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Ether lipid transfer across the blood-brain and placental barriers does not improve by inactivation of the most abundant ABC transporters

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ABSTRACT

Phospholipid transport from the periphery to the brain is an understudied topic. When certain lipid species are deficient due to impaired synthesis, though, transfer across the blood-brain barrier is essential for replenishing lipids in the brain. For example, the deficiency in plasmalogens, the most abundant ether lipids in mammals, has detrimental effects on the brain, which is a major issue in inherited peroxisomal disorders but also contributes to more common disorders like Alzheimer's disease. Oral administration of alkylglycerols like batyl alcohol, which carry a pre-formed ether bond, enables replenishment of ether lipids in various peripheral tissues. However, plasmalogen deficiency in the brain cannot be overcome by this approach. Here, we tried to increase cerebral plasmalogen uptake by modulating the efflux transport across the blood-brain barrier. We hypothesized, based on previous literature, that at least some ether lipid species readily enter endothelial cells of the barrier through the transporter MFSD2A but are re-exported by ATP-binding cassette (ABC) transporters. By crossbreeding *Mdr1a*$^{-/-}$/*Mdr1b*$^{-/-}$/*Bcrp*$^{-/-}$ and ether lipid-deficient *Gnpat*$^{-/-}$ mice as well as pharmacological inhibition with MK-571 to inactivate the major ABC transporters at the blood-brain barrier, we evaluated the potential of combined ABC transporter inhibition and oral batyl alcohol administration for the treatment of plasmalogen deficiency. We found that even in the absence of the most abundant ABC transporters, batyl alcohol supplementation did not restore plasmalogen levels in the brain, despite the presence of a wide spectrum of ether lipid subspecies in the plasma as demonstrated by lipidomic analysis. Surprisingly, batyl alcohol treatment of pregnant *Gnpat*$^{+/-}$ dams had beneficial effects on the plasmalogen levels of *Gnpat*$^{-/-}$ offspring with defective ether lipid biosynthesis, independently of ABC transporter status at the placental barrier. Our results underline the autonomy of brain lipid homeostasis and indicate that peripheral supplementation of ether lipids is not sufficient to supply the brain with larger amounts of plasmalogens. Yet, the findings suggest that alkylglycerol treatment during pregnancy may pose a viable option to ameliorate some of the severe developmental defects of inborn ether lipid deficiency.

Abbreviations: ABC, ATP-binding cassette; BA, batyl alcohol; DHA, docosahexaenoic acid; BCRP, breast cancer resistance protein; DMA, dimethylacetal; FAME, fatty acid methyl ester; Gnpat, glyceronephosphate O-acyltransferase; KO, knockout; LPC-O, ether-linked lysophosphatidylcholine; LPC-P, vinyl ether-linked lysophosphatidylcholine; LPE-O, ether-linked lysophosphatidylethanolamine; LPE-P, vinyl ether-linked lysophosphatidylethanolamine; MRP, multidrug resistance-associated protein; PA, phosphatidic acid; MDR, multidrug-resistance protein; PAF, platelet-activating factor; PC-O, ether-linked phosphatidylcholine; PC-P, vinyl ether-linked phosphatidylcholine (choline plasmalogen); PE-O, ether-linked phosphatidylethanolamine; PE-P, vinyl ether-linked phosphatidylethanolamine (ethanolamine plasmalogen); PGP, P-glycoprotein; RCDP, rhizomelic chondrodysplasia punctata; WT, wild type.

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1. Introduction

In mammals, entry of molecules and cells into the brain from the periphery is highly restricted. A border made up of endothelial cells lining the cerebral vasculature together with pericytes and the end-feet of astrocytes forms the so-called blood-brain barrier, which regulates access of solutes and compounds of higher molecular weight to the brain (Zlokovic, 2008). While the blood-brain barrier is a crucial protective mechanism, it also poses a problem in the delivery of (potential) therapeutic substances to the brain. A class of compounds, whose transfer to the brain is highly restricted by the blood-brain barrier, are lipids, which are of utmost importance for proper brain function; for example, as a major portion of myelin, they ensure fast nerve cell conduction. Most lipid components can be produced by brain tissue autonomously. However, essential fatty acids, namely linoleic and α-linolenic acid as precursors for the various n-6 and n-3 polyunsaturated fatty acids, must be provided by the periphery (Bruce et al., 2017; Yehuda et al., 2005). Also in case of certain genetic defects in lipid metabolism, the central nervous system depends on supply from outside the brain. One such deficiency concerns ether lipids, a particular subgroup of phospholipids distinguished by the nature of the substituent at the *sn*-1 position of the glycerol backbone. Here, a fatty alcohol is linked to the backbone via an ether bond instead of an ester bond, where the former is generated by the sequential activity of two enzymes located in peroxisomes. Inherited ether lipid deficiency in humans manifests in a dramatic neurological phenotype (Bams-Mengerink et al., 2013, 2006; Dorninger et al., 2017) and severe deficiencies at birth. Accordingly, the success of any treatment approach heavily relies on its ability to replenish ether lipids, particularly plasmalogens, the most abundant subtype of ether lipids, in the brain. In contrast to ether lipids with a simple ether bond, plasmalogens carry a vinyl ether bond, which is a double bond adjacent to the ether bond that is introduced by the action of a desaturase (Gallego-Garcia et al., 2019; Werner et al., 2020). So far, it is largely unclear, if and how physiologically relevant levels of lipids can be transferred from the periphery across the blood-brain barrier. Previous studies have mainly focused on brain supply with fatty acids like docosahexaenoic acid (DHA, 22:6 n-3) (Hachem et al., 2020; Pifferi et al., 2021), which have been identified as vital for brain development and function (Bazinet and Laye, 2014; McNamara et al., 2018). However, in the case of ether lipid shortage, also transport of the specific phospholipid backbone to the brain is essential, because the ether bond confers crucial biophysical and physiological properties to the molecule and cannot be remodeled locally. A potential way for phospholipids to cross the blood-brain barrier may involve transfer of lipoproteins, as has been shown in *Drosophila* (Brankatschk and Eaton, 2010). However, a corresponding proof in mammals is still lacking and the relevance of this route *in vivo* remains uncertain. Alternatively, active transport of phospholipid molecules across the blood-brain barrier via selective transport proteins like Major facilitator superfamily-domain containing 2a (MFSD2A), a sodium-dependent lipid transporter highly expressed in endothelial cells (He et al., 2022; Wood et al., 2021), could represent a viable option. Originally described as a key determinant of blood-brain barrier integrity and function (Ben-Zvi et al., 2014), MFSD2A was also revealed to be a transporter supplying the brain with DHA as part of lysophosphatidylcholine (LPC) (Nguyen et al., 2014). Interestingly, more detailed competition assays pinpointed ether lipids, for example lyso-plasmalogen or platelet-activating factor (PAF; 1-alkyl-2-acetyl--phosphocholine), as efficient substrates for MFSD2A (Nguyen et al., 2014), thus indicating a potential *in vivo* entry point for ether lipids into the brain. Nevertheless, oral application of the ether lipid precursor batyl alcohol (BA) does not lead to efficient replenishment of plasmalogens in the brain of ether lipid-deficient mice, in spite of readily restoring plasmalogens in various peripheral tissues (Brites et al., 2011), suggesting the existence of a mechanism that prevents the brain entry of ether lipids through MFSD2A.

