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Title	Acyl-CoA Reductase Far1 Deficiency Impairs Ether Lipid Production and Hypomyelination in Mouse Brains
Author(s)	Takahashi, Tenga; Otsuka, Kento; Sassa, Takayuki et al.
Citation	Molecular and cellular biology, 45, 495-510 https://doi.org/10.1080/10985549.2025.2548234
Issue Date	2025-09-11
Doc URL	https://hdl.handle.net/2115/98118
Type	journal article
File Information	Takahashi T et al MCB.pdf



Acyl-CoA reductase *Far1* deficiency impairs ether lipid production and hypomyelination in mouse brains

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Abstract

Mammalian cell membranes contain ether lipids, which include an alkyl chain derived from a fatty alcohol that is produced by fatty acyl-CoA reductases (FARs). There are two mammalian *FAR* genes, *FAR1* and *FAR2*, and mutations in *FAR1* cause the peroxisomal fatty acyl-CoA reductase 1 disorder (PFCRD), which is accompanied by various symptoms, including neurological disorders. To date, the contributions of FAR1 and FAR2 to brain ether lipid production and the molecular mechanism of PFCRD have remained unknown. To investigate these, we analyzed knockout (KO) mice of *Far1* and *Far2*. In the brain, the expression levels of *Far1* were higher than those of *Far2*, and *Far1* was widely expressed. Lipidomics analyses showed that the quantity of ether lipids ethanolamine-plasmalogens was reduced in *Far1* KO mice, with a complementary increase in diacyl-type phosphatidylethanolamines, but not in *Far2* KO mice. Electron microscope analysis of the corpus callosum revealed reductions in the percentage of myelinated axons and myelin thickness in *Far1* KO mice relative to WT mice. In conclusion, FAR1 is the major FAR isozyme involved in ether lipid synthesis in the brain, and its deficiency causes hypomyelination. We speculate that this hypomyelination is one of the causes of the neurological symptoms of PFCRD.

Keywords

ether lipid; FAR1; fatty acyl-CoA reductase; myelin; plasmalogen

Introduction

Mammalian cell membranes contain ether lipids, which include a fatty alcohol-derived alkyl moiety attached to the *sn*-1 position of the glycerol backbone via an ether or vinyl ether bond.¹⁻

⁴ The ether lipids include plasmalogens, glycosylphosphatidylinositol anchors, and seminolipids.⁵⁻⁷ Diacyl-type phosphatidylethanolamines (PEs) and phosphatidylcholines (PCs) contain two ester-linked fatty acid (FA) moieties in the *sn*-1 and *sn*-2 positions, respectively, and a phosphoethanolamine or phosphocholine moiety in the *sn*-3 position. Plasmalogens, comprising ethanolamine-plasmalogen/plasmenylethanolamine (PE[P]) and choline-plasmalogen/plasmenylcholine (PC[P]), differ in having a vinyl ether-linked alkyl moiety in the *sn*-1 position.^{4,6,8} Although plasmalogens are thought to exhibit antioxidative effects and pool polyunsaturated FAs in the *sn*-2 position as lipid mediator precursors, their function remains incompletely understood.⁹⁻¹³ However, mutations in the genes encoding peroxisomal enzymes involved in the synthesis of ether lipids cause severe peroxisomal disorders.^{8,14}

The initial two reactions of ether lipid synthesis occur in peroxisomes.^{4,6,15} The first reaction is the formation of acyl-glycerone-phosphate via the acylation of glycerone phosphate (dihydroxyacetone phosphate), catalyzed by glycerone phosphate *O*-acyltransferase (GNPAT) (Figure 1).¹⁶⁻¹⁸ In the second reaction, acyl-glycerone-phosphate is converted to alkyl-glycerone-phosphate by alkylglycerone-phosphate synthase (AGPS), which catalyzes the substitution of the acyl moiety with an ether-linked alkyl moiety.^{16,19,20} This alkyl moiety is derived from a fatty alcohol, which is produced in peroxisomes via the reduction of fatty acyl-CoA, catalyzed by fatty acyl-CoA reductases (FARs).^{18,21-23} There are two FAR isozymes, FAR1 and FAR2, in mammals.²¹ The third reaction is the reduction of alkyl-glycerone-phosphate to 1-alkyl-glycerol-3-phosphate. This reaction occurs predominantly in peroxisomes, although it has also been detected in mitochondria and the endoplasmic reticulum.^{16,24,25} The next reaction is acylation at the *sn*-2 position of 1-alkyl-glycerol-3-phosphate to form 1-alkyl-2-acyl-glycerol-3-phosphate, followed by dephosphorylation to generate 1-alkyl-2-acyl-glycerol. These reactions and the subsequent reactions for the production of ether phospholipid syntheses take place in the endoplasmic reticulum.⁴ In the synthesis of ethanolamine-type ether

phospholipids, the phosphoethanolamine head group is attached to the *sn*-3 position of 1-alkyl-2-acyl-glycerol,²⁶ resulting in the production of plasmalylethanolamine (PE[O]).^{4,27} The ether bond in the PE[O] is converted to a vinyl ether bond via desaturation,²⁸⁻³⁰ resulting in the synthesis of PE[P]. In addition to those with an ethanolamine head group, ether phospholipids with a choline head group also exist; these include plasmalylcholine (PC[O]) and PC[P], the latter being less abundant.^{6,31,32}

Mutations in *GNPAT*, *AGPS*, *PEX7*, or in a long isoform of *PEX5*, the latter two of which are involved in the transport of proteins with peroxisome targeting signals into peroxisomes, cause *rhizomelic chondrodysplasia punctata* (RCDP), a disorder characterized by reduced plasmalogen levels in the blood and tissues, shortening of humerus and femur (*rhizomelia*), punctate calcifications in epiphyseal cartilage (*chondrodysplasia punctata*), mental retardation, epilepsy, cataracts, and growth retardation.³³⁻³⁶ Mutations in *FAR1* cause peroxisomal fatty acyl-CoA reductase 1 disease (PFCRD), which has symptoms similar to those of RCDP but differs in that it is not accompanied by *rhizomelia* or *chondrodysplasia punctata*.³⁷⁻⁴⁰ Thus, the clinical features shared by RCDP and PFCRD may be caused by impaired synthesis of ether lipids in peroxisomes, although the molecular mechanism underlying the pathogenesis remains undetermined and the reason for the differences between the two conditions is unclear.

Several mouse models of RCDP—*Pex7* knockout (KO) mice, *Gnpat* KO mice, and *Agps* hypomorphic mice—have been reported.⁴¹⁻⁴⁴ In these mouse models, the levels of plasmalogens were severely reduced and most animals did not reach adulthood due to embryonic or neonatal lethality and growth retardation.⁴¹⁻⁴⁴ In the adult brains of *Pex7* KO mice and *Gnpat* KO mice, reduced levels of staining for myelin markers were observed in the *corpus callosum* and cerebellar white matter,⁴⁵⁻⁴⁷ and electron microscope analysis showed impaired myelination and reduced myelin thickness in the *corpus callosum*, internal capsule, and cerebellar white matter in *Gnpat* KO mice.⁴⁷

Although both FAR1 and FAR2 localize in the peroxisomes, their substrate specificities and tissue distributions are different. FAR1 and FAR2 exhibit high activity toward C16–C18 acyl-CoAs and $\geq$ C20 acyl-CoAs, respectively.^{21,48} In mice, *Far1* is most highly expressed in

the preputial gland, followed by kidney, testis, and brain.²¹ Similar to RCDP model mice, most *Far1* KO mice cannot survive to adulthood, and the few that do survive exhibit male infertility due to impaired seminolipid production.^{49,50} *Far2* is most highly expressed in the meibomian glands, followed by skin containing sebaceous glands, small intestine, and brain.²¹ Lipids secreted from the meibomian glands (collectively known as meibum lipids) constitute the tear film lipid layer and play an important role in protecting the cornea.⁵¹ *Far2* is involved in the production of fatty alcohol-containing meibum and sebum lipids such as wax esters, and *Far2* KO mice exhibit dry eye and *cicatricial alopecia*.^{48,52} To date, no studies have analyzed the brains of *Far1* KO or *Far2* KO mice, so the contributions of *Far1* and *Far2* to the synthesis of ether lipids in the brain remain unclear. In this study, we analyzed *Far1* KO and *Far2* KO mice to resolve this question and the pathogenic mechanism of PFCRD.

Results

Expression and distribution of *Far1* in the brain

To reveal the contributions of *Far1* and *Far2* to ether lipid synthesis in the mouse brain, the expression levels of their mRNAs were examined via quantitative reverse-transcription polymerase chain reaction (RT-PCR). We found that both *Far1* and *Far2* were expressed in the brain, but the expression levels of *Far1* were 2.0- and 3.1-fold those of *Far2* in males and females, respectively (Figure 2A), although there were no significant differences in *Far1* or *Far2* expression between males and females. We then examined the distribution of *Far1* mRNA in the brain via *in situ* hybridization. Compared with the samples without the probe, the *Far1* probe generated stronger signals in the cerebral cortex, hippocampus, and cerebellum (Figure 2B). *Far1* mRNA was expressed in the neurons constituting the granular layer of the cerebellar cortex and the CA1–CA3 regions and *dentate gyrus* of the hippocampus. In addition, *Far1* was expressed in white matter such as the *corpus callosum*. Since the predominant constituent cells of white matter are oligodendrocytes, which form the myelin sheath, *Far1* is likely to be expressed in oligodendrocytes as well as neurons.

Impaired ossification in embryonic *Far1* KO mice

Although the symptoms of PFCRD caused by *FAR1* mutations show many similarities to those of RCDP, PFCRD is not accompanied by *rhizomelia* or *chondrodysplasia punctata*.^{38,40} To investigate the reason for this and reveal the phenotype caused by *Far1* deficiency in mice, we analyzed *Far1* KO mice on embryonic day 18.5. The *Far1* KO mice, which have a 22 bp deletion encompassing the start codon, were generated in an earlier study via the CRISPR-Cas9 system.⁴⁵ *Far1* KO mice were identified via genomic PCR, followed by agarose gel electrophoresis (Supplementary Figure S1). Although there was no obvious difference in their appearance from wild-type (WT) mice (Figure 3A), *Far1* KO mice had a slightly lighter body weight than WT mice (Figure 3B). Staining of hard bones and cartilage with alizarin red and alcian blue, respectively, revealed that *Far1* KO mice showed impaired hard bone formation (Figure 3C and D). In WT mice, ossification of approximately eight segments was observed

from the base to the tip of the tail, whereas only four or five segments were ossified in the *Far1* KO mice. A similar phenotype was observed in *Pex7* KO mice,⁴¹ a mouse model of RCPD, suggesting that *Far1* deficiency causes RCPD-like ossification abnormalities, at least in mice.

