ΔMMSE±1), and worsening (ΔMMSE≤-2).

14.4.2 Results

14.4.2.1 Change in Mini-Mental State Examination

The MMSE score in moderate AD made a significant improvement by 1.72 points. A MMSE domain-specific analysis showed a statistically significant improvement in orientation to time, orientation to place, and three-word recall. No significant improvement in the MMSE score was observed in severe AD (Table 14.6).

As illustrated in Fig. 14.5, more than half of moderate AD improved in the post-treatment MMSE score. According to the degree of improvement, 31% of moderate AD were classified as remarkable improvement, 21% as improvement, 36% as no change, and 12% as worsening. Although no remarkable improvement was noted in severe AD, 29% of them improved.

14.4.2.2 Change in the Blood Level of PlsPE

Orally administered Pls to moderate AD resulted in a significant elevation of the PlsPE levels in both plasma and erythrocyte, whereas there was no significant change after a 12-week treatment in severe AD (Table 14.6).

When compared to age-matched healthy controls, the baseline levels of erythrocyte PlsPE were markedly significantly decreased, while those of plasma PlsPE were significantly lower than the controls (Fig. 14.3).

14.4.3 Discussion

These results provide a strong indication that Pls administration could enhance cognitive function in moderate AD. However, this study was open-labeled without a control group, and some of the observed improvement may have been due to the placebo effect. In this regard, it could be

Table 14.6 Mean difference from baseline (moderate AD and severe AD)

Variable	Moderate AD ($n = 57$) mean (95% CI)		Severe AD ($n = 18$), mean (95% CI)		P value[a]
MMSE	1.72	(0.88: 2.56)	0.28	(−0.71: 1.27)	0.07
1. Orientation to time	0.51	(0.15: 0.87)	−0.11	(−0.27: 0.05)	0.06
2. Orientation to place	0.37	(0.02: 0.72)	0.17	(−0.18: 0.52)	0.54
3. Three-word registration	0.04	(−0.12: 0.19)	0.17	(−0.29: 0.63)	0.47
4. Attention and calculation	0.23	(−0.13: 0.59)	0.17	(−0.09: 0.42)	0.85
5. Three-word recall	0.42	(0.08: 0.76)	−0.17	(−0.42: 0.09)	0.06
6. Language (naming)	0.04	(−0.08: 0.15)	−0.11	(−0.56: 0.34)	0.35
7. Language (repeating)	0.05	(−0.05: 0.16)	0.11	(−0.05: 0.27)	0.57
8. Language (three-step command)	0.00	(−0.13: 0.13)	0.11	(−0.50: 0.72)	0.58
9. Language (reading)	0.04	(−0.04: 0.11)	−0.06	(−0.17: 0.06)	0.20
10. Language (writing)[b]	−0.02	(−0.13: 0.09)	0.00	–	0.85
11. Visual construction[b]	0.05	(−0.07: 0.17)	0.00	–	0.61
Erythrocyte PlsPE	0.20	(0.00: 0.40)	0.14	(−0.33: 0.61)	0.79
Plasma PlsPE[c]	0.30	(0.02: 0.58)	0.42	(−0.20: 1.04)	0.68

MMSE = Mini-Mental State Examination
PlsPE = ethanolamine plasmalogens
[a]Unpaired t-test for mean
[b]Number of patients: n = 56 in moderate AD and n = 18 in severe AD
[c]Number of patients: n = 45 in moderate AD and n = 18 in severe AD