Like the adult mammalian brain, also the developing fetus is shielded from potentially harmful substances. This is accomplished by highly regulated transport across a layer of syncytiotrophoblasts, a type of epithelium, which separates fetal capillaries from the maternal circulation, thus forming the blood-placenta barrier or simply placental barrier (Tetro et al., 2018). The blood-brain barrier and placental barrier have in common high expression of transporters that ensure the efflux of potentially dangerous compounds like drugs or (feto)toxic metabolites (Vahakangas and Myllynen, 2009). Here, a major role is ascribed to ATP-binding cassette (ABC) transporters at the luminal and apical membrane of endothelial cells and syncytiotrophoblasts, respectively (Loscher and Potschka, 2005; Tetro et al., 2018), where they utilize ATP to pump a wide range of substrates back into the bloodstream. In mammals, the most abundant ABC transporter subtypes at both the blood-brain and the placental barrier include P-glycoprotein (PGP, also named multidrug-resistance protein 1, MDR1, or ABCB1), the breast cancer resistance protein (BCRP, also named ABCG2) and members of the ABCC (multidrug resistance-associated protein, MRP) subfamily (Dauchy et al., 2008; Miller, 2015; Strazielle and Ghersi-Egea, 2015).

Remarkably, several studies have identified ether lipids as substrates of ABC transporters, most prominently PGP. For example, *Leishmania* strains overexpressing a PGP-like protein turned out to be resistant against anti-Leishmanian alkyl-lysophospholipids like edelfosine or miltefosine (Perez-Victoria et al., 2001). Another study demonstrated that PAF, a pro-inflammatory ether lipid, as well as its lyso-form (lyso-PAF) systematically belonging to the group of ether-linked lysophosphatidylcholines (LPC-O), is efficiently transported by PGP in various cultured mammalian, including human, cell types (Ernest and Bello-Reuss, 1999; Raggers et al., 2001) as well as in proteoliposomes (Eckford and Sharom, 2006). The fact that many of the ABC transporters at the blood-brain and placental barriers exhibit overlapping substrate specificities (Erdo and Krajcsi, 2019) further suggests that also transporters other than PGP may be capable of shuttling ether lipids.

Here, we hypothesized that highly abundant ABC transporters at the blood-brain and the placental barrier are the key components that regulate and prevent entry of ether lipids, and probably also other essential lipids, into the adult brain and the developing fetus, respectively. In order to test this hypothesis, we generated mice with a genetic defect in ether lipid biosynthesis (*Gnpat*$^{-/-}$) and a concomitant deficiency in the main ABC transporters (*Mdr1a*$^{-/-}$/*Mdr1b*$^{-/-}$/*Bcrp*$^{-/-}$) to determine if exogenous ether lipid supplementation can rescue brain ether lipid levels under these conditions.

2. Materials and methods

2.1. Mice

Mice with a targeted inactivation (knockout, KO) of the *Gnpat* gene (*Gnpat*^{tm1Just}) have been described previously (Rodemer et al., 2003). The strain was maintained on an outbred C57BL/6 x CD1 background and experimental cohorts with *Gnpat*$^{-/-}$ (KO) and *Gnpat*$^{+/+}$ (wild type, WT) littermates were obtained by mating heterozygous animals. Genotypes were determined at weaning by polymerase chain reaction (PCR) as described previously (Rodemer et al., 2003) and confirmed after sacrifice. Mice with a targeted inactivation of *Mdr1a/b* (Schinkel et al., 1997) and *Bcrp* (Jonker et al., 2002) have been described previously and *Mdr1a*$^{-/-}$/*Mdr1b*$^{-/-}$/*Bcrp*$^{-/-}$ mice on an FVB genetic background were obtained commercially (Taconic Biosciences) and cross-bred with the *Gnpat* KO strain to generate *Gnpat*$^{-/-}$/*Mdr1a*$^{-/-}$/*Mdr1b*$^{-/-}$/*Bcrp*$^{-/-}$ mice.

Mice were fed standard chow and water *ad libitum* and were housed in a temperature- and humidity-controlled room with 12:12 h light-dark cycle and a low level of acoustic background noise at the local animal facility of the Medical University of Vienna.

In all experiments, age- and sex-matched WT animals, when possible from the same litters, were used as controls to minimize variability, except for the experiments involving embryonic tissue, in which the sex was not determined.

Experiments were carried out in compliance with the 3Rs of animal welfare (replacement, reduction, refinement), and the number of animals was reduced to the estimated minimum necessary to obtain clear-cut, statistically significant results. Whenever required, approval for individual experiments was obtained from the Institutional Animal Care and Use Committee of the Medical University of Vienna and the Austrian Federal Ministry of Science, Research and Economy (BMWFW-66.009/0147-WF/II/3b/2014 and BMBWF-66.009/0174-V/3b/2019).

2.2. Treatment with batyl alcohol and MK-571

Adult *Gnpat* KO and WT mice, with and without combined *Mdr1* and *Bcrp* deficiency, were randomly assigned to either the treatment or the control group. In the oral treatment regime, the treatment group received a standard diet (ssniff-Spezialdiäten GmbH) supplemented with 2% (w/w) 1-O-*rac*-octadecylglycerol (batyl alcohol, BA; Biotain Pharma Co, Ltd) for one month. The purity of BA was confirmed by nuclear magnetic resonance spectroscopy prior to treatment experiments (Department of Chemistry, University of Natural Ressources and Life Sciences Vienna). Control animals received the same chow without BA.

For the experiments involving the MRP inhibitor MK-571, mice were placed on BA diet 3 days prior to the onset of MK-571 application. MK-571 (sodium salt; MedChemExpress, article no. HY-19989A) was dissolved in tap water (working solution 5 mg/ml) and administered orally (by gavage) at a dose of 25 mg/kg/day for 14 days.

2.3. Tissue processing

Blood was collected into EDTA tubes by cardiac puncture following euthanasia with an overdose of CO_2. A crude preparation of red blood cells (RBCs) was performed as described previously (Steinberg et al., 2008). After separation of RBCs, blood plasma was subjected to centrifugation (1,500*xg*, 10 min, 4 °C) for purification. Mouse tissue samples were flash-frozen in liquid N_2 after dissection and processed after thawing by a two-step homogenization procedure depending on the type of tissue.

Liver (20 volumes (20x) PBS based on sample weight): Glass-Teflon tissue grinder (Potter-Elvehjem homogenizer, 1 stroke) followed by centrifugation (1000*xg*, 5 min, 4 °C) and additional 10 strokes to homogenize the pellet. Supernatants obtained after centrifugation were combined. Brain (10x PBS): Glass-Teflon tissue grinder (10 strokes) followed by three passages through a 27 G needle. Heart (8x PBS): Tissue disperser (Polytron PT3100 equipped with a PT-DA 3012/2 S aggregate, Kinematica; 10 s at 15,000 rpm) followed by centrifugation (1000*xg*, 5 min, 4 °C) and additional 10 strokes using a Glass-Teflon tissue grinder. Supernatants obtained after centrifugation were combined. Embryonic heart and kidney (12x PBS): Sonication (5 s) followed by three passages through a 27 g needle. Embryonic tissue was pooled according to genotype for homogenization in order to obtain enough material for lipid analysis.

For all tissues, the supernatants obtained after centrifugation (1000*xg*, 5 min, 4 °C) were stored at -80 °C until lipid analysis.

2.4. Lipid analysis

Plasmalogen levels were determined by gas chromatographic detection of dimethylacetals (DMA) after acidic methanolysis as described previously (Dacremont and Vincent, 1995). For selected samples, duplicate determinations (deriving from the same biological samples) were performed to confirm an appropriate assay variability. Lipidomic analysis was performed as described (Herzog et al., 2016). The HPLC system consisted of an Ultimate 3000 binary HPLC pump, a vacuum degasser, a column temperature controller, and an auto sampler (Thermo Scientific, Waltham, MA, USA). The column temperature was maintained at 25 °C. The lipid extract was injected onto a "normal phase column" LiChrospher 2 × 250-mm silica-60 column, 5 μm particle diameter (Merck, Darmstadt, Germany) and a "reverse phase column" Acquity UPLC HSS T3, 1.8 μm particle diameter (Waters, Milford Massachusetts, USA). A Q Exactive Plus Orbitrap (Thermo Scientific) mass spectrometer was used in the negative and positive electrospray ionization mode. Nitrogen was used as the nebulizing gas. The spray voltage used was 2500 V, and the capillary temperature was 256 °C. S-lens RF level: 50, auxiliary gas: 11, auxiliary temperature 300 °C, sheath gas: 48, sweep cone gas: 2. In both the negative and positive ionization mode, mass spectra of the lipid species were obtained by continuous scanning from *m/z* 150 to *m/z* 2000 with a resolution of 280,000 full width at half maximum (FWHM).