Hypomyelination in the brain of *Far1* KO mice

To gain insight into the molecular mechanisms underlying the neurological symptoms of PFCRD, we analyzed the brains of *Far1* KO mice. Hematoxylin and eosin staining of the sagittal sections of the brain revealed that *Far1* KO mice showed no obvious histological abnormalities in the cerebral cortex, hippocampus, or cerebellum (Figure 4A). Next, considering that white matter abnormalities have been reported in PFCRD patients,^{38,40} the *corpus callosum* of *Far1* KO mice was analyzed via electron microscopy. We found that *Far1* KO mice had fewer myelinated axons than WT mice did, especially among axons with radii of $< 0.5 \mu\text{m}$, and more unmyelinated axons (Figure 4B, C, and D), although total axon numbers were similar (Figure 4C). The ratio of myelinated axons to total axons was approximately half in *Far1* KO mice of the ratio in WT mice (Figure 4E). The *g*-ratio value, an index of myelin sheath thickness in myelinated axons, was greater in *Far1* KO mice than in WT mice, indicating thinner myelin sheaths in *Far1* KO mice (Figure 4F). Immunoblotting revealed that the quantity of myelin basic protein, the oligodendrocyte marker, in *Far1* KO mice was 73% of that in WT mice (Figure 4G). However, there was no significant difference in the quantity of the neuron marker NeuN between WT and *Far1* KO mice. The *Far1* KO mice therefore exhibited hypomyelination.

We then examined the expression levels of cell type-specific markers in the brain on embryonic day 18.5 via quantitative RT-PCR. The mRNA levels of the neuron marker *Rbfox3* (encoding the NeuN protein), the astrocyte marker *Gfap*, the microglia marker *Tmem119*, the oligodendrocyte precursor cell (OPC) marker *Cspg4*, and the oligodendrocyte marker *Cnp* were similar in WT and *Far1* KO mice (Figure 4H). In contrast, the mRNA levels of *Pdgfra*, another OPC marker, were lower in *Far1* KO mice than in WT mice. These results suggest that the initial differentiation of various cell types in the embryonic brain proceeds normally in

Far1 KO mice, although a subtle change in OPC differentiation may be present.

Reduced quantities of ether lipids in the brain of *Far1* KO mice

To examine the effect of *Far1* deficiency on ether lipid synthesis in the brain, lipids were extracted from the brains of WT and *Far1* KO mice, treated with alkali (NaOH) or acid (HCl), and separated via thin-layer chromatography (TLC). Alkalis hydrolyze ester bonds but not ether bonds, whereas acids hydrolyze vinyl ether bonds in plasmalogens (Figure 5A). For example, PE[P]s are converted to lysoPE[P]s (LPE[P]s) and lysoPEs (LPEs) via alkali and acid treatment, respectively. When lipids prepared from WT mouse brain were separated via TLC without alkali or acid treatment and stained with copper phosphate, several lipid bands were observed, including cholesterol/FAs (cholesterol and FAs migrated to similar positions under the TLC conditions used), hexosylceramides (HexCers; mostly galactosylceramides in the brain), PE[P]s/PEs, phosphatidylinositols (PIs)/phosphatidylserines (PSs), PCs, and sphingomyelins (SMs) (Figure 5B). A similar band pattern was observed for *Far1* KO mice. However, differences in lipid patterns appeared when the alkali- or acid-treated samples were separated via TLC. Alkali treatment converted ester-type glycerophospholipids (PEs, PIs, PSs, and PCs) and PE[P]s into FAs and LPE[P]s, respectively. The LPE[P]s observed in WT mice were barely detectable in *Far1* KO mice. When treated with acid, LPEs were generated in WT mice, but their levels were barely detectable in *Far1* KO mice. These results indicate that PE[P] levels were substantially reduced in the brains of *Far1* KO mice compared with WT mice. Separation of cholesterol and FAs using a more hydrophobic resolving solvent revealed that the quantities of FAs and cholesterol were similar in the brains of WT and *Far1* KO mice (Figure 5C).

Next, PEs, PE[P]s, PCs, PC[O]s, PIs, HexCers, and SMs were measured more quantitatively via liquid chromatography–tandem mass spectrometry (LC-MS/MS). Of the total lipid quantities measured, PE[P]s and PEs accounted for 10.6% and 8.6%, respectively, in the brain of WT mice (Figure 5D). In *Far1* KO mice, the quantities of PE[P]s were greatly reduced (0.9% of total lipids), whereas those of PEs were increased to 20.9% of total lipids.

The percentages of PEs and PE[P]s combined were similar in WT and *Far1* KO mice (Figure 5E). In mammals, FAR2 exists as another FAR. We also quantified PE[P]s and PEs in *Far2* KO mice, generated in an earlier study via the CRISPR-Cas9 system,⁵⁰ and found that both quantities were similar to those in WT mice (Figure 5F).

In WT mice, PC[O]s were also present, although in much lower quantities than PE[P]s (Figure 5D). In *Far1* KO mice, PC[O]s were almost entirely absent. No significant differences were observed in the levels of PCs, or HexCers between WT and *Far1* KO mice. In contrast, the levels of PIs and SMs were slightly increased (1.4-fold and 1.8-fold, respectively) in *Far1* KO mice relative to WT mice.

Regarding the composition of the alkyl chain at the *sn*-1 position and the acyl chain at the *sn*-2 position of PE[P]s in the WT mouse brain, the predominant alkyl chains were C16:0, C18:0, and C18:1 (Figure 5G). Hereafter, alkyl chains will be represented as “P-” (e.g., “P-C16:0”) to distinguish them from acyl chains.²⁷ The most abundant acyl chain was C18:1, followed by C22:6, C20:4, C22:4, C16:0, and C18:0. In *Far1* KO mice, most of these species were absent, but small amounts of P-C18:0 species (P-C18:0/C18:1, P-C18:0/C20:4, P-C18:0/C22:4, and P-C18:0/C22:6) were present. There were no significant changes in the quantities of PE[P] species in *Far2* KO mice relative to WT mice (Supplementary Figure S2A).

In the WT mouse brain, the PEs included several species, with the most abundant ones being C18:0–C20:4, C18:0–C22:6, C18:1–C18:1, C18:0–C18:1, C16:0–C18:1, and C18:0–C22:4 (Figure 5H). In *Far1* KO mice, the levels of many PE species, except for C18:0–C22:6, were increased compared with WT mice, probably due to altered metabolic flux caused by impaired PE[P] production. PE species levels did not differ significantly between WT and *Far2* KO mice (Supplementary Figure S2B). In summary, *Far1* plays a central role in fatty alcohol production for ether lipids in the brain.

Discussion

In the present study, we revealed that of the two FAR isozymes, FAR1 is mainly responsible for the production of fatty alcohols used in ether lipid synthesis in the brain. PE[P]s were almost absent in the brains of *Far1* KO mice, with the exception of some residual P-C18:0 species (Figure 5G), which are probably produced by FAR2. The dominant contribution of FAR1 over FAR2 to brain ether lipid production can be attributed to its substrate specificities. We previously reported that FAR1 and FAR2 show high activity toward C16–C20 and $\geq$ C20 acyl-CoAs, respectively.⁴⁸ Most of the brain PE[P]s have a C16–C18 alkyl chain at the *sn*-1 position (Figure 5G).

In *Far1* KO mice, the reduction in PE[P] levels was accompanied by a compensatory increase in the levels of most of the PE species (Figure 5G and H). However, the levels of PE species containing C22:6 (docosahexaenoic acid) were less affected, and those of PE species containing C18:0–C22:6 were unchanged. This may be explained by the observation that C22:6-containing PE[P]s were present in neurons and astrocytes but not in oligodendrocytes, whereas others, including C20:4 (arachidonic acid)-containing species, were present in all three cell types.⁵³ Oligodendrocytes may be more sensitive to reduced ether lipid levels than neurons and astrocytes and may compensate for reduced ether lipid levels with increased PE levels.

White matter abnormalities have been observed in PFCRD and RCDP patients via MRI analyses.^{54,55} Here, we found that *Far1* KO mice had fewer myelinated axons and thinner myelin sheaths than WT mice via electron microscopy of the *corpus callosum* (Figure 4B–F). Since the analyses were performed on six-month-old mice, it remains unclear whether the hypomyelination in *Far1* KO mice was due to impaired myelin formation or to demyelination following initially normal myelination. Similar myelination defects have been observed in the spinal cord and optic nerves of *Gnpat* KO mice, from as early as postnatal day 21 to 1.5 years of age,⁴⁷ suggesting that both the initial myelination and myelin maintenance phases are defective. Consistent with the possibility of impaired myelin formation, patients with *Far1* mutations exhibit neurological abnormalities early after birth; they are hypotonic in the neonatal period and developed epileptic seizures around one year of age.³⁸

The reduced myelinated axons and thinning of myelin sheaths in the *Far1* KO mouse brain could be related to a reduced myelination capacity of oligodendrocytes, as demonstrated via *in vitro* co-culture of neurons and oligodendrocytes from *Gnpat* KO mice.⁴⁷ We speculate that oligodendrocyte myelination capacity depends on the unique structure of PE[P]s. The *sn*-1 alkyl chain, which has a vinyl ether bond, and the *sn*-2 acyl-chain (often polyunsaturated FAs) enable PE[P] to form a cone-shaped structure with a wider tail than PE, which has the same ethanolamine as a polar head group.^{56,57} This structure may be suitable as a building block for highly curved membranes in myelin sheaths, such as those responsible for the tip wrapping of axons and paranodes, which are adjacent to the nodes of Ranvier (Figure 6). Indeed, in *Far1* KO mice, a smaller proportion of axons with shorter radii, i.e., higher curvature, were myelinated (Figure 4D). It is also possible that the reduced curvature of the myelin sheath in *Far1* KO mice causes impaired adhesion between the myelin sheath and the axon at the paranodes (Figure 6).

The antioxidative properties of PE[P]s may also be important for myelin formation and maintenance. In WT mice, isolated sciatic nerves exhibit compaction of the myelin membrane stack upon treatment with reactive oxygen species; sciatic nerves from *Pex7* KO mice are more vulnerable to this treatment than those from WT mice.⁹ Thus, PE[P] may protect myelin membranes from damage by neutralizing reactive oxygen species.

Mutations in the genes involved in plasmalogen synthesis generally cause RCDP, which includes *rhizomelia* and *chondrodysplasia punctata*, whereas mutations in *FAR1* cause PFCDP, which does not.^{38,40} However, we observed that ossification of the tail was impaired in *Far1* KO mice (Figure 3C and D), which may be related to the *rhizomelia* and *chondrodysplasia punctata* in human RCDP patients. Although the exact reason for this discrepancy between humans and mice remains unknown, it is possible that the contribution of *FAR2* to bone formation in humans is greater than it is in mice and that this compensates for the effects of *FAR1* deficiency.