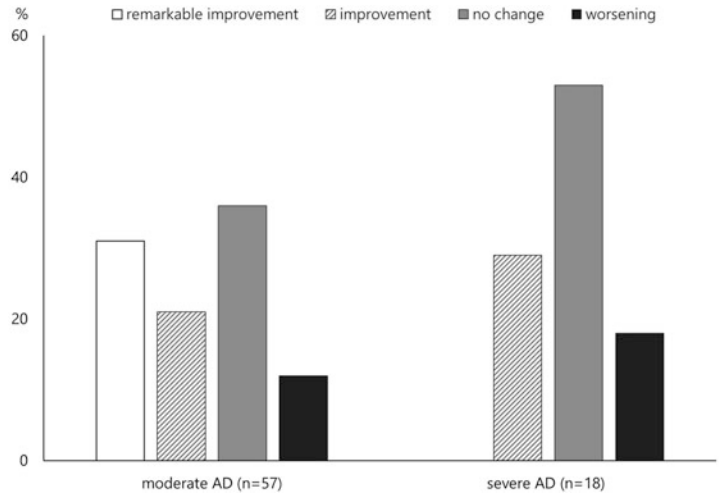

Fig. 14.5 Categorical distribution of MMSE improvement in moderate and severe Alzheimer's disease *MMSE* Mini-Mental State Examination, *AD* Alzheimer's disease. Remarkable improvement (ΔMMSE≥4), improvement (ΔMMSE 2 to 3), no change (ΔMMSE±1), worsening (ΔMMSE≤-2)

presumed that the placebo effect is unlikely to occur in moderate-to-severe AD in such a way as observed in general physical illnesses and symptoms. If based on the premise that the placebo effect is brought about by patients' *expectation* and *hope*, those suffering from moderate-to-severe AD are less likely to develop the conceptions of *expectation* and *hope* because of their cognitive dysfunction.

To deal with the magnitude of the placebo effect, we searched and retrieved relevant studies from PubMed and CiNii database. The placebo

group in each study targeting moderate-to-severe AD presented cognitive deterioration consistently. One of them reported a decrease of 0.3 points in MMSE after a 24-week placebo treatment period [30]. It is unlikely that the natural course of MMSE in moderate to severe AD patients is improved by the placebo effect, although no direct evidence is available that placebo does not make improvement of the MMSE score with a 12-week treatment duration. Putting together these findings, it could be concluded that scallop-derived Pls had therapeutic efficacy for moderate-to-severe AD.

Only 1.0 mg/day of Pls showed efficacy on cognitive function in AD and MCI. It is unclear how its physiological mechanism with such a small amount of administered Pls works. One hypothesis is that Pls may act through some receptors like hormones. Lipid rafts in the cell membrane are considered to be associated with cell signaling [23, 31]. Some studies reported that lipid rafts are abundant in PlsPE [32]. G-protein-coupled receptors (GPCR) are also localized to lipid rafts and caveolae [33]. These findings may indicate the possibility of Pls functioning as a ligand of GPCR. The concentration of PlsPE in human plasma is about 100 μmol/l, but plasma PlsPE may circulate as lipoproteins. It is unlikely that Pls in lipoproteins solely act as a ligand of receptors, but it is possible for free Pls derived from oral administration, even in a small amount, to work as a ligand of some receptors at intestinal cells before becoming lipoproteins. It is well-known that the intestine is in close communication with the brain through neural, endocrine, and immune pathways [34].

There are naturally concerns that orally administered Pls might be destroyed by stomach acid because of their sensitivity to it. Despite such concerns, our experiments in mice [35] and clinical trials in human [24, 26, 27] showed that the levels of Pls were increased in plasma and erythrocyte membrane by oral administration of Pls (1 mg/day/person). These results suggest that Pls might reach the intestines without being destroyed by stomach acid in an effective amount as a hormone when being ingested with a meal.

Interestingly enough, after a 12-week of treatment, the improvement of the MMSE total score in moderate AD was more remarkable than that in mild AD and MCI with the same dosage of Pls (MCI: 0.32 points, mild AD: 0.50 points, moderate AD: 1.72 points) [24]. It could be possible that patients with moderate AD have relatively less normal brain tissue and more damaged tissue due to the brain atrophy than those with mild AD and MCI. Therefore, the former may demand and consume less hormone-like substance, Pls, than the latter. Additionally, the brain activity of the latter is almost as high as that of healthy individuals, and thus consumes as much Pls as healthy individuals do. On the other hand, the brain activity of moderate AD is lower and consumes less Pls than that of healthy individuals. This hypothetical reasoning leads to a conclusion that even a very small amount of Pls is effective for moderate AD. The reason for no significant improvement in severe AD is presumably that they have a smaller number of normal brain tissues than other diseases and therefore do not respond to small doses of Pls. If a larger amount of Pls (i.e., 2–3 mg per day) is administered to mild AD, MCI, and severe AD, the same effect as moderate AD might be obtained. Further research on the dose-response effect is needed to determine the influence of severity.