In total 2310 different lipid species were identified, including 854 ether lipids within 12 different ether lipid subclasses. For the final analyses, di- and tri-glyceride ether lipids were excluded and all other ether lipid species (278) were normalized to the sum of all phospholipids to reduce variability within the experimental groups. All lipid analyses were done with investigators blinded to genotype or treatment condition. Differences between genotypes and treatment groups on individual lipid species level were visualized using the heatmap feature of the Omics Explorer (version 3.7; Qlucore) software. All raw data derived from lipidomic analysis as well as the filtered and normalized data for ether lipid species, as used for calculations, are provided in the Supplementary Material.

2.5. Assessment of hyperactivity

Locomotor activity was examined using an open field activity meter (30 × 30 cm; Opto Varimex, Columbus Instruments, Columbus, Ohio), as described previously (Dorninger et al., 2019). Ambulatory and non-ambulatory movements were recorded automatically throughout a trial period of 41 min. The first minute was excluded from analyses due to large inter-individual variations of test animals exploring their novel environment.

3. Results

3.1. Optimization of oral batyl alcohol treatment regime and time course

Before initiating further experiments, we sought to investigate the kinetics of plasmalogen replacement upon treatment of ether lipid-deficient mice with BA. Previous studies had routinely applied BA treatment of ether lipid-deficient mice for 2 months (Brites et al., 2011); however, it was not stated whether also shorter treatment durations were sufficient to restore plasmalogens to WT levels. We treated *Gnpat* KO mice with BA for 4, 7, 14, 20, 30 and 40 days and analyzed the levels of C18:0 and C16:0 plasmalogens in liver, heart and brain tissue as well as in erythrocytes. In all peripheral tissues investigated, the treatment had a clear impact on C18:0 plasmalogen levels already after a few days. The effect was most pronounced in the liver (Fig. 1A), where the amount of C18:0 plasmalogens rose to more than five times the normal WT level after 4 days of treatment and kept on increasing upon prolonged BA supplementation. Similar continuous increases of C18:0 plasmalogen levels with treatment duration were observed in the heart and erythrocytes of BA-treated *Gnpat* KO animals as well as WT controls (analyzed at the 20- and 40-day time points only), and the untreated WT level was strongly exceeded in all three tissues after 40 days of treatment (Fig. 1A-C). As (biochemically) expected and described previously, BA treatment did not have any influence on C16:0 plasmalogen levels in *Gnpat* KO mice (Supp. Fig. 1). However, particularly in heart and liver of WT controls, there was a trend towards decreasing C16:0 plasmalogen levels upon longer BA treatment periods (Supp. Fig. 1A,B), presumably as a compensation for the increased levels of C18:0 plasmalogens. Based on these findings in the peripheral tissues and considering that *Gnpat* KO mice lack not only C18:0 plasmalogens but also C16:0 and C18:1 plasmalogens, we opted for a treatment regime of 30 days in all further experiments in order to guarantee full restoration of total plasmalogen

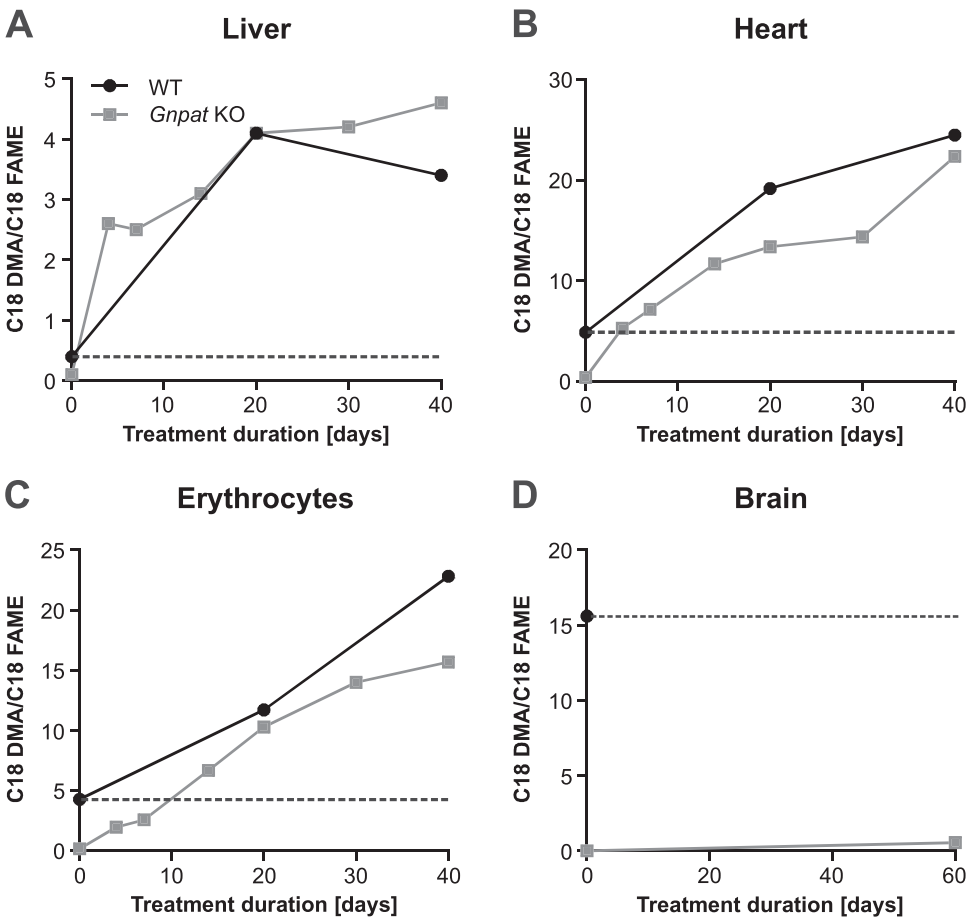

Fig. 1. *Time course of plasmalogen restoration upon BA treatment.* WT and *Gnpat* KO mice were treated orally with BA (2% in standard mouse chow) for 4, 7, 14, 20, 30, 40 or 60 days and C18:0 plasmalogens were detected as C18:0 dimethylacetals (DMA) by capillary gas chromatography. DMA levels were determined in the liver (A), heart (B), erythrocytes (C) and brain (D) of treated animals and results are depicted as C18:0 DMA related to C18:0 fatty acid methyl esters (FAME, derived from fatty acids incorporated into phospholipids). The horizontal dashed line represents the average value of untreated WT mice. The corresponding values for C16:0 DMA are shown in Supp. Fig. 1.

levels, while shortening the originally described treatment duration by a month.

In line with previous observations (Brites et al., 2011), we did not find any substantial plasmalogen replacement in the brain of *Gnpat* KO mice, even after an extended treatment period of 60 days (Fig. 1D and Supp. Fig. 1D). Accordingly, we did not observe any improvement of the hyperactive phenotype after BA treatment as indicated by the open field test (Supp. Fig. 2), a measure that we have described previously in *Gnpat* KO mice (Dorninger et al., 2019).