In the present study, we revealed that FAR1 plays an essential role in ether lipid synthesis and myelination in the brain. We also found compensatory changes in lipid composition due

to the deficiency in ether phospholipid synthesis, such as increases in PEs, PIs, and SMs. These findings provide clues that will help with the elucidation of the molecular mechanisms of the pathogenesis of PFCRD and RCDP and with the development of therapeutic drugs.

Materials and Methods

Mice

C57BL/6J mice were purchased from Sankyo Labo Service (Sapporo, Japan). *Far1* KO and *Far2* KO mice used in this study were generated from C57BL/6J mice, as reported previously.^{48,50} Genotyping of *Far1* KO mice was performed by PCR using genomic DNA obtained from the tail, using the following allele-specific primer pairs: WT allele, 5'-ATGGTTTCAATCCCAGAATACTACG-3' and 5'-GGGATCCGTGAGTGATTTGTCTGATATGATCC-3'; KO allele, 5'-CATGTTTTTCTCAGTCAGGTTTCAGG-3' and 5'-GGGATCCGTGAGTGATTTGTCTGATATGATCC-3'. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Genotyping of *Far2* KO mice was performed as previously described.⁴⁸ Mice were housed under specific pathogen-free conditions at a room temperature of 23 ± 1 °C and a humidity of $50 \pm 10\%$, with a 12 h light and 12 h dark cycle and water and food (Rodent Diet CE-2; CLEA Japan, Tokyo, Japan) available *ad libitum*. Animal experiments were approved (No. 22-0036) by the Institutional Animal Care and Use Committee of Hokkaido University.

Quantitative RT-PCR

Total RNAs from adult or embryonic day 18.5 mouse brains were isolated using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and converted to first-strand cDNAs using the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan), following the manufacturer's instructions. The first-strand cDNAs were mixed with gene-specific primer pairs (Table 1) and KOD SYBR qPCR Mix (Toyobo, Osaka, Japan), which contains SYBR Green I fluorophore and KOD polymerase, and subjected to real-time quantitative PCR on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The PCR conditions were as follows: 98 °C for 2 min; 40 cycles of 98 °C for 10 sec, 60 °C for 10 sec, and 68 °C for 10 sec. The mRNA levels were normalized with respect to *Gapdh* and *Hprt1* for embryonic day 18.5 and adult mouse brains, respectively.

In situ hybridization

The brains of two-month-old C57BL/6J mice were fixed via cardiac perfusion of 4% paraformaldehyde in sodium phosphate buffer (pH 7.4), removed from the skull, and post-fixed in the same fixative for 24 h at 4 °C. The brains were frozen in Tissue-Tek OCT compound (Sakura Finetek Japan, Tokyo, Japan), cut into 30 µm-thick sections using a cryostat (CM3050S; Leica Biosystems, Wetzlar, Germany), allowed to float freely, and treated with 10 µg/ml proteinase K in Tris-HCl buffer (pH 8.0) containing 5 mM EDTA for 10 min at room temperature. Preparation of digoxigenin-labeled antisense *Far1* probes, hybridization of probes with sections, signal detection, and image acquisition were performed as described previously.⁵⁰

Hematoxylin and eosin staining

The brains of two-month-old male WT and *Far1* KO mice were fixed in Super Fix (Kurabo, Osaka, Japan) for 24 h at 4 °C. The fixed brains were embedded in paraffin using Tissue-Tek VIP6 and Tissue-Tek TEC Plus (Sakura Finetek Japan), cut into 4 µm-thick sections using microtome (REM-701; Yamato Kohki, Saitama, Japan), stained with hematoxylin and eosin using an automated staining system (TissueTek Prisma 2000; Sakura Finetek Japan), and mounted using Tissue-Tek Glas g2 (Sakura Finetek Japan). Bright-field images were captured using a Leica DM5000 B microscope equipped with a DFC295 digital color camera (Leica Microsystems, Wetzlar, Germany).

Transmission electron microscopy

The *corpus callosum* of six-month-old male WT and *Far1* KO mice were subjected to transmission electron microscopy. Sample preparation and image acquisition of parasagittal planes were performed as described previously.⁵⁸ The number of myelinated and unmyelinated axons in 12 randomly captured images per genotype were counted using the ImageJ software.⁵⁹ For the calculation of the g-ratio—the ratio of the axon diameter to the outer fiber (axon plus

myelin) diameter—the areas of each axon and its outer fiber were measured using ImageJ, and the diameters of axon and outer fiber were calculated under the assumption that they were exact circles.

Immunoblotting

Each brain tissue sample (about 20 mg) from six-month-old female WT and *Far1* KO mice was homogenized in a tube containing zirconia beads and lysis buffer (50 mM HEPES-NaOH [pH 7.4], 150 mM NaCl, 10% glycerol [v/v], 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1×cOmplete EDTA-free protease inhibitor cocktail [Merck, Darmstadt, Germany]) via vigorous mixing using a Micro Smash MS-100 (TOMY Seiko, Tokyo, Japan) at 4,500 rpm for 1 min at 4 °C. After centrifugation ($800 \times g$, 3 min, 4 °C), the supernatant was recovered, and proteins were precipitated by adding trichloroacetic acid to a final concentration of 5% and then dissolved in 1×SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol).

Immunoblotting was performed as described previously,⁶⁰ using anti- α -tubulin monoclonal antibody 10G10 (0.5 μ g/ml; FUJIFILM Wako Pure Chemical, Osaka, Japan), anti-NeuN monoclonal antibody EPR12763 (1:1,000; Abcam, Cambridge, UK), and anti-myelin basic protein monoclonal antibody D8X4Q (1:1,000; Cell Signaling Technology, Danvers, MA, USA). Labeling was detected using a chemiluminescent reagent, prepared by mixing equal volumes of solution A (100 mM Tris-HCl [pH 8.5], 0.4 mM *p*-coumaric acid [Merck], 5 mM luminol [FUJIFILM Wako Pure Chemical]) and solution B (100 mM Tris-HCl [pH 8.5], 0.04% hydrogen peroxide), and the chemiluminescence was imaged using an Amersham Imager 600 (Cytiva, Little Chalfont, UK).

Lipid extraction

Each brain tissue sample (about 10 mg) from six- or seven-week-old WT, *Far1* KO, and *Far2* KO mice was mixed in a tube containing zirconia beads, chloroform/methanol (1:2, v/v), and the following deuterium (*d*)-labeled internal standards (500 pmol each; C16:0 glucosylceramide [d18:1/C16:0-*d*₃] and C16:0 SM [d18:1/C16:0-*d*₉]; Cayman Chemical, Ann

Arbor, MI, USA) and subjected to lipid extraction via vigorous mixing using a Micro Smash MS-100 at 4,500 rpm for 1 min at 4 °C. The samples were centrifuged ($20,400 \times g$, 3 min, 4 °C) and separated into supernatant and pellet. The pellet was subjected to second extraction with 300 μ l of chloroform/methanol (1:2, v/v), followed by centrifugation ($20,400 \times g$, 3 min, 4 °C). The supernatant was recovered, combined with the previous supernatant, mixed with 200 μ l of chloroform and 360 μ l of water, and vigorously mixed. After centrifugation ($20,400 \times g$, 5 min, room temperature), the resulting lower (organic) phase was recovered and dried.

Treatment of lipids with alkali or acid was performed as described below. The dried lipids dissolved in 200 μ l of chloroform/methanol (1:2, v/v) were mixed with 5 μ l of 4 M NaOH for the alkali treatment, 5 μ l of 1.6 M HCl for the acid treatment, and 5 μ l of water for the control treatment and incubated for 1 h at 37 °C. The samples were then neutralized by adding 5 μ l of 4 M formic acid for the alkali treatment, 5 μ l of 1.6 M NaOH for the acid treatment, and 5 μ l of water for the control treatment. To increase the extraction efficiency of the glycerophospholipids, the samples were acidified by mixing them with 2 μ l of 4 M formic acid. The samples were sequentially mixed with 400 μ l of chloroform/methanol (1:2, v/v), 200 μ l of chloroform, and 360 μ l of water and centrifuged ($20,400 \times g$, 5 min, room temperature). The resulting lower (organic) phase was recovered and dried.

Lipid analysis via TLC

Lipids (from 0.25 mg of brain tissue) treated with alkali, acid, or water (control) were spotted onto a TLC plate (HPTLC Silica gel 60, Merck) and separated with 1-butanol/acetic acid/water (3:1:1, v/v) or hexane/diethyl ether/acetic acid (20:80:1, v/v) as the solvent system. The TLC plate was dried and the lipids were stained with a cupric acetate/phosphoric acid solution.

Lipid analysis via LC-MS/MS

We used a Xevo TQ-XS LC-coupled triple quadrupole mass spectrometer (Waters, Milford, MA, USA) for this analysis. The samples dissolved in chloroform/methanol (1:2, v/v) were separated using an ACQUITY UPLC CSH C18 reversed-phase column (1.7 μ m particle size,

2.1 mm inner diameter, 100 mm length; Waters). The LC flow rate was 0.3 ml/min in a binary gradient system using mobile phase A (acetonitrile/water [3:2, v/v] containing 5 mM ammonium formate) and mobile phase B (acetonitrile/2-propanol [1:9, v/v] containing 5 mM ammonium formate). The gradient steps were as follows: 0 min, 40% B; 0–18 min, linear gradient to 100% B; 18–23 min, 100% B; 23–23.1 min, step to 40% B; and 23.1–25 min, 40% B. Alkali-treated lipids were used for the analysis of sphingolipids (HexCer and SM) to reduce the ion-suppression effect and unrelated peaks resulting from the presence of glycerophospholipids. For the analysis of glycerophospholipids (PCs, PC[O]s, PEs, PE[P]s, and PIs), control-treated lipids were used. Lipids were ionized via electrospray ionization and analyzed in positive ion mode (for the analysis of PCs, PC[O]s, HexCers, and SMs) or negative ion mode (for the analysis of PEs, PE[P]s, and PIs). MS/MS analysis was performed in multiple reaction monitoring mode using the m/z values of the precursor (Q1) and product (Q3) ions specific to each lipid species and optimized collision energies (Supplementary Table S1). Data analysis was performed using the MassLynx software (Waters). The quantity of each HexCer and SM species was calculated from its peak area as its ratio to the value of the corresponding internal standards: HexCer, d18:1/C16:0- d_3 ; SM, d18:1/C16:0- d_9 . The quantity of each PC, PC[O], PE, PE[P], and PI species was calculated in the same way using the corresponding external standards: PC, 18:0/18:1; PC[O], O-18:1/18:1; PE, 16:0/18:1; PE[P], P-18:0/18:1; PI, 16:0/18:1 (all from Avanti Research, Alabaster, AL, USA).