14.5 Efficacy in Patients with Parkinson's Disease [27]

14.5.1 Study Methods

To assess the efficacy of Pls in patients with Parkinson's disease (PD), 14 participants were recruited, of which 7 had a history of deep brain stimulation (DBS) treatment. Finally, 10 of them completed the trial. The baseline characteristics are summarized in Table 14.7. The participants taking anti-Parkinson drugs (including dopamine agonists) had no regimen changes in the preceding 1 month and kept unchanged during the study period. Participants ingested 1 mg/day of scallop-derived Pls for a 24-week treatment period

Table 14.7 Baseline characteristics of open trial (PD) [27]

Variable	Parkinson's disease ($n = 10$), mean		Normal control ($n = 39$), mean		P value[a]
Male, n (%)	3	(30.0%)	13	(33.3%)	1.000
Age in year	67.80	(7.41)	71.87	(5.50)	0.058
MMSE	28.56	(2.13)	29.90	(0.31)	0.000
Plasma PlsPE	2.97	(0.76)	4.27	(1.07)	0.001
Erythrocyte PlsPE (%)	7.67	(0.78)	8.56	(0.94)	0.008

Values are mean (SD) unless otherwise specified
MMSE = Mini-Mental State Examination
PlsPE; ethanolamine plasmalogen
[a]Chi-square test for proportion and paired t-test for mean

followed by a 4-week observation period. Parkinson's Disease Questionnaire-39, which evaluates PD-specific health-related quality of life with a higher score indicating lower quality of life, and the levels of Pls in plasma and erythrocyte were checked at 0, 4, 12, 24, and 28 weeks of treatment. The blood levels of Pls in patients with PD were compared with those of 39 age-matched normal controls.

14.5.2 Results

14.5.2.1 Change in Parkinson's Disease Questionnaire-39

Oral administration of Pls improved some clinical symptoms in patients with PD concomitantly with an increase of Pls in the peripheral blood (Table 14.8). This clinical improvement was almost parallel to the increase of erythrocyte PlsPE (Fig. 14.6).

14.5.2.2 Change in the Blood Level of Ethanolamine Plasmalogen

Baseline levels in plasma and erythrocyte PlsPE in patients with PD were significantly lower than those of normal elderly controls (Fig. 14.3). The levels in both plasma and erythrocyte PlsPE, however, increased rapidly after ingestion of Pls, reached almost the normal range at 12 weeks, and were maintained until 24 weeks. Four weeks after the end of the administration period, the levels of both plasma PlsPE and erythrocyte PlsPE showed a tendency to decrease (Fig. 14.6).

14.5.3 Discussion

It is well-known that the vinyl ether bond at the sn-1 position makes Pls more susceptible to oxidative stress than corresponding ester-bonded glycerophospholipids [6, 36–38]. Therefore, Pls may act as an antioxidant and protect cells against oxidative stress [37, 38]. Some studies suggested that increased oxidative stress might be present in the peripheral blood of PD [39]. The increase in oxidized form of coenzyme Q10 in the plasma of PD may indicate elevated systemic oxidative stress in PD [40]. Actually, Dragonas et al. reported that a decrease in plasma Pls served as a marker of increased systemic oxidative stress in PD [41]. In their study, plasma Pls were measured by transesterification of plasma phospholipids and Pls-derived dimethyl acetal (DMA) was measured with gas chromatography. Several reports indicated the presence of neuroinflammation in PD [42] and α-synuclein dimerization in erythrocyte of PD [43]. The decrease of Pls in the peripheral blood of PD might be in part due to high oxidative stress in PD.