3.2. Lipidomic analysis after BA treatment

In order to prove that BA treatment not only rescued total plasmalogens with a certain *sn*-1 chain (as covered by analysis of the DMA form) but all major ether lipid subspecies that are potential targets of ABC transporters at the blood-brain barrier, we examined the lipidome of plasma from WT and BA-treated or untreated *Gnpat* KO mice by using HPLC-MS-MS. As expected, all ether lipid subspecies were almost completely absent in the plasma of untreated *Gnpat* KO animals. Targeted analyses showed that all main ether lipid species containing C18:0 at *sn*-1 were readily restored in the treated animals, whereas species with C16:0, C18:1 and C20:0 chains at *sn*-1 did not respond to BA treatment (Fig. 2 and Supp. Fig. 3). The treatment effect was visible in all the different ether lipid subclasses including alkyl (non-vinyl ether; PE-O and PC-O) and alkenyl (vinyl ether; PE-P and PC-P) lipids as well as their lyso-forms (LPE-O, LPE-P, LPC-O, LPC-P). Not only was the spectrum of different individual species restored, but the total level of ether lipids in each of the main classes was either similar or even increased in BA-treated KO mice as compared with untreated WT levels (Fig. 3). This was also true for ether-bonded phosphatidic acid (PA-O+P) and mono-alk(en)ylglycerols (MG-O+P), which include BA itself (Fig. 3). The latter are relatively rare also in WT plasma but the levels are markedly raised after ingestion of BA. Accordingly, the levels of total plasmalogens, total alkyl (non-plasmalogen ether) lipids, total ethanolamine ether lipids and total choline ether lipids were elevated to those of WT or higher by the treatment (Supp. Fig. 4). Compared to the WT levels, the increase after treatment appeared more pronounced for the species with simple ether bonds (“O”) than for those with vinyl ether bonds (“P”). Overall, this indicates that elevated amounts of C18:0-containing ether lipids compensate for the lack of species with other *sn*-1 chains following oral BA supplementation.

3.3. Generation and BA treatment of ether lipid-deficient mice with inactivated ABC transporters

In order to establish, whether ABC transporters at the blood-brain barrier are responsible for or involved in the inefficiency of oral BA treatment at restoring brain plasmalogens, we applied a previously generated mouse model lacking ABCB1A/B (also known as MDR1 or PGP) and BCRP (Jonker et al., 2005), which are the major ABC transporters at the blood-brain barrier. We crossed *Mdr1*$^{-/-}$ /*Bcrp*$^{-/-}$ mice with the ether lipid-deficient *Gnpat* KO strain to generate animals with combined ether lipid and ABC transporter deficiency (“triple KO”). Adult triple KO mice displayed a phenotype that was not overtly different from that of *Gnpat* single KO mice; however, survival beyond the weaning period was reduced, in line with previous observations showing that a considerable fraction of ether lipid-deficient mice die before or immediately after birth in certain background strains ((Liegel et al., 2014) and own unpublished observation).

To investigate how the absence of MDR1 and BCRP affects the ability of oral BA treatment to restore brain plasmalogen levels, we treated the ether lipid-deficient triple KO and control mice with BA-supplemented

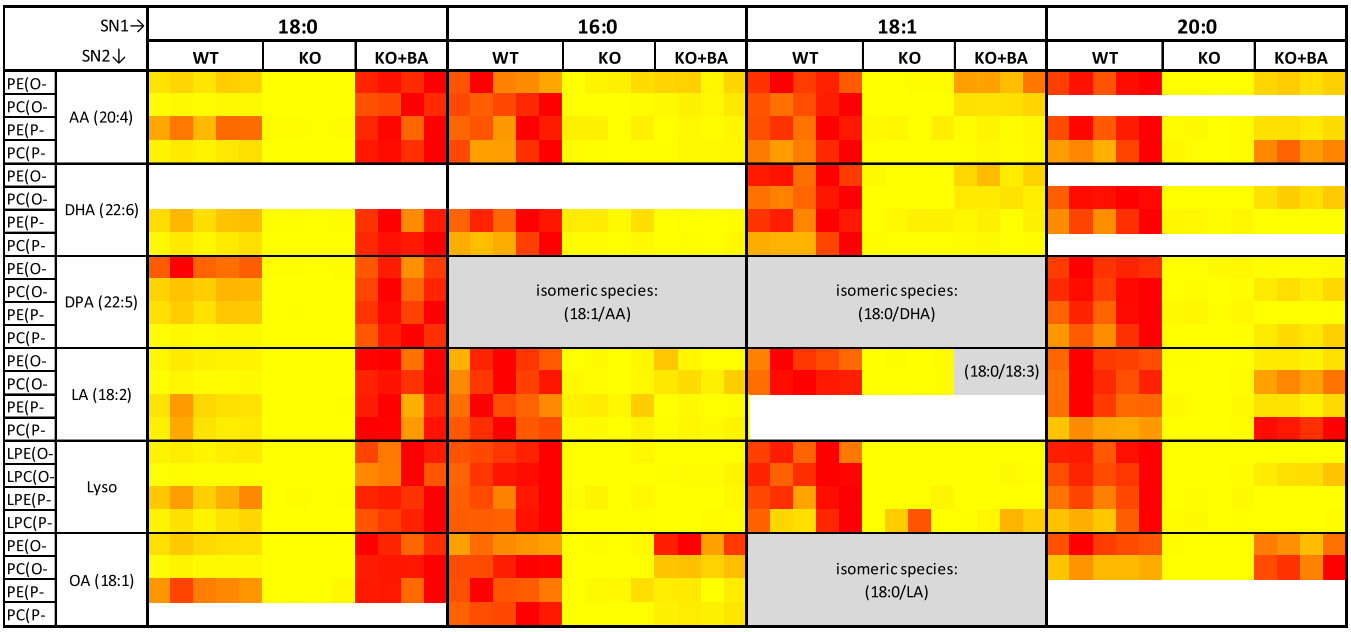

Fig. 2. *Ether lipid species identified by lipidomic analysis assigned to a matrix based on their sn-1 and sn-2 side chains*. Lipidomic analysis was performed in plasma of untreated WT ($n = 5$), *Gnpat* KO ($n = 4$) and BA-treated *Gnpat* KO (KO+BA, $n = 4$) animals. Although our lipidomic approach can discriminate between isomeric alkyl (O) and alkenyl (P) species, it is not able to distinguish between isomeric lipid species with different chain lengths at *sn*-1 and *sn*-2. Therefore, we assigned the identified metabolites to a matrix covering most relevant ether lipid species, i.e., carrying C16:0, C18:0 C18:1 or C20:0 at *sn*-1 and being present either in the lyso form or with a (poly)unsaturated fatty acid at *sn*-2: arachidonic acid (AA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), linoleic acid (LA) or oleic acid (OA). For lipid species with more isomeric species, these were assigned to the more abundant *sn*-2 side chain (e.g., PC-P-38:5 is depicted as PC-P-18:1/20:4 although also the less abundant isomeric species PC-P-16:0/22:5 is included). The validity of this approach is underlined by the fact that summed up, the depicted lipid species account for 89% (WT), 86% (KO) and 88% (KO+BA) of all detected ether lipids. The color coding reflects differences between different animals and genotypes individually for each lipid species with yellow indicating minimal values and red maximal values. White areas represent lipid species not identified in the lipidomic approach. The apparent BA-induced increase in lipid species with assigned *sn*-1 chain lengths other than C18:0 is caused by accumulation of minor isomeric species with C18:0 at *sn*-1. This is most obvious for PE-O-16:0/18:1 and several species with C20:0 at *sn*-1. LPC-O, ether-linked lysophosphatidylcholine; LPC-P, vinyl ether-linked lysophosphatidylcholine; LPE-O, ether-linked lysophosphatidylethanolamine; LPE-P, vinyl ether-linked lysophosphatidylethanolamine; PC-O, ether-linked phosphatidylcholine; PC-P, vinyl ether-linked phosphatidylcholine (choline plasmalogen); PE-O, ether-linked phosphatidylethanolamine; PE-P, vinyl ether-linked phosphatidylethanolamine (ethanolamine plasmalogen).

chow for one month. Whereas C18:0-containing plasmalogens were readily replaced in cardiac tissue of *Gnpat/Mdr1/Bcrp* triple KO mice after treatment, brain levels were barely detectable (Fig. 4A,B). As anticipated, C16:0 plasmalogens were not restored in any tissue after BA treatment in all genotypes (Supp. Fig. 5). In line with the lack of cerebral plasmalogen replacement in the brain, no functional improvement of the hyperactive phenotype, as measured by open field experiments, was observed in triple KO animals following BA supplementation for 30 days (Supp. Fig. 6).