Skeleton analysis via bone staining

Staining of cartilage and ossified bones from WT and *Far1* KO mice at embryonic day 18.5 was performed as follows at room temperature. All tissues except the cartilage and ossified bones were removed from the body with forceps, and the samples were fixed in ethanol for 96 h. The cartilage was stained in solution A (0.15 mg/ml alcian blue 8GX [Merck], 20% acetic acid [v/v], and 75% ethanol [v/v]) for 24 h. After washing twice with 95% ethanol (v/v), the samples were incubated in 2% KOH (w/v) overnight, then the ossified bones were stained in solution B (5 mg/ml of alizarin red [Merck] in 1% KOH [w/v]) for 24 h. Nonspecific staining

was removed via incubation in a solution containing 1% KOH (w/v) and 20% glycerol (v/v) for 3–7 days. The samples were sequentially incubated for each 24 h in the following three solutions: 20% glycerol (v/v) and 20% ethanol (v/v), 50% glycerol (v/v) and 50% ethanol (v/v), and 100% glycerol (v/v). Bright-field images were captured using a HC-210M digital microscope (Sato Shouji, Kanagawa, Japan).

Statistical analysis

Microsoft Excel (Microsoft, Redmond, USA) and JMP 13 (SAS Institute, Cary, USA) were used to analyze the data using Welch's *t*-test and Dunnett's test, respectively.

Disclosure statement

The authors have no conflicts of interest to declare.

Funding

This work was supported by KAKENHI (grant numbers: JP22H04986 to A.K. and JP23K24020 to T.S.) from the Japan Society for the Promotion of Science (JSPS).

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Data availability statement

The data that support the findings of this study are available within the article and its supplementary materials.

Author contributions

T. T., K. O., and T. S. investigation; T. S. and A. K. writing—original draft; T. S. and A. K. supervision; A. K. writing—review and editing; A. K. conceptualization; A. K. project administration; T. S. and A. K. funding acquisition.

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Figure legends

Figure 1. Synthetic pathway of PE[P]. In the first reaction, a FA is esterified to glycerone-phosphate by GNPAT. This FA is then replaced by an ether-linked fatty alcohol to form alkyl-glycerone-phosphate. AGPS catalyzes this reaction, and the substrate, fatty alcohol, is generated from fatty acyl-CoA by FARs. Two FAR isozymes, FAR1 and FAR2, exist in mammals. Subsequent reduction of the carbonyl group to a hydroxy group, esterification of a FA to the *sn*-2 position, and removal of a phosphate group generate 1-alkyl-2-acyl-glycerol. The addition of phosphoethanolamine to 1-alkyl-2-acyl-glycerol generates plasmanylethanolamine (1-alkyl-2-acyl-glycerol-3-phosphoethanolamine; PE[O]). Conversion of the ether bond in PE[O] to a vinyl ether bond generates PE[P]. Etn, ethanolamine; P (encircled), phosphate.

Figure 2. *Far1* expression in the mouse brain. (A) Total RNAs were extracted from the brains of eight-week-old male and female C57BL/6 mice and subjected to quantitative RT-PCR using specific primers for *Far1*, *Far2*, or the housekeeping gene *Hprt1*. Values presented are means + SD of each mRNA quantity relative to that of *Hprt1* (n = 4). (B) Fresh frozen coronal sections containing cerebral cortex, hippocampus, cerebellar cortex, and *corpus callosum* were prepared from the brains of seven-week-old male C57BL/6 mice and subjected to *in situ* hybridization using digoxigenin-labeled antisense RNA probes against *Far1*. Probe (–) represents a negative control experiment without probes. Scale bars: 30 μ m, cerebral cortex and *corpus callosum*; 50 μ m, hippocampus and cerebellar cortex. CA1 and CA3, CA1 and CA3 subregions of the hippocampus, respectively; CC, *corpus callosum*; DG, *dentate gyrus*; GCL, granule cell layer; ML, molecular layer.

Figure 3. Impaired bone ossification in *Far1* KO mice. (A) The appearance of WT and *Far1* KO mouse embryos at embryonic day 18.5. Scale bars, 5 mm. (B) The body weights of WT, *Far1* heterozygous KO (HET), and *Far1* KO mouse embryos at embryonic day 18.5. (C and D) The tails of WT and *Far1* KO mouse embryos at embryonic day 18.5 were stained with

alcian blue (cartilages) and alizarin red (hard bones) (C), and the number of ossified segments (arrows) was counted (D). Scale bars, 1 mm. Values presented are means + SD (n = 3). Statistically significant differences are indicated (B, Dunnett's test; D, Welch's *t* test; * *p* < 0.05, ** *p* < 0.01).

Figure 4. Impaired myelination in *Far1* KO mice. (A) Images of sagittal sections of brains from 10-week-old male WT and *Far1* KO mice stained with hematoxylin and eosin. Scale bars, 5 mm. (B) Representative transmission electron microscopy images of transverse sections of the corpus callosum from six-month-old male WT and *Far1* KO mice. The lower images are enlarged views of the areas denoted by the white rectangles in the upper images. Scale bars, 1 μ m. (C) Average numbers of myelinated axons, unmyelinated axons, and their sum per μ m², calculated from the analysis of 12 independent images. (D) Composition of axon radii in myelinated axons. (E) Ratio of myelinated axons to total axons. (F) *G*-ratio, the ratio of the axon diameter to the outer fiber (axon plus myelin) diameter, calculated from myelinated axons (WT, n = 113; *Far1* KO, n = 98). Values presented are means + SD. Statistically significant differences are indicated (Welch's *t*-test; * *p* < 0.05, ** *p* < 0.01). (G) Total lysates prepared from the brains of six-month-old female WT and *Far1* KO mice (n = 3) were separated via SDS-PAGE and subjected to immunoblotting using anti-myelin basic protein (MBP, an oligodendrocyte marker), anti-neuronal nuclei (NeuN, a neuronal marker), and anti- α -tubulin (loading control) antibodies. The relative levels of MBP and NeuN proteins to α -tubulin are shown in right graphs. Values presented are means + SD. Statistically significant differences are indicated (Welch's *t*-test; * *p* < 0.05). (H) Total RNAs prepared from the brains of embryonic day 18.5 WT and *Far1* KO mice (n = 5–8) were subjected to quantitative RT-PCR using primers specific to *Rbfox3* (encoding NeuN), *Gfap* (an astrocyte marker), *Tmem119* (a microglia marker), *Pdgfra* and *Cspg4* (OPC markers), *Cnp* (an oligodendrocyte marker), and *Gapdh* (a housekeeping gene). Values presented are means + SD of each mRNA relative to that of *Gapdh*. Statistically significant differences are indicated (Welch's *t*-test; * *p* < 0.05).

Figure 5. Decrease in PE[P] levels and compensatory increase in PE levels in *Far1* KO mice. (A) Schematic diagram of alkali and acid treatment. PE[P] is degraded to lysoPE[P] (LPE[P]) and lysoPE (LPE) by alkali (NaOH) and acid (HCl) treatments, respectively. Etn, ethanolamine; P (encircled), phosphate. (B and C) Lipids were extracted from the brains of six- or seven-week-old female WT and *Far1* KO mice ($n = 3$). Untreated lipids and lipids treated with NaOH or HCl were separated via TLC using (B) 1-butanol/acetic acid/water = 3:1:1 (v/v) and (C) hexane/diethyl ether/acetic acid = 20:80:1 (v/v) as the solvent systems and stained with a cupric acetate/phosphoric acid solution. Chol, cholesterol. (D, E, G, and H) Lipids were extracted from the brains of six- or seven-week-old female WT and *Far1* KO mice ($n = 3$), and PCs, PC[O]s, PEs, PE[P]s, PIs, HexCers, and SMs were quantified via LC-MS/MS. Values presented are means + SD of the ratio of each lipid class (D) or the sum of PE[P]s and PEs (E) to all lipids measured, or the ratio of each PE[P] (G) or PE (H) species to the sum of PE[P]s and PEs. The use of a hyphen in the PE species indicates that the two acyl chains were not specific to the *sn*-1 or *sn*-2 position. (F) Lipids were extracted from the brains of six- or seven-week-old female WT and *Far2* KO mice ($n = 3$) and PE[P]s and PEs were quantified via LC-MS/MS. Values presented are means + SD. Statistically significant differences are indicated (Welch's *t*-test; * $p < 0.05$, ** $p < 0.01$).

Figure 6. Possible role for PE[P]s in myelination in the brain. The membranes of a myelinating oligodendrocyte form a highly curved structure with adjacent plasma membranes and narrow cytoplasm between them. The structure of PE[P] is cone-shaped, with a wider tail than PE, which has the same ethanolamine as a polar head group, making it suitable for forming curved membrane structures. This highly curved membrane structure of the myelin sheath may be necessary for wrapping around short-radius axons (upper left panel, transverse sectional view) and for forming close contact with axons at the paranodes (lower left panel, longitudinal sectional view). In *Far1* KO mice, reduced quantities of PE[P]s result in a less curved structure, leading to impaired myelination (right images).