This study showed that oral administration of purified Pls derived from scallop increased plasma and erythrocyte Pls in patients with PD as well as concomitant improvement in some PD-specific clinical symptoms. Some studies using animal models of PD have suggested efficacy of Pls for symptoms of PD. Gregoire et al. reported that Pls precursor analog treatment reduced levodopa-induced dyskinesia in parkinsonian monkeys [44] and that Pls augmentation

Table 14.8 Improvement of clinical symptoms of Parkinson's disease after oral administration of scallop-derived ether phospholipids [27]

PDQ-39	Before ($n = 10$), mean scores		After 24 weeks ($n = 10$), mean scores		P value[a]
Total	273.5	(145.2)	188.8	(93.8)	0.02
Mobility	50.5	(29.0)	48.8	(22.9)	0.66
Daily activity	48.8	(23.7)	35.4	(16.3)	0.05
Emotional Well-being	40.4	(27.5)	26.3	(19.1)	0.07
Stigma	23.1	(17.9)	15.0	(17.7)	0.05
Social support	17.5	(18.2)	6.7	(12.3)	0.03
Cognitions	40.7	(19.8)	25.0	(16.4)	0.02
Communication	35.8	(33.8)	22.5	(26.4)	0.07
Bodily discomfort	16.7	(11.1)	9.2	(10.0)	0.03

Values are mean (SD) unless otherwise specified
[a]Paired t-test for mean

with a Pls precursor reversed striatal dopamine loss in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice [45].

14.6 Proposal of a New Hypothesis for the Etiology of Alzheimer's Disease and a Future Study

It is common knowledge that AD is characterized pathologically by senile plaques which are mainly composed of amyloid-beta (Aβ) protein. The amyloid cascade hypothesis, widely supported at the present time, proposes that the deposition of Aβ is a hallmark pathological lesion observed at the earliest stage of AD, that aggregated Aβ induces neuronal toxicity, and that deficiency in the production and deposition of Aβ correlates well with the onset of AD based on the genetic analysis of familial AD.

Aβ, fragments of amyloid precursor protein (APP), is produced and secreted when cleaved by β- and γ-secretase. It is, therefore, considered essential to regulate the activity of secretase and activate the pathway of Aβ degradation as an AD treatment strategy. Meanwhile, some studies reported that there were many patients who did not develop any AD symptoms despite high Aβ deposition in the postmortem brain and that by contrast some patients with serious AD symptoms turned out at autopsy to be mild cases [46]. Thus far major pharmaceutical companies have been enthusiastic about developing new antidementia drugs, none of which works. To take an example of Aβ-targeting AD drugs, no beneficial effects were achieved by administration to human patients [47, 48]. From these results, it seems reasonable to conclude that the amyloid cascade hypothesis of AD has problems in itself.

Amid such circumstances, strong evidence supporting the neuroinflammation hypothesis is gradually accumulating. Our animal studies demonstrated that neuroinflammation-induced Aβ deposition caused AD and, furthermore, that Pls administration inhibited the deposition of Aβ and prevented the onset of AD as detailed in the former chapter. These findings strongly suggest that neuroinflammation is one of the main causes of AD.

Hossain et al. revealed that Pls administration to AD animal models improved spatial memory and concurrently increased the number of dendrites and spines in the hippocampus (manuscript in preparation), which indicates that Pls are effective for the improvement of neural network failure. Actually, improvement in cognitive function was observed among patients ranging from MCI to severe AD as described above. It is worth noting that spatial memory improvement in animal models is well consistent with significant improvement in orientation to place (one of the MMSE domains) among MCI patients in the Pls-treated group compared to the placebo group.

Taken together, a new hypothesis is proposed here to explain AD pathogenesis as illustrated in Fig. 14.7.