As so far, our experimental strategy addressed specifically PGP/MDR1 and BCRP, we wanted to exclude that other ABC transporters are preventing the entry of ether lipids into the brain, thus evading our experimental strategy. Because the major additional ABC transporters at the blood-brain barrier belong to the ABCC subclass, we treated *Gnpat/Mdr1/Bcrp* triple KO mice with MK-571, an inhibitor of several ABCC transporters including the abundant ABCC1 (also termed multidrug resistance-associated protein 1, MRP1), in combination with the BA diet. However, no increase in C18:0 plasmalogens in brain homogenates was detected after the combined treatment (Fig. 4A), whereas cardiac plasmalogen levels, measured as a control for recovery in the periphery, were restored (Fig. 4B). Taken together, these results demonstrate a lack of any substantial treatment effect in the adult brain even in the context of MDR, BCRP and ABCC deficiency or blockade.

3.4. Targeting the placental barrier in triple KO mice

Similarly to the blood-brain barrier, the placental barrier poses also a major obstacle in the treatment of ether lipid deficiency, as it presumably prevents the transfer of ether lipids from maternal tissue to fetal tissue in developing ether lipid-deficient individuals. This was previously deduced from the observation that *Gnpat* KO pups present with a severe phenotype already at birth, even though the heterozygote dams have tissue plasmalogen levels similar to those of WT mice (Fig. 5A,B and Supp Fig. 7). In order to evaluate whether fetal plasmalogen deficiency can be overcome by eliminating the major ABC transporters at the placental barrier, we treated pregnant *Gnpat*$^{+/-}$ and ABC transporter-deficient (*Mdr1*$^{-/-}$/*Bcrp*$^{-/-}$) females, which had been mated with *Gnpat*$^{+/-}$ and ABC transporter-deficient males, with BA diet from the onset of pregnancy and determined plasmalogen levels in WT and *Gnpat* KO embryos and newborns. As control, we performed the same experiment in a background of normal ABC transporter expression. Surprisingly, already in these control animals (non-ABC transporter-deficient), BA treatment had a pronounced effect on plasmalogen levels in the *Gnpat* KO progeny. Compared with the *Gnpat* KO embryos and pups of untreated *Gnpat*$^{+/-}$ dams, the *Gnpat* KO offspring of treated dams had considerable amounts of C18:0 plasmalogens in the heart (~40% of untreated WT levels; Fig. 5D), liver (~30–40% of untreated WT levels; Fig. 5E) and kidneys (~10–15% of untreated WT levels; Fig. 5F). A similar, albeit smaller effect was seen in the brain (~7–10% of untreated WT levels; Fig. 5C). The ABC transporter-deficient background did not lead to any additional increase in C18:0 plasmalogen levels (Fig. 5C-F). C16:0 plasmalogens were not, or just barely, detectable in any of the analyzed embryonic tissues from *Gnpat* KO mice, independent of the treatment condition (Supp. Fig. 8).