Figure 1

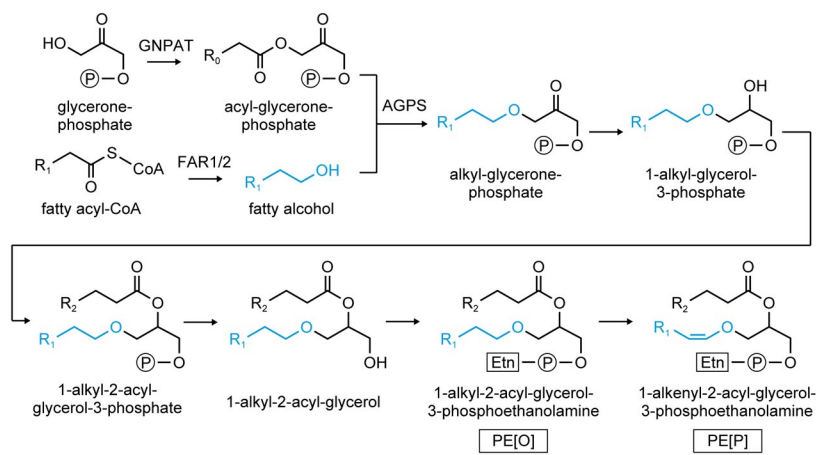


Figure 2

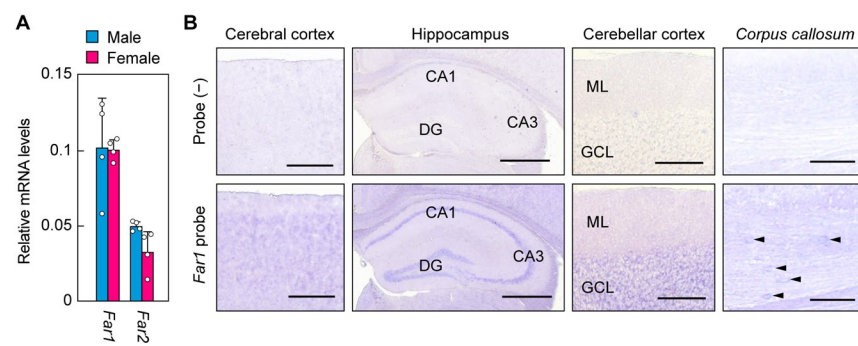


Figure 3

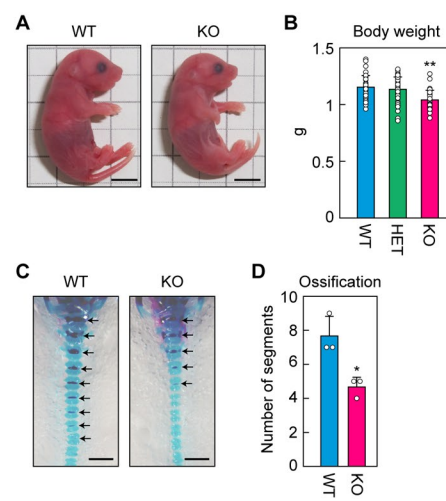


Figure 4

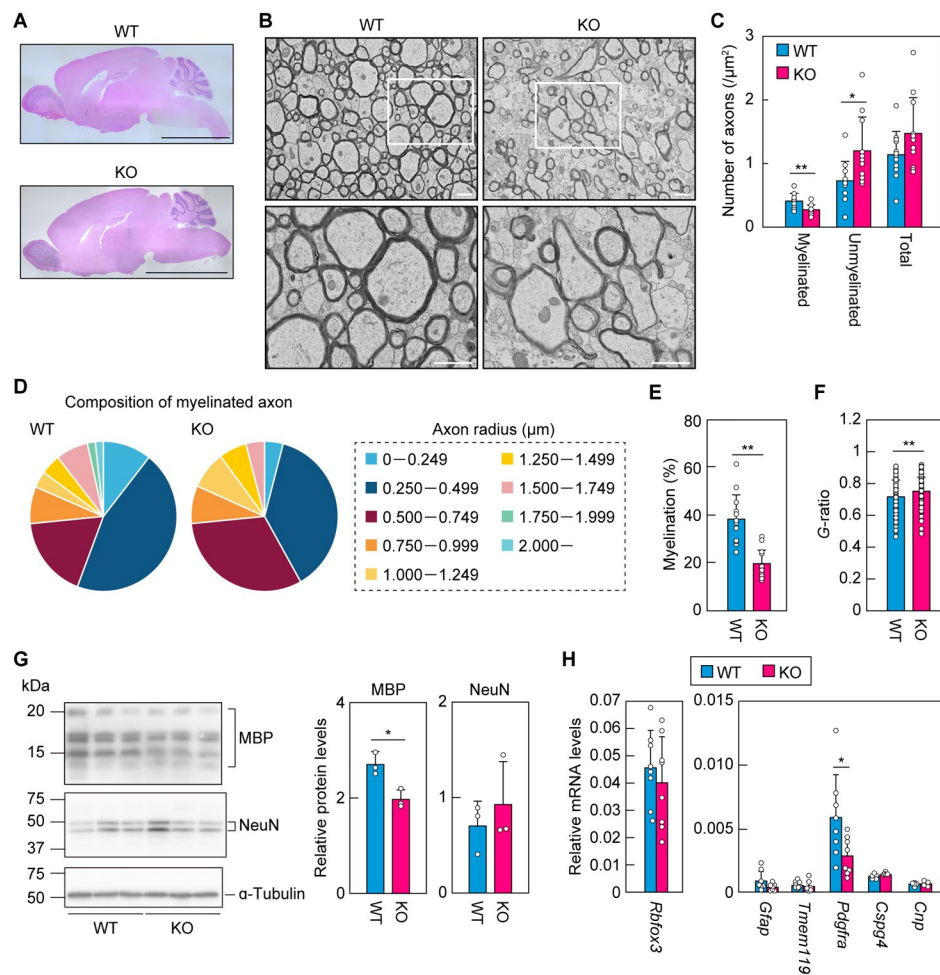


Figure 5

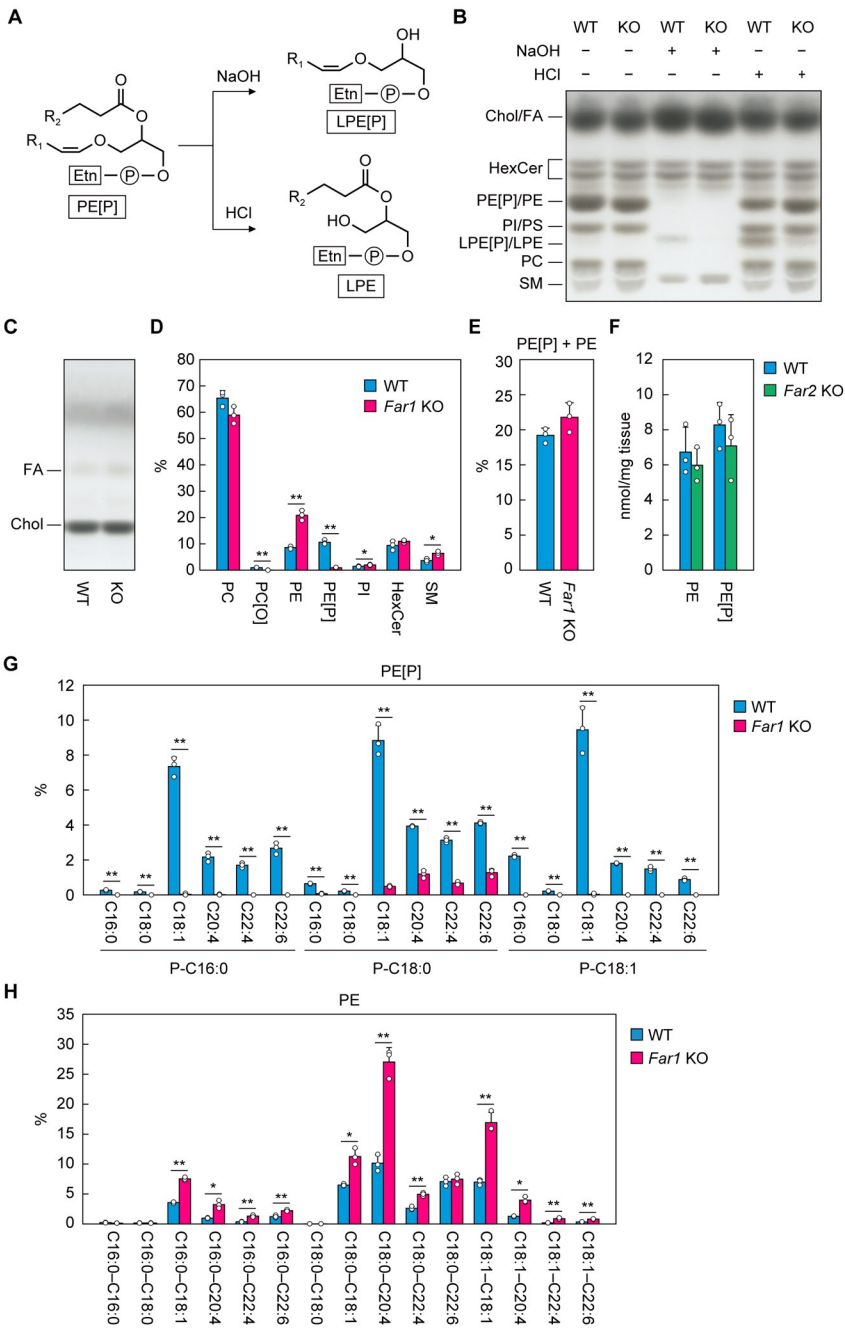


Figure 6

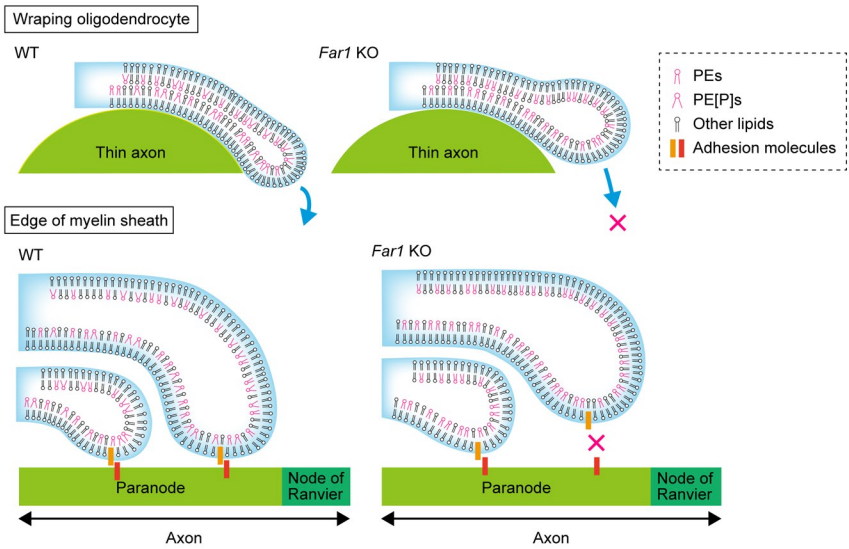


Table 1

Oligonucleotide primers used in quantitative RT-PCR

Primer name	Nucleotide sequence (5' to 3')
Cnp-F	CTGCCACATGCTAGGGACAA
Cnp-R	CCGCCCAGTAAATAGCCCAA
Cspg4-F	TGGACGTGGTGAACCGTAAG
Cspg4-R	GATGATGCGAGGTGGATCGT
Far1-F	GTTGCCATTGTAAGGCCGTC
Far1-R	ATTGTTGGAGGCACGCATTG
Far2-F	ACCAGCAATTTACCAACCCA
Far2-R	AGCCGGTTCATGAGCTTCAG
Gapdh-F	CCAAGGTCATCCATGACAACTTTGG
Gapdh-R	GGTCCACCACCCTGTTGCTGTAGCC
Gfap-F	AGGAGTACCAGGATCTACTCAACG
Gfap-R	GAAAGTCTGTACAGGAATGGTGATG
Hprt1-F	GCTGACCTGCTGGATTACATTAAAG
Hprt1-R	CTTAACCATTTTGGGGCTGTACTGC
Pdgfra-F	GGTCGAAGGTACAGCTTATGGATTA
Pdgfra-R	AGGTGAGTCATTATCTTCAGCTCAG
Rbfox3-F	CAGGATGGATTTTATGGTGCTGAGA
Rbfox3-R	CGGCATAGACTCTACCATAACTGTC
Tmem119-F	GTATGTGGGGTCACTGAAGAGAAG
Tmem119-R	GATTCCTGGGCTAAGGAGAAAGAC

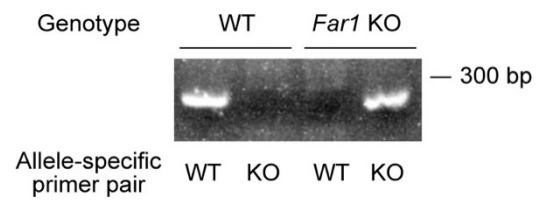


Figure S1. Genotyping of *Far1* KO mice. Genomic DNA was obtained from the tails of WT and *Far1* KO mice and subjected to PCR using allele-specific primer pairs. The PCR products were analyzed via agarose gel electrophoresis.