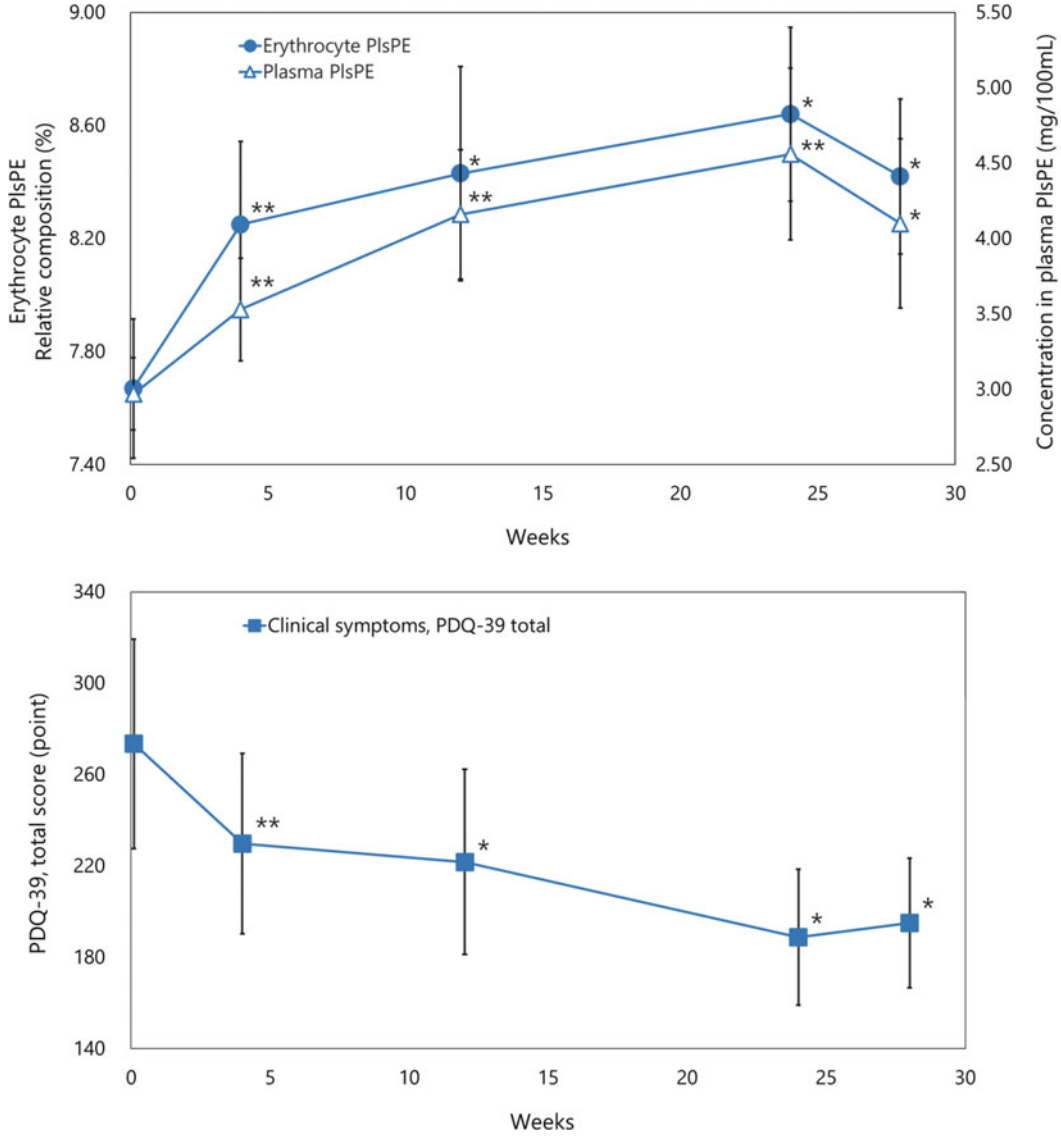

Fig. 14.6 Increase in erythrocyte PlsPE (relative composition) and plasma PlsPE (concentration) and improvement in clinical symptoms of PD (PDQ-39) [27] $^{**}p \prec 0.01$, $^{*}p \prec 0.05$ error bars indicate standard error. *PlsPE* ethanolamine plasmalogen. *PDQ-39* Parkinson's Disease Questionnaire-39. The last 4 weeks (24 to 28 weeks) were the observation period without administration. Differences from immediate before trial

When the brain is exposed to risk factors including mental stress, infection, metabolic syndrome and aging, neuroinflammation (microglial activation) is caused. This neuroinflammation leads to the malfunction of peroxisome and endoplasmic reticulum, and then causes an immediate decrease in Pls. Conversely, the low levels of Pls induce neuroinflammation. Such low-level Pls are caused when Pls are overconsumed due to overstress, or the production of peroxisome and endoplasmic is suppressed by aging. To put it differently, physiological phenomenon "neuroinflammation" and biochemical phenomenon "decreased Pls" are opposite sides of the same AD pathogenesis. Such