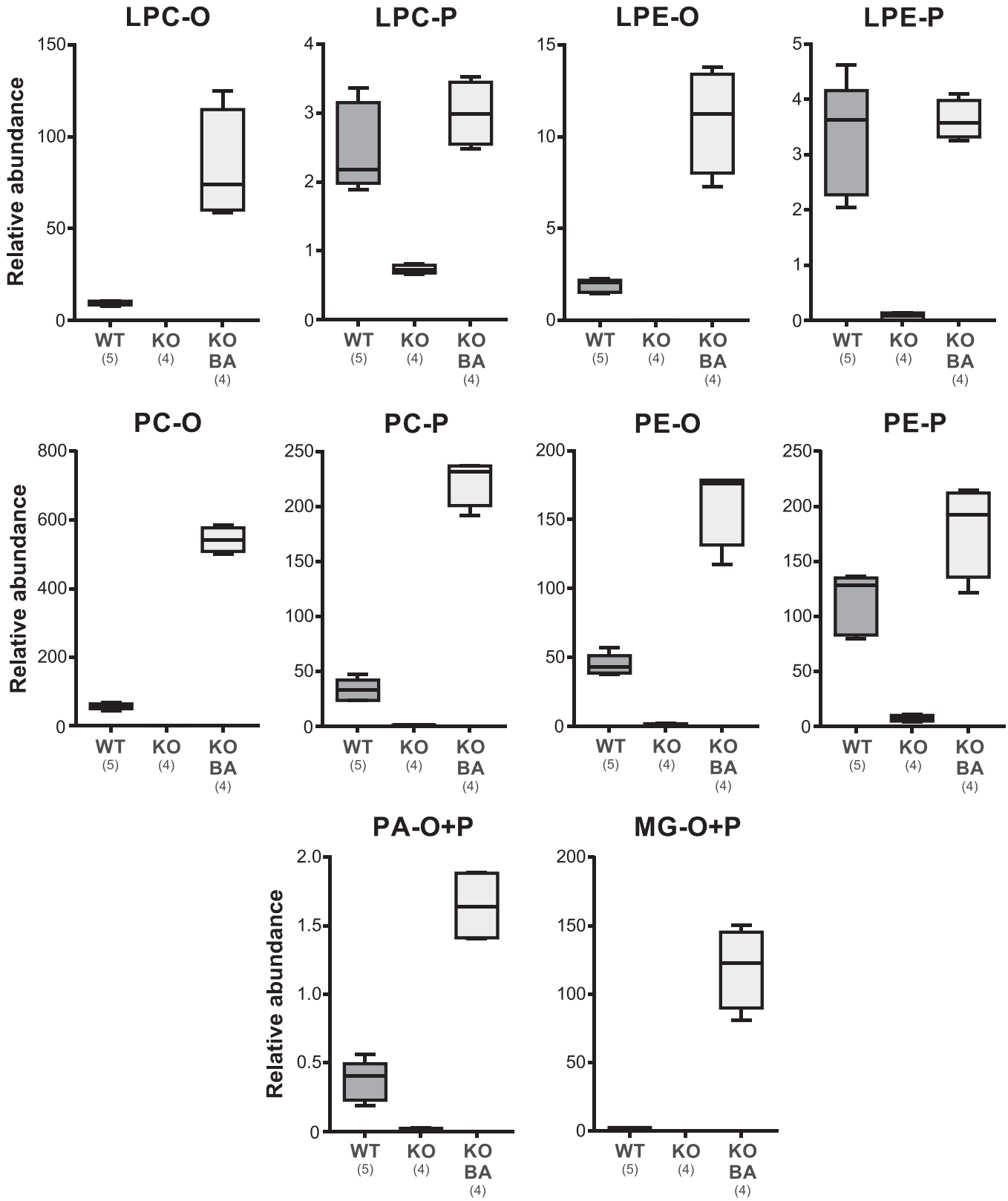

Fig. 3. *Restoration of the major ether lipid classes in plasma of Gnpat KO mice after BA treatment.* Lipidomic analysis was performed in plasma of WT, *Gnpat* KO and BA-treated *Gnpat* KO (KO BA) animals. The values for each of the main ether lipid subclasses were obtained by summing up the relative abundance of individual species belonging to the corresponding subclass. Note that for phosphatidic acid (PA) and monoalkyl-/monoalkenyl-glycerol (MG), no distinction between alkyl (O) and alkenyl (P) species was made in our lipidomics approach and both were assigned to the subclass (O+P). Data are shown as box plots and whiskers indicate minimal and maximal values. The number of analyzed animals is given in brackets. LPC-O, ether-linked lysophosphatidylcholine; LPC-P, vinyl ether-linked lysophosphatidylcholine; LPE-O, ether-linked lysophosphatidylethanolamine; LPE-P, vinyl ether-linked lysophosphatidylethanolamine; PC-O, ether-linked phosphatidylcholine; PC-P, vinyl ether-linked phosphatidylcholine (choline plasmalogen); PE-O, ether-linked phosphatidylethanolamine; PE-P, vinyl ether-linked phosphatidylethanolamine (ethanolamine plasmalogen).

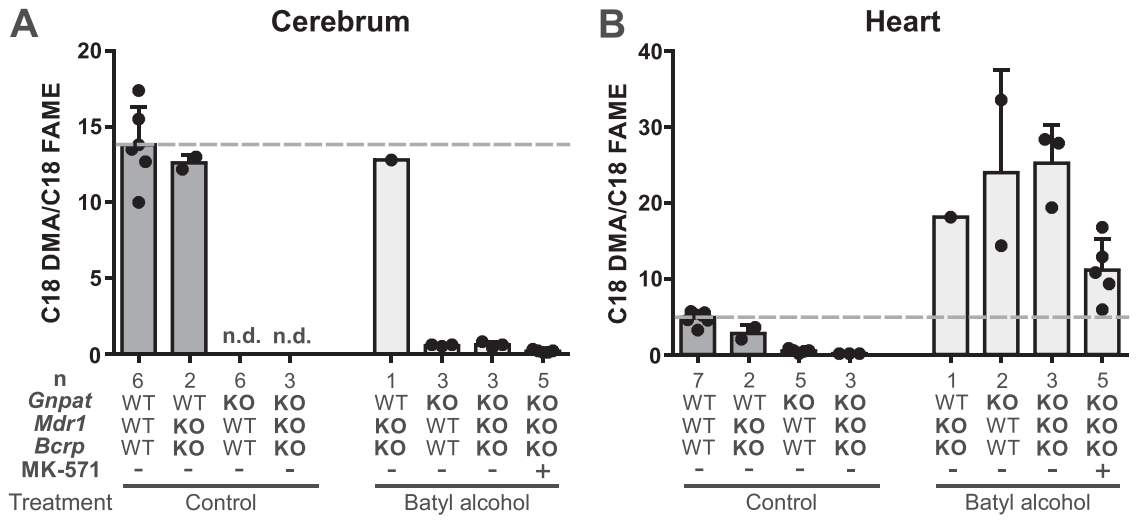

Fig. 4. *Plasmalogen precursor treatment in ABC transporter- and Gnpat-deficient animals.* Adult mice with genetic deficiencies in *Mdr1*, *Bcrp* and/or *Gnpat* were treated with either BA (2% w/w) or control chow. At the endpoint, plasmalogen levels were determined by gas chromatography in the cerebrum (A) and, as a peripheral tissue control, heart (B). Results are depicted as C18 DMA related to C18 FAME. Pharmacological treatment with MK-571 (25 mg/kg/day) was employed to inhibit transporters of the ABCC subfamily. Note that the treatment duration with MK-571 + BA was shorter (17 days, thereof day 4–17 including MK-571) than with BA alone (30 days). The horizontal dashed line represents the average value for untreated WT mice and bars represent means ± SD. The corresponding values for C16:0 DMA are shown in Supp. Fig. 5. *n.d.*, not detected.

4. Discussion

In the present manuscript, we addressed the hypothesis that the main ABC transporters at the blood-brain barrier counteract the uptake of ether lipids into the brain and, thus, jeopardize strategies aiming to increase brain plasmalogen levels by dietary supplementation of plasmalogens or their precursors. It has long been known that the most abundant ABC transporters in endothelial cells, MDR1 and BCRP, transport a wide variety of lipid-based substrates *in vitro* (Bosch et al., 1997; Sharom, 2014; van Helvoort et al., 1996). Accordingly, translocation of labelled phosphatidylcholine was affected in erythrocytes from *Mdr1a/b*$^{-/-}$ mice (Kalin et al., 2004). Particularly the fact that numerous therapeutics are exported by these transporters complicates the delivery of these agents to the brain in different diseases, most importantly cancer (Borst et al., 2000; Schinkel et al., 1996; Tarling et al., 2013; van Helvoort et al., 1996). The identification of ether lipids as substrates for MDR1 *in vitro* appeared to explain why previous efforts to restore plasmalogen levels of ether lipid-deficient individuals by dietary supplementation of precursors were effective in the periphery, but unsuccessful in the brain (Brites et al., 2011; Das and Hajra, 1988; Das et al., 1992). Here, the results from our approach, involving knockout mouse models deficient for the most abundant ABC transporters and pharmacological inhibition, did not support the hypothesis. Rather, the absence of transport mediated by MDR1, BCRP or ABCC transporters did not lead to any substantial replacement of brain plasmalogens in ether lipid-deficient mice, even though all ether lipid species that could serve as plasmalogen precursors after transport across the blood-brain barrier were abundantly present in the plasma, often at considerably higher amounts than in WT mice. This included also a number of potential substrates for MFSD2A, like for example LPC-O (lyso-PAF). Thus, our findings make ABC transporters unlikely as the cause for the inability to rescue brain plasmalogens by applying ether lipid precursors in the periphery.

We are certainly aware that inhibition of ABC transporters is a difficult strategy to implement in clinical practice (Kalvass et al., 2013). Nevertheless, the molecular understanding of the failure of previous treatment regimens is paramount for the development of further therapeutic approaches. Actually, our results represent a considerable setback for efforts targeting an increase in plasmalogens in the brain by means of oral ingestion. Currently, such approaches to restore cerebral plasmalogen levels are not only discussed in the context of inborn errors of ether lipid deficiency like rhizomelic chondrodysplasia punctata (RCDP) or Zellweger syndrome, a disease with deficiency in peroxisome biogenesis, but have also been proposed in common diseases (Bozelli and Epand, 2021) like Alzheimer's disease, for which reduced plasmalogen levels have been repeatedly reported (Han, 2010; Han et al., 2001; Kou et al., 2011) and even suggested to be critically involved in disease etiology (Senanayake and Goodenowe, 2019). Recently, several different groups have reported considerable effects of oral plasmalogen or precursor treatment on brain-related parameters, like neuroinflammation or even cognitive function and behavior (Fallatah et al., 2020b; Fujino et al., 2017; Hossain et al., 2022; Yamashita et al., 2017), indicating that also plasmalogen replacement in the periphery may be beneficial in neurological diseases. Potential mechanisms could involve direct or indirect effects on inflammatory mediators, systemic oxidative stress, a modulation of the intestinal microbiome or the provision of polyunsaturated fatty acids, which can be released from the *sn*-2 position and transported to the brain independently of plasmalogens. In addition, we cannot exclude that minimal amounts of plasmalogens or other ether lipids do cross the blood-brain barrier and reach the brain after BA treatment, as observed in a recent study using a modified plasmalogen precursor (Smith et al., 2022), although these are not reflected in elevated total DMA levels. Also small amounts of ether lipids can have a major impact, as indicated by the genotype-phenotype correlation in a series of mouse models with graded ether lipid deficiency (Fallatah et al., 2022). In addition, traces of specific, less abundant ether lipids may restore important functions in the brain via pre- and postsynaptic mechanisms, for example in signal transduction (Dorninger et al., 2020), and could, therefore, contribute to the beneficial results of dietary ether lipid treatment as observed in other studies. Our data, however, indicate no improvement in behavioral function, specifically hyperactivity, after BA supplementation. In view of these conflicting results, the detailed lipidomic investigation of brain ether lipids after BA treatment is an important subject of future studies. However, for an effective and sustained treatment strategy that also targets myelin abnormalities, which are prominent in patients with ether lipid deficiency (Bams-Mengerink et al., 2006; Sztriha et al., 2000), the reconstitution of brain tissue plasmalogens pre- and postnatally is probably indispensable. A remarkable finding in this respect is the observation that an alkylglycerol with only 14 carbon atoms (tetradecylglycerol) has been