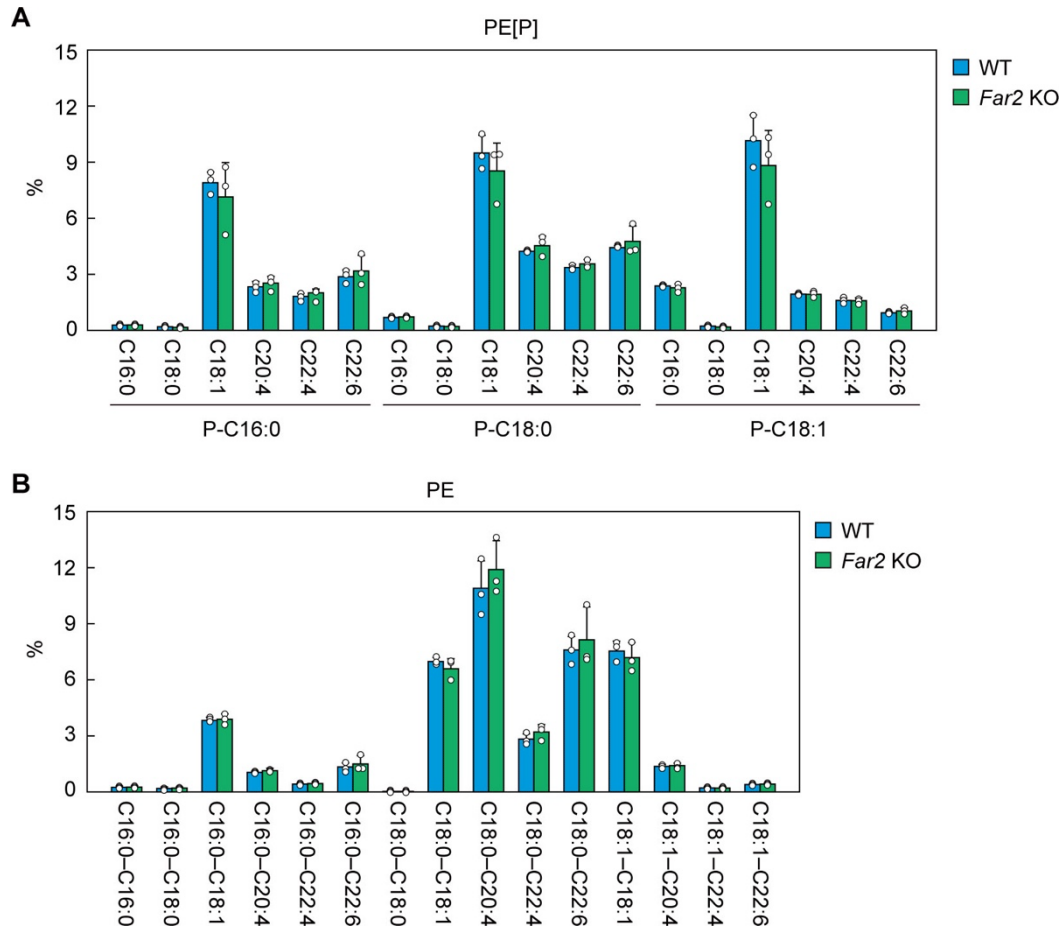


Figure S2. PE[P] and PE levels did not differ significantly between *Far2* KO mice and WT mice. Lipids were extracted from the brains of six- or seven-week-old female WT and *Far2* KO mice (n = 3) and PE[P]s and PEs were quantified via LC-MS/MS. Values presented are means + SD of the ratio of each PE[P] (A) or PE (B) species to the sum of PE[P]s and PEs. The inclusion of a hyphen in the PE species name indicates that the two acyl chains were not specific to the *sn*-1 or *sn*-2 position. No statistically significant differences were found between the WT and *Far2* KO mice (Welch's *t*-test).

Table S1. LC-MS/MS precursor and product ion m/z values and collision energies

Lipid species	Precursor ions (Q1)			Product ions (Q3)	Collision energies (eV)
	$[M+H]^+$	$[M-H_2O+H]^+$	$[M-H]^-$		
PC (C30:0)	720.37			184.00	32
PC (C30:1)	718.32			184.00	32
PC (C30:2)	716.30			184.00	32
PC (C30:3)	714.29			184.00	32
PC (C32:0)	734.35			184.00	32
PC (C32:1)	732.34			184.00	32
PC (C32:2)	730.32			184.00	32
PC (C32:3)	728.30			184.00	32
PC (C32:4)	726.29			184.00	32
PC (C32:5)	724.27			184.00	32
PC (C34:0)	762.38			184.00	32
PC (C34:1)	760.37			184.00	32
PC (C34:2)	758.35			184.00	32
PC (C34:3)	756.34			184.00	32
PC (C34:4)	754.32			184.00	32
PC (C34:5)	752.30			184.00	32
PC (C36:0)	790.42			184.00	32
PC (C36:1)	788.40			184.00	32
PC (C36:2)	786.38			184.00	32
PC (C36:3)	784.37			184.00	32
PC (C36:4)	782.35			184.00	32
PC (C36:5)	780.34			184.00	32
PC (C36:6)	778.32			184.00	32
PC (C38:0)	818.45			184.00	32
PC (C38:1)	816.43			184.00	32
PC (C38:2)	814.42			184.00	32
PC (C38:3)	812.40			184.00	32
PC (C38:4)	810.38			184.00	32
PC (C38:5)	808.37			184.00	32
PC (C38:6)	806.35			184.00	32
PC (C38:7)	804.34			184.00	32
PC (C38:8)	802.32			184.00	32
PC (C40:0)	846.48			184.00	32
PC (C40:1)	844.46			184.00	32
PC (C40:2)	842.45			184.00	32
PC (C40:3)	840.43			184.00	32
PC (C40:4)	838.42			184.00	32
PC (C40:5)	836.40			184.00	32
PC (C40:6)	834.38			184.00	32
PC (C40:7)	832.37			184.00	32
PC (C40:8)	830.35			184.00	32
PC (C42:0)	874.51			184.00	32
PC (C42:1)	872.50			184.00	32
PC (C42:2)	870.48			184.00	32
PC (C42:3)	868.46			184.00	32
PC (C42:4)	866.45			184.00	32
PC (C42:5)	864.43			184.00	32
PC (C42:6)	862.42			184.00	32

PC (C42:7)	860.40			184.00	32
PC (C42:8)	858.38			184.00	32
PC (C42:9)	856.37			184.00	32
PC (C42:10)	854.35			184.00	32
PC (C42:11)	852.34			184.00	32
PC (C42:12)	850.32			184.00	32
PC (C44:0)	902.54			184.00	32
PC (C44:1)	900.53			184.00	32
PC (C44:2)	898.51			184.00	32
PC (C44:3)	896.50			184.00	32
PC (C44:4)	894.48			184.00	32
PC (C44:5)	892.46			184.00	32
PC (C44:6)	890.45			184.00	32
PC (C44:7)	888.43			184.00	32
PC (C44:8)	886.42			184.00	32
PC (C44:9)	884.40			184.00	32
PC (C44:10)	882.38			184.00	32
PC (C44:11)	880.37			184.00	32
PC (C44:12)	878.35			184.00	32
PC[O] (C30:0)	706.35			184.00	32
PC[O] (C30:1)	704.34			184.00	32
PC[O] (C30:2)	702.32			184.00	32
PC[O] (C30:3)	700.30			184.00	32
PC[O] (C32:0)	720.34			184.00	32
PC[O] (C32:1)	718.35			184.00	32
PC[O] (C32:2)	716.34			184.00	32
PC[O] (C32:3)	714.32			184.00	32
PC[O] (C32:4)	712.30			184.00	32
PC[O] (C32:5)	710.29			184.00	32
PC[O] (C34:0)	748.40			184.00	32
PC[O] (C34:1)	746.38			184.00	32
PC[O] (C34:2)	744.37			184.00	32
PC[O] (C34:3)	742.35			184.00	32
PC[O] (C34:4)	740.34			184.00	32
PC[O] (C34:5)	738.32			184.00	32
PC[O] (C36:0)	776.43			184.00	32
PC[O] (C36:1)	774.42			184.00	32
PC[O] (C36:2)	772.40			184.00	32
PC[O] (C36:3)	770.38			184.00	32
PC[O] (C36:4)	768.37			184.00	32
PC[O] (C36:5)	766.35			184.00	32
PC[O] (C36:6)	764.34			184.00	32
PC[O] (C38:0)	804.46			184.00	32
PC[O] (C38:1)	802.45			184.00	32
PC[O] (C38:2)	800.43			184.00	32
PC[O] (C38:3)	798.42			184.00	32
PC[O] (C38:4)	796.40			184.00	32
PC[O] (C38:5)	794.38			184.00	32
PC[O] (C38:6)	792.37			184.00	32
PC[O] (C38:7)	790.35			184.00	32
PC[O] (C38:8)	788.34			184.00	32
PC[O] (C40:0)	832.50			184.00	32