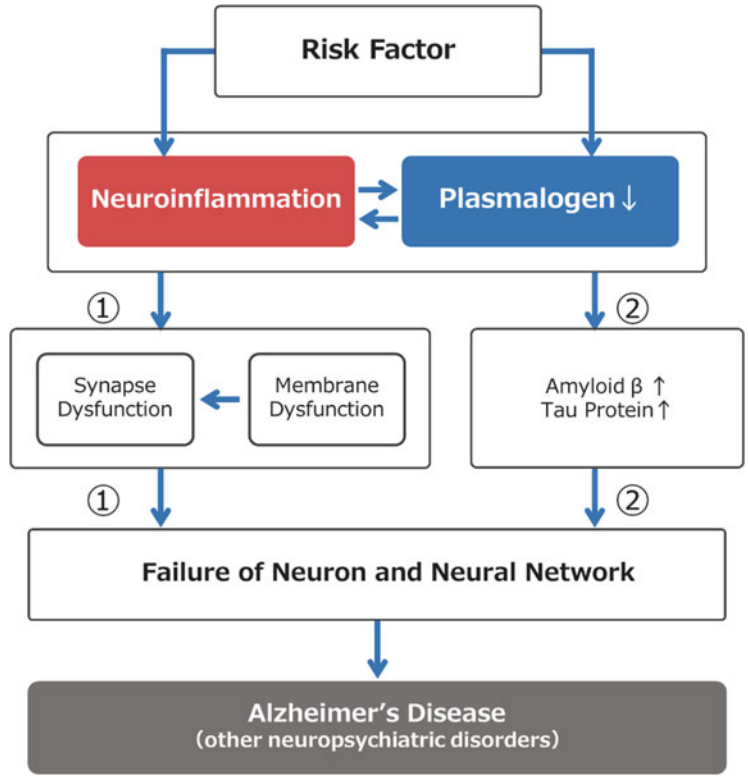

Fig. 14.7 A new hypothesis for the etiology of AD pathogenesis

interactivity between "neuroinflammation" and "decreased Pls" results in functional and structural defects in neuronal membrane and synapse [49]. On the other hand, microglial activation causes activation of ɤ-secretase, accumulates Aβ, and then increases tau proteins [50].

Such membrane dysfunction and synapse dysfunction causes the failure of neurons and disturbs the neural network through Route 1 shown in Fig. 14.7. Likewise, accumulation of Aβ and tau proteins affects the neurons and neural network through Route 2. Consequently, AD occurs. Route 1 and Route 2 presumably progress in parallel. Route 2, however, may not be a main route, because it has been reported that no accumulation of Aβ was observed in some patients with AD as results of an autopsy [46] and any inhibitor of Aβ accumulation has little effect in development of new drugs [47, 48].

It is not difficult to imagine that the failure of neuron and neural network, which occurs in various areas of the brain, causes other different diseases than AD (e.g., PD, schizophrenia, or depression) [51]. As stated in Sect. 14.5, some clinical symptoms of PD were significantly improved by Pls administration concomitantly with an increase of the Pls levels. Some other studies reported that Pls were decreased in patients with schizophrenia [52, 53], which is mainly considered to occur as a result of neuroinflammation. These findings strongly support our new hypothesis of pathogenesis.

Thus far we have examined the efficacy of Pls, which reduce neuroinflammation, in patients with MCI, AD, and PD. Further clinical trials by other research teams are required to verify the effects on these diseases. In addition to that, new trials are highly needed in patients with neuropsychiatric disorders including schizophrenia, depression, and others. Moreover, the measurement of the blood Pls should be also investigated in the clinical trials from now on, because our present study and other reports suggest [18, 54] that the blood levels of Pls are a new biomarker for the severity of diseases including MCI, AD, PD, and other neuropsychiatric disorders

[51]. We strongly hope that this new hypothesis will make a contribution to the elucidation of the pathogenesis of neuropsychiatric disorders and to the establishment of their therapeutic and preventive methods.

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