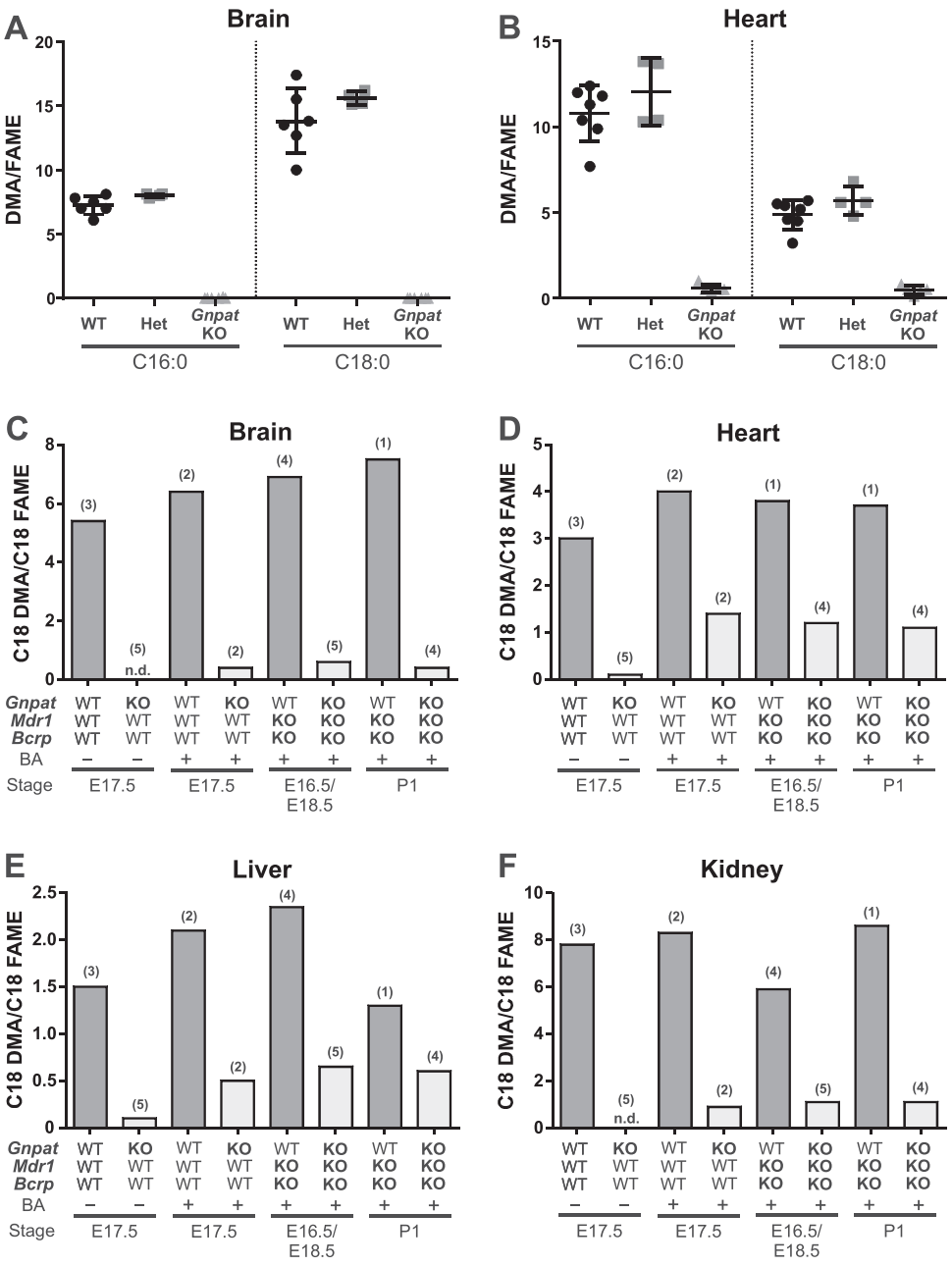

Fig. 5. *Effect of plasmalogen precursor treatment of pregnant dams on ether lipid-deficient embryos and pups.* (A, B) Plasmalogen levels were determined in the brain (A) and heart (B) of WT, *Gnpat* heterozygous (Het) and KO mice. Data are expressed as C16 DMA related to C16 FAME (left part of each panel) or C18 DMA related to C18 FAME (right part) and means ± SD as well as individual values are shown. (C-F) Plasmalogens were determined in the brain (C), heart (D), liver (E) and kidney (F) of wild type (WT) and *Gnpat* KO embryos or newborns with or without ABC transporter (*Mdr1, Bcrp*)-deficient background after BA treatment of pregnant dams. Tissue from several animals was pooled to obtain sufficient material for analysis (number of animals given in brackets) and results are depicted as C18 DMA related to C18 FAME. The corresponding values for C16:0 DMA are shown in Supp. Fig. 8. *n.d.*, not detected; E, embryonic day; P, postnatal day.

reported to improve myelination under conditions of ether lipid deficiency *in vitro* and *in vivo* (Malheiro et al., 2019). Given that C14 alkyl residues at the *sn*-1 position of ether lipids are normally very rare in mammals, the exact molecular mechanism remains unclear and an actual impact on brain plasmalogen levels is yet to be shown. Our results, though, suggest that exogenous supplementation of ether lipid precursors is unlikely to accomplish the goal of plasmalogen restoration in the brain. In the case of congenital ether lipid deficiency, alternative treatment strategies are scarce. In these rare diseases, therapy is mainly symptomatic and life expectancy is generally low (Duker et al., 2020). One compound suggested to ameliorate some of the molecular downstream effects of plasmalogen deficiency in mice is lithium (da Silva et al., 2014). However, no beneficial effects have yet been shown in human trials and a number of caveats are involved with its use in clinical practice (McKnight et al., 2012). Apart from inborn ether lipid deficiency, the situation is certainly different in diseases with reported partial plasmalogen deficits, like Alzheimer's disease, for which the role of plasmalogens in the disease course is not yet fully established and numerous other potential therapeutic targets have been proposed.