PC[O] (C40:1)	830.48			184.00	32
PC[O] (C40:2)	828.46			184.00	32
PC[O] (C40:3)	826.45			184.00	32
PC[O] (C40:4)	824.43			184.00	32
PC[O] (C40:5)	822.42			184.00	32
PC[O] (C40:6)	820.40			184.00	32
PC[O] (C40:7)	818.38			184.00	32
PC[O] (C40:8)	816.37			184.00	32
PC[O] (C42:0)	860.53			184.00	32
PC[O] (C42:1)	858.51			184.00	32
PC[O] (C42:2)	856.50			184.00	32
PC[O] (C42:3)	854.48			184.00	32
PC[O] (C42:4)	852.46			184.00	32
PC[O] (C42:5)	850.45			184.00	32
PC[O] (C42:6)	848.43			184.00	32
PC[O] (C42:7)	846.42			184.00	32
PC[O] (C42:8)	844.40			184.00	32
PC[O] (C42:9)	842.38			184.00	32
PC[O] (C42:10)	840.37			184.00	32
PC[O] (C42:11)	838.35			184.00	32
PC[O] (C42:12)	836.34			184.00	32
PC[O] (C44:0)	888.56			184.00	32
PC[O] (C44:1)	886.54			184.00	32
PC[O] (C44:2)	884.53			184.00	32
PC[O] (C44:3)	882.51			184.00	32
PC[O] (C44:4)	880.50			184.00	32
PC[O] (C44:5)	878.48			184.00	32
PC[O] (C44:6)	876.46			184.00	32
PC[O] (C44:7)	874.45			184.00	32
PC[O] (C44:8)	872.43			184.00	32
PC[O] (C44:9)	870.42			184.00	32
PC[O] (C44:10)	868.40			184.00	32
PC[O] (C44:11)	866.38			184.00	32
PC[O] (C44:12)	864.37			184.00	32
PE (C16:0–C16:0)			690.30	255.20	38
PE (C16:0–C18:0)			718.40	283.20	38
PE (C16:0–C18:1)			716.30	281.20	38
PE (C16:0–C18:2)			714.30	279.20	38
PE (C16:0–C20:3)			740.30	305.60	32
PE (C16:0–C20:4)			738.30	303.60	32
PE (C16:0–C20:5)			736.30	301.60	32
PE (C16:0–C22:4)			766.30	331.60	32
PE (C16:0–C22:5)			764.30	329.60	32
PE (C16:0–C22:6)			762.30	327.60	32
PE (C16:1–C16:0)			688.30	255.20	38
PE (C16:1–C18:0)			716.30	283.20	38
PE (C16:1–C18:1)			714.30	281.20	38
PE (C16:1–C18:2)			712.30	279.20	38
PE (C16:1–C20:3)			738.30	305.60	32
PE (C16:1–C20:4)			736.30	303.60	32
PE (C16:1–C20:5)			734.30	301.60	32
PE (C16:1–C22:4)			764.30	331.60	32

PE (C16:1–C22:5)	762.30	329.60	32
PE (C16:1–C22:6)	760.30	327.60	32
PE (C16:2–C16:0)	686.30	255.20	38
PE (C16:2–C18:0)	714.30	283.20	38
PE (C16:2–C18:1)	712.30	281.20	38
PE (C16:2–C18:2)	710.30	279.20	38
PE (C16:2–C20:3)	736.30	305.60	32
PE (C16:2–C20:4)	734.30	303.60	32
PE (C16:2–C20:5)	732.30	301.60	32
PE (C16:2–C22:4)	762.30	331.60	32
PE (C16:2–C22:5)	760.30	329.60	32
PE (C16:2–C22:6)	758.30	327.60	32
PE (C18:0–C18:0)	746.40	283.20	38
PE (C18:0–C18:1)	744.40	281.20	38
PE (C18:0–C18:2)	742.40	279.20	38
PE (C18:0–C20:3)	768.30	305.60	32
PE (C18:0–C20:4)	766.30	303.60	32
PE (C18:0–C20:5)	764.30	301.60	32
PE (C18:0–C22:4)	794.40	331.60	32
PE (C18:0–C22:5)	792.30	329.60	32
PE (C18:0–C22:6)	790.30	327.60	32
PE (C18:1–C18:1)	742.40	281.20	38
PE (C18:1–C18:2)	740.30	279.20	38
PE (C18:1–C20:3)	766.30	305.60	32
PE (C18:1–C20:4)	764.30	303.60	32
PE (C18:1–C20:5)	762.30	301.60	32
PE (C18:1–C22:4)	792.30	331.60	32
PE (C18:1–C22:5)	790.30	329.60	32
PE (C18:1–C22:6)	788.30	327.60	32
PE (C18:2–C18:2)	738.30	279.20	38
PE (C18:2–C20:3)	764.30	305.60	32
PE (C18:2–C20:4)	762.30	303.60	32
PE (C18:2–C20:5)	760.30	301.60	32
PE (C18:2–C22:4)	790.30	331.60	32
PE (C18:2–C22:5)	788.30	329.60	32
PE (C18:2–C22:6)	786.30	327.60	32
PE (C20:0–C16:0)	746.40	255.20	38
PE (C20:0–C18:0)	774.40	283.20	38
PE (C20:0–C18:1)	772.40	281.20	38
PE (C20:0–C18:2)	770.40	279.20	38
PE (C20:0–C20:3)	796.40	305.60	32
PE (C20:0–C20:4)	794.40	303.60	32
PE (C20:0–C20:5)	792.30	301.60	32
PE (C20:0–C22:4)	822.40	331.60	32
PE (C20:0–C22:5)	820.40	329.60	32
PE (C20:0–C22:6)	818.40	327.60	32
PE (C20:1–C16:0)	744.40	255.20	38
PE (C20:1–C18:0)	772.40	283.20	38
PE (C20:1–C18:1)	770.40	281.20	38
PE (C20:1–C18:2)	768.40	279.20	38
PE (C20:1–C20:3)	794.40	305.60	32
PE (C20:1–C20:4)	792.30	303.60	32

PE (C20:1–C20:5)	790.30	301.60	32
PE (C20:1–C22:4)	820.40	331.60	32
PE (C20:1–C22:5)	818.40	329.60	32
PE (C20:1–C22:6)	816.30	327.60	32
PE (C20:2–C16:0)	742.40	255.20	38
PE (C20:2–C18:0)	770.40	283.20	38
PE (C20:2–C18:1)	768.40	281.20	38
PE (C20:2–C18:2)	766.40	279.20	38
PE (C20:2–C20:3)	792.30	305.60	32
PE (C20:2–C20:4)	790.30	303.60	32
PE (C20:2–C20:5)	788.30	301.60	32
PE (C20:2–C22:4)	818.40	331.60	32
PE (C20:2–C22:5)	816.30	329.60	32
PE (C20:2–C22:6)	814.30	327.60	32
PE (C22:0–C16:0)	774.40	255.20	38
PE (C22:0–C18:0)	802.50	283.20	38
PE (C22:0–C18:1)	800.40	281.20	38
PE (C22:0–C18:2)	798.40	279.20	38
PE (C22:0–C20:3)	824.40	305.60	32
PE (C22:0–C20:4)	822.40	303.60	32
PE (C22:0–C20:5)	820.40	301.60	32
PE (C22:0–C22:4)	850.40	331.60	32
PE (C22:0–C22:5)	848.40	329.60	32
PE (C22:0–C22:6)	846.40	327.60	32
PE (C22:1–C16:0)	772.40	255.20	38
PE (C22:1–C18:0)	800.40	283.20	38
PE (C22:1–C18:1)	798.40	281.20	38
PE (C22:1–C18:2)	796.40	279.20	38
PE (C22:1–C20:3)	822.40	305.60	32
PE (C22:1–C20:4)	820.40	303.60	32
PE (C22:1–C20:5)	818.40	301.60	32
PE (C22:1–C22:4)	848.40	331.60	32
PE (C22:1–C22:5)	846.40	329.60	32
PE (C22:1–C22:6)	844.40	327.60	32
PE (C22:2–C16:0)	770.40	255.20	38
PE (C22:2–C18:0)	798.40	283.20	38
PE (C22:2–C18:1)	796.40	281.20	38
PE (C22:2–C18:2)	794.40	279.20	38
PE (C22:2–C20:3)	820.40	305.60	32
PE (C22:2–C20:4)	818.40	303.60	32
PE (C22:2–C20:5)	816.30	301.60	32
PE (C22:2–C22:4)	846.40	331.60	32
PE (C22:2–C22:5)	844.40	329.60	32
PE (C22:2–C22:6)	842.40	327.60	32
PE (C24:0–C16:0)	802.50	255.20	38
PE (C24:0–C18:0)	830.50	283.20	38
PE (C24:0–C18:1)	828.50	281.20	38
PE (C24:0–C18:2)	826.50	279.20	38
PE (C24:0–C20:3)	852.40	305.60	32
PE (C24:0–C20:4)	850.40	303.60	32
PE (C24:0–C20:5)	848.40	301.60	32
PE (C24:0–C22:4)	878.50	331.60	32

PE (C24:0–C22:5)			876.40	329.60	32
PE (C24:0–C22:6)			874.40	327.60	32
PE (C24:1–C16:0)			800.40	255.20	38
PE (C24:1–C18:0)			828.50	283.20	38
PE (C24:1–C18:1)			826.50	281.20	38
PE (C24:1–C18:2)			824.40	279.20	38
PE (C24:1–C20:3)			850.40	305.60	32
PE (C24:1–C20:4)			848.40	303.60	32
PE (C24:1–C20:5)			846.40	301.60	32
PE (C24:1–C22:4)			876.40	331.60	32
PE (C24:1–C22:5)			874.40	329.60	32
PE (C24:1–C22:6)			872.40	327.60	32
PE (C24:2–C16:0)			798.40	255.20	38
PE (C24:2–C18:0)			826.50	283.20	38
PE (C24:2–C18:1)			824.40	281.20	38
PE (C24:2–C18:2)			822.40	279.20	38
PE (C24:2–C20:3)			848.40	305.60	32
PE (C24:2–C20:4)			846.40	303.60	32
PE (C24:2–C20:5)			844.40	301.60	32
PE (C24:2–C22:4)			874.40	331.60	32
PE (C24:2–C22:5)			872.40	329.60	32
PE (C24:2–C22:6)			870.40	327.60	32
PE[P] (P-C16:0/C16:0)			674.40	255.20	34
PE[P] (P-C16:0/C18:0)			702.40	283.20	34
PE[P] (P-C16:0/C18:1)			700.40	281.20	34
PE[P] (P-C16:0/C18:2)			698.40	279.20	34
PE[P] (P-C16:0/C20:3)			724.30	305.20	28
PE[P] (P-C16:0/C20:4)			722.30	303.20	28
PE[P] (P-C16:0/C20:5)			720.30	301.10	28
PE[P] (P-C16:0/C22:4)			750.30	331.20	28
PE[P] (P-C16:0/C22:5)			748.30	329.20	28
PE[P] (P-C16:0/C22:6)			746.30	327.20	28
PE[P] (P-C16:1/C16:0)			672.40	255.20	34
PE[P] (P-C16:1/C18:0)			700.40	283.20	34
PE[P] (P-C16:1/C18:1)			698.40	281.20	34
PE[P] (P-C16:1/C18:2)			696.40	279.20	34
PE[P] (P-C16:1/C20:3)			722.30	305.20	28
PE[P] (P-C16:1/C20:4)			720.30	303.20	28
PE[P] (P-C16:1/C20:5)			718.30	301.10	28
PE[P] (P-C16:1/C22:4)			748.30	331.20	28
PE[P] (P-C16:1/C22:5)			746.30	329.20	28
PE[P] (P-C16:1/C22:6)			744.30	327.20	28
PE[P] (P-C16:2/C16:0)			670.30	255.20	34
PE[P] (P-C16:2/C18:0)			698.40	283.20	34
PE[P] (P-C16:2/C18:1)			696.40	281.20	34
PE[P] (P-C16:2/C18:2)			694.30	279.20	34
PE[P] (P-C16:2/C20:3)			720.30	305.20	28
PE[P] (P-C16:2/C20:4)			718.30	303.20	28
PE[P] (P-C16:2/C20:5)			716.30	301.10	28
PE[P] (P-C16:2/C22:4)			746.30	331.20	28
PE[P] (P-C16:2/C22:5)			744.30	329.20	28
PE[P] (P-C16:2/C22:6)			742.30	327.20	28