From a basic science perspective, the question remains why exogenous supplementation rescues plasmalogens in peripheral tissues but not in the brain, if the specific efflux transporters at the blood-brain barrier are not the obstacle. One possibility would be that brain tissue is intrinsically programmed to rely on cell-autonomous biosynthesis and does not accept complex lipids from external sources. Alternatively, the low turnover of plasmalogens in brain tissue could account for the findings. A large fraction of brain plasmalogens is part of the myelin sheath, which is known for its metabolic stability. Accordingly, two different pools of plasmalogens exist in the brain: a dynamic pool, mainly consisting of plasmalogens located in neurons (i.e., the gray matter), and the pool of white matter plasmalogens characterized by low turnover (Rosenberger et al., 2002). The latter pool might simply not be amenable to exogenous supply with plasmalogens or their precursors within the observation period of our study. In this case, presumably a

longer duration of the BA supplementation would enhance the therapeutic effect. Contradicting this hypothesis, a prolonged treatment period of 4 months only minimally increased the plasmalogen levels in nervous tissue of ether lipid-deficient mice in a previous study (Brites et al., 2011). Finally, we cannot fully rule out that a component of the blood-brain barrier that we did not address in our experiments precludes ether lipid entrance into the brain, which is a limitation of our study. Specifically, we cannot exclude that another ABC transporter, although expressed at lower abundance, is responsible for ether lipid export at the blood-brain barrier and/or that upregulation of the expression could occur in response to the inactivation of MDR1, BCRP and the ABCC class. Obvious candidates in this respect are ABCG1 and ABCG4, belonging to the same ABC transporter subclass as BCRP. These transporters have also been associated with the export of lipids, most prominently cholesterol and other sterols, and thus were implicated in regulating the homeostasis of these compounds in the brain (Bojanic et al., 2010; Kobayashi et al., 2006; Kober et al., 2017; Wang et al., 2008; Xu et al., 2022). However, both ABCG1 and ABCG4 are present are very low levels in murine endothelial cells as compared to MDR1, BCRP and also other ABC transporters (Warren et al., 2009). Accordingly, at their normal expression level, these transporters are rather unlikely to prevent the brain entry of ether lipids at the concentrations present in the blood after BA supplementation. It is conceivable, though, that upon deficiency of the main ABC transporters there is compensatory upregulation of less common transporters at the blood-brain barrier. Whereas such compensation has been repeatedly shown for other organs like liver or kidney, where particularly ABCC transporters are upregulated in MDR1 KO mice (Tiwari et al., 2013), results for the brain and endothelial cells are conflicting: Some authors find upregulated *Abcg2* expression in brain capillaries of *Mdr1a* KO mice (Cisternino et al., 2004). However, a recent study in rats concluded that expression of various blood-brain barrier transporters, including several ABC transporters, is unaltered upon MDR1 deficiency (Liang et al., 2019).

In addition to showing that a broad variety of ether lipid species is restored by BA treatment, our lipidomic analysis in the plasma revealed several interesting details of ether lipid metabolism. For example, we found that plasmalogen species with a C20:0 fatty alkyl chain at *sn*-1 are present at considerable levels in the plasma. Commonly, C16:0, C18:0 and C18:1 have been viewed as the only residues found at *sn*-1 due to the substrate specificity of fatty acyl-CoA reductase (FAR) (Braverman and Moser, 2012; Honsho et al., 2010), the enzyme providing the fatty alcohols for plasmalogen biosynthesis. Very recently, analytic studies demonstrated the existence of longer fatty alkyl chains in plasmalogens of human neutrophils (Amunugama et al., 2021) and murine brain (Azad et al., 2021). Here, we add to these findings by showing that also in murine plasma, species with longer chain lengths, particularly those with 20 carbon atoms, are abundant. Another interesting aspect is that BA supplementation did not only restore ether lipids in the plasma to WT levels, but led to a considerable overshoot of all ether lipid subgroups, thus potentially modulating the overall lipid composition. This observation is in line with a recent investigation, showing that the levels of total ether lipids can be increased in various tissues of WT mice by oral administration of an alkylglycerol mix (Paul et al., 2021). In our study, the increase was even more drastic for non-vinyl ether species suggesting that excess amounts of these lipids are not necessarily promptly degraded, e.g. by alkylglycerol monooxygenase, but likely transported to peripheral organs.

Another line of our experiments targeted the question, if ABC transporter deficiency of dams receiving BA treatment during pregnancy would enhance the plasmalogen supply to ether lipid-deficient embryos and pups. To our surprise, we found that already BA supplementation of female mice heterozygous for *Gnpat*, without any genetic modification of ABC transporters, has a considerable beneficial effect on ether lipid-deficient pups leading to detectable plasmalogen levels in all tissues examined, including the brain. However, similar to our results from experiments addressing transport across the blood-brain barrier in adult mice, we did not find any influence of the *Mdr1/Bcrp* genotype on the results indicating that these transporters do not have any impact on the transfer of plasmalogens from the maternal tissue to the embryo. Our findings come unexpectedly, as *Gnpat* KO pups are born to heterozygous mothers, which have normal levels of plasmalogens. Nevertheless, we show here for the first time that also *Gnpat* KO embryos of untreated dams have no or barely detectable plasmalogen levels in a variety of peripheral tissues, for which exogenous supplementation works effectively in adult animals. This implies that ether lipids, and maybe also other types of phospholipids, are normally not efficiently transported from maternal circulation to the embryo. However, apparently, by increasing the freely available amounts of ether lipid precursors or plasmalogens themselves, beneficial effects can still be reached as indicated by our findings upon BA supplementation of pregnant dams. Clinically, these results could be of importance for children affected by inherited ether lipid deficiency, as they suggest that also in these disorders an increase in plasmalogen levels can be achieved by early treatment with plasmalogen precursors like BA. Although also partially reduced plasmalogen levels are associated with severe disease in humans, it has been shown repeatedly that in the human disease RCDP, increased plasmalogen levels directly correlate with a milder disease course (Duker et al., 2017; Fallatah et al., 2020a). Accordingly, an increase in plasmalogen levels, like that observed in *Gnpat* KO embryos after BA treatment, may bring some relief to patients with this devastating disease. On the other hand, care must be taken to rule out potential adverse effects, if BA treatment during pregnancy is considered. While no negative consequences have been identified for supplementation with individual fatty acids like DHA, experience with BA in humans is limited, particularly with the high amounts likely required to reach therapeutic value. Certainly, early detection of the genetic defect is a prerequisite to allow for timely treatment initiation, which may be easier to achieve in the future given recent advances in the development of whole genome or whole exome sequencing techniques, for example in the context of newborn screening programs.

5. Conclusions

In general, little is known about the transfer of phospholipids across the blood-brain barrier. Research has mainly focused on brain supply with essential fatty acids, rather than the residual parts of complex lipid molecules, from which these can be derived. This is not surprising given that the glycerol backbone, as found in diacyl phospholipids, represents highly abundant metabolites. However, this does not apply to the backbone of ether lipids, which needs to be specifically generated by peroxisomal biosynthesis. Even though our results did not support our original hypothesis, the data add important information to our understanding of ether lipid supply of the brain and should be considered, when developing treatment approaches aiming at a modulation of brain plasmalogen levels. On the one hand, transfer of peripheral ether lipids to the brain occurs only to a very low extent, even when reverse transport at the blood-brain barrier is prevented. On the other hand, we show that increasing the availability of ether lipids in pregnant dams enables the transmission of limited amounts of these lipids to the developing embryo. Our results may have important implications for the treatment of inborn ether lipid deficiency but also for more common disorders involving alterations of ether lipid levels, like Alzheimer's disease. Furthermore, they substantiate the importance of brain-autonomous ether lipid biosynthesis and the brain's independence from exogenous ether lipid supply thus adding an important puzzle piece to our understanding of brain lipid homeostasis.

Declaration of Interest

None.

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Author contributions

Fabian Dorninger: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft. **Frédéric M. Vaz:** Methodology, Validation, Data curation, Supervision. **Hans R. Waterham:** Resources, Supervision. **Jan B. van Klinken:** Methodology, Investigation, Data curation. **Gerhard Zeitler:** Investigation. **Sonja Forss-Petter:** Investigation, Writing – review & editing. **Johannes Berger:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Christoph Wiesinger:** Conceptualization, Formal analysis, Visualization, Funding acquisition, Writing – review & editing.

All authors read and approved the final manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.brainresbull.2022.08.006.

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