PE[P] (P-C18:0/C16:0)	702.40	255.20	34
PE[P] (P-C18:0/C18:0)	730.40	283.20	34
PE[P] (P-C18:0/C18:1)	728.40	281.20	34
PE[P] (P-C18:0/C18:2)	726.40	279.20	34
PE[P] (P-C18:0/C20:3)	752.40	305.20	28
PE[P] (P-C18:0/C20:4)	750.30	303.20	28
PE[P] (P-C18:0/C20:5)	748.30	301.10	28
PE[P] (P-C18:0/C22:4)	778.40	331.20	28
PE[P] (P-C18:0/C22:5)	776.40	329.20	28
PE[P] (P-C18:0/C22:6)	774.30	327.20	28
PE[P] (P-C18:1/C16:0)	700.40	255.20	34
PE[P] (P-C18:1/C18:0)	728.40	283.20	34
PE[P] (P-C18:1/C18:1)	726.40	281.20	34
PE[P] (P-C18:1/C18:2)	724.40	279.20	34
PE[P] (P-C18:1/C20:3)	750.30	305.20	28
PE[P] (P-C18:1/C20:4)	748.30	303.20	28
PE[P] (P-C18:1/C20:5)	746.30	301.10	28
PE[P] (P-C18:1/C22:4)	776.40	331.20	28
PE[P] (P-C18:1/C22:5)	774.30	329.20	28
PE[P] (P-C18:1/C22:6)	772.30	327.20	28
PE[P] (P-C18:2/C16:0)	698.40	255.20	34
PE[P] (P-C18:2/C18:0)	726.40	283.20	34
PE[P] (P-C18:2/C18:1)	724.40	281.20	34
PE[P] (P-C18:2/C18:2)	722.40	279.20	34
PE[P] (P-C18:2/C20:3)	748.30	305.20	28
PE[P] (P-C18:2/C20:4)	746.30	303.20	28
PE[P] (P-C18:2/C20:5)	744.30	301.10	28
PE[P] (P-C18:2/C22:4)	774.30	331.20	28
PE[P] (P-C18:2/C22:5)	772.30	329.20	28
PE[P] (P-C18:2/C22:6)	770.30	327.20	28
PE[P] (P-C20:0/C16:0)	730.40	255.20	34
PE[P] (P-C20:0/C18:0)	758.50	283.20	34
PE[P] (P-C20:0/C18:1)	756.40	281.20	34
PE[P] (P-C20:0/C18:2)	754.40	279.20	34
PE[P] (P-C20:0/C20:3)	780.40	305.20	28
PE[P] (P-C20:0/C20:4)	778.40	303.20	28
PE[P] (P-C20:0/C20:5)	776.40	301.10	28
PE[P] (P-C20:0/C22:4)	806.40	331.20	28
PE[P] (P-C20:0/C22:5)	804.40	329.20	28
PE[P] (P-C20:0/C22:6)	802.40	327.20	28
PE[P] (P-C20:1/C16:0)	728.40	255.20	34
PE[P] (P-C20:1/C18:0)	756.40	283.20	34
PE[P] (P-C20:1/C18:1)	754.40	281.20	34
PE[P] (P-C20:1/C18:2)	752.40	279.20	34
PE[P] (P-C20:1/C20:3)	778.40	305.20	28
PE[P] (P-C20:1/C20:4)	776.40	303.20	28
PE[P] (P-C20:1/C20:5)	774.30	301.10	28
PE[P] (P-C20:1/C22:4)	804.40	331.20	28
PE[P] (P-C20:1/C22:5)	802.40	329.20	28
PE[P] (P-C20:1/C22:6)	800.40	327.20	28
PE[P] (P-C22:0/C16:0)	758.50	255.20	34
PE[P] (P-C22:0/C18:0)	786.50	283.20	34

PE[P] (P-C22:0/C18:1)			784.50	281.20	34
PE[P] (P-C22:0/C18:2)			782.50	279.20	34
PE[P] (P-C22:0/C20:3)			808.40	305.20	28
PE[P] (P-C22:0/C20:4)			806.40	303.20	28
PE[P] (P-C22:0/C20:5)			804.40	301.10	28
PE[P] (P-C22:0/C22:4)			834.40	331.20	28
PE[P] (P-C22:0/C22:5)			832.40	329.20	28
PE[P] (P-C22:0/C22:6)			830.40	327.20	28
PE[P] (P-C22:1/C16:0)			756.40	255.20	34
PE[P] (P-C22:1/C18:0)			784.50	283.20	34
PE[P] (P-C22:1/C18:1)			782.50	281.20	34
PE[P] (P-C22:1/C18:2)			780.40	279.20	34
PE[P] (P-C22:1/C20:3)			806.40	305.20	28
PE[P] (P-C22:1/C20:4)			804.40	303.20	28
PE[P] (P-C22:1/C20:5)			802.40	301.10	28
PE[P] (P-C22:1/C22:4)			832.40	331.20	28
PE[P] (P-C22:1/C22:5)			830.40	329.20	28
PE[P] (P-C22:1/C22:6)			828.40	327.20	28
PE[P] (P-C24:0/C16:0)			786.50	255.20	34
PE[P] (P-C24:0/C18:0)			814.50	283.20	34
PE[P] (P-C24:0/C18:1)			812.50	281.20	34
PE[P] (P-C24:0/C18:2)			810.50	279.20	34
PE[P] (P-C24:0/C20:3)			836.50	305.20	28
PE[P] (P-C24:0/C20:4)			834.40	303.20	28
PE[P] (P-C24:0/C20:5)			832.40	301.10	28
PE[P] (P-C24:0/C22:4)			862.50	331.20	28
PE[P] (P-C24:0/C22:5)			860.50	329.20	28
PE[P] (P-C24:0/C22:6)			858.40	327.20	28
PE[P] (P-C24:1/C16:0)			784.50	255.20	34
PE[P] (P-C24:1/C18:0)			812.50	283.20	34
PE[P] (P-C24:1/C18:1)			810.50	281.20	34
PE[P] (P-C24:1/C18:2)			808.50	279.20	34
PE[P] (P-C24:1/C20:3)			834.40	305.20	28
PE[P] (P-C24:1/C20:4)			832.40	303.20	28
PE[P] (P-C24:1/C20:5)			830.40	301.10	28
PE[P] (P-C24:1/C22:4)			860.50	331.20	28
PE[P] (P-C24:1/C22:5)			858.40	329.20	28
PE[P] (P-C24:1/C22:6)			856.40	327.20	28
PI (C16:0–C18:0)			837.30	255.10	48
PI (C16:0–C18:1)			835.30	255.10	48
PI (C18:0–C18:0)			865.30	283.10	52
PI (C18:0–C18:1)			863.30	283.20	48
PI (C18:0–C20:4)			885.30	283.20	42
PI (C18:1–C18:1)			861.30	281.10	52
PI (C18:1–C20:4)			883.30	281.30	42
HexCer (d18:1/C16:0- <i>d</i> ₃)		685.60		264.30	40
HexCer (d18:1/C16:0)		682.60		264.30	40
HexCer (d18:1/C18:0)		710.60		264.30	40
HexCer (d18:1/C20:0)		738.60		264.30	40
HexCer (d18:1/C22:0)		766.60		264.30	40
HexCer (d18:1/C23:0)		780.70		264.30	40
HexCer (d18:1/C24:0)		794.70		264.30	40

HexCer (d18:1/C24:1)		792.70		264.30	40
HexCer (d18:1/C25:0)		808.70		264.30	40
HexCer (d18:1/C25:1)		806.70		264.30	40
HexCer (d18:1/C26:0)		822.70		264.30	40
HexCer (d18:1/2-OH C16:0)		698.60		264.30	40
HexCer (d18:1/2-OH C18:0)		726.60		264.30	40
HexCer (d18:1/2-OH C20:0)		754.60		264.30	40
HexCer (d18:1/2-OH C22:0)		782.60		264.30	40
HexCer (d18:1/2-OH C24:0)		810.70		264.30	40
HexCer (d18:1/2-OH C24:1)		808.70		264.30	40
HexCer (d18:1/2-OH C26:0)		838.70		264.30	40
HexCer (d18:1/2-OH C26:1)		836.70		264.30	40
SM (d18:1/C16:0- <i>d</i> ₉)	712.70			184.20	30
SM (d18:1/C16:0)	703.70			184.20	30
SM (d18:1/C16:1)	701.70			184.20	30
SM (d18:1/C16:2)	699.70			184.20	30
SM (d18:1/C18:0)	731.73			184.20	30
SM (d18:1/C18:1)	729.73			184.20	30
SM (d18:1/C18:2)	727.73			184.20	30
SM (d18:1/C20:0)	759.76			184.20	30
SM (d18:1/C20:1)	757.76			184.20	30
SM (d18:1/C20:2)	755.76			184.20	30
SM (d18:1/C22:0)	787.80			184.20	30
SM (d18:1/C22:1)	785.80			184.20	30
SM (d18:1/C22:2)	783.80			184.20	30
SM (d18:1/C23:0)	801.81			184.20	30
SM (d18:1/C23:1)	799.81			184.20	30
SM (d18:1/C23:2)	797.81			184.20	30
SM (d18:1/C24:0)	815.83			184.20	30
SM (d18:1/C24:1)	813.83			184.20	30
SM (d18:1/C24:2)	811.83			184.20	30
SM (d18:1/C24:3)	809.83			184.20	30
SM (d18:1/C25:0)	829.84			184.20	30
SM (d18:1/C25:1)	827.84			184.20	30
SM (d18:1/C25:2)	825.84			184.20	30
SM (d18:1/C26:0)	843.86			184.20	30
SM (d18:1/C26:1)	841.86			184.20	30
SM (d18:1/C26:2)	839.86			184.20	30