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Disturbed brain phospholipid and docosahexaenoic acid metabolism in calcium-independent phospholipase A_2-VIA (iPLA$_2\beta$)-knockout mice

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ABSTRACT

Calcium-independent phospholipase A_2 group VIA (iPLA$_2\beta$) releases docosahexaenoic acid (DHA) from phospholipids *in vitro*. Mutations in the iPLA$_2\beta$ gene, *PLA2G6*, are associated with dystonia-parkinsonism and infantile neuroaxonal dystrophy. To understand the role of iPLA$_2\beta$ in brain, we applied our *in vivo* kinetic method using radiolabeled DHA in 4 to 5-month-old wild type (iPLA$_2\beta^{+/+}$) and knockout (iPLA$_2\beta^{-/-}$) mice, and measured brain DHA kinetics, lipid concentrations, and expression of PLA_2, cyclooxygenase (COX), and lipoxygenase (LOX) enzymes. Compared to iPLA$_2\beta^{+/+}$ mice, iPLA$_2\beta^{-/-}$ mice showed decreased rates of incorporation of unesterified DHA from plasma into brain phospholipids, reduced concentrations of several fatty acids (including DHA) esterified in ethanolamine- and serine-glycerophospholipids, and increased lysophospholipid fatty acid concentrations. DHA turnover in brain phospholipids did not differ between genotypes. In iPLA$_2\beta^{-/-}$ mice, brain levels of iPLA$_2\beta$ mRNA, protein, and activity were decreased, as was the iPLA$_2\gamma$ (Group VIB PLA_2) mRNA level, while levels of secretory sPLA$_2$-V mRNA, protein, and activity and cytosolic cPLA$_2$-IVA mRNA were increased. Levels of COX-1 protein were decreased in brain, while COX-2 protein and mRNA were increased. Levels of 5-, 12-, and 15-LOX proteins did not differ significantly between genotypes. Thus, a genetic iPLA$_2\beta$ deficiency in mice is associated with reduced DHA metabolism, profound changes in lipid-metabolizing enzyme expression (demonstrating lack of redundancy) and of phospholipid fatty acid content of brain (particularly of DHA), which may be relevant to neurologic abnormalities in humans with *PLA2G6* mutations.

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1. Introduction

In vitro studies have demonstrated that the group VI Ca^{2+}-independent phospholipases A_2 (iPLA$_2$, EC 3.1.1.4) hydrolyze docosahexaenoic acid (DHA) from the stereospecifically numbered (*sn*)-2 position of phospholipids [1,2]. This is consistent with reduced brain DHA metabolism in unanesthetized iPLA$_2\beta$-knockout mice [3]. Of known iPLA$_2$ isoforms, iPLA$_2\beta$ is designated *PARK14*, *PNPLA9*, *PLA2G6* or iPLA$_2$-VIA, and iPLA$_2\gamma$ is designated PNPLA8 or iPLA$_2$-VIB. Both isoforms are found post-synaptically in brain [4] and in the cytosol of resting cells [4–7], and can be activated and undergo membrane association by stimuli that induce release of Ca^{2+} from intracellular stores, *e.g.*, muscarinic or serotonergic G-protein-coupled neuroreceptor signaling [3,8–11]. iPLA$_2\beta$, and to a lesser extent iPLA$_2\gamma$, also can hydrolyze arachidonic acid (AA, 20:4n−6) from phospholipids [12–15].

Humans with *PLA2G6* mutations may show progressive regression of cognitive and motor skills, as manifested in the disorders infantile neuroaxonal dystrophy, idiopathic neurodegeneration with brain iron accumulation, dystonia-parkinsonism, and cerebellar cortical atrophy with gliosis [16–19]. In mice, mutations in iPLA$_2\gamma$ or iPLA$_2\beta$ genes cause cognitive deficits and motor abnormalities over time [14,20,21]. iPLA$_2\beta$ knockout mice display neuropathology characterized by swollen axons and vacuoles [20,21], protein misfolding and aggregation [21], and reduced mitochondrial function [14,22] by age 13 months. Other studies have demonstrated a role for iPLA$_2\beta$ in maintaining axonal membrane stability [20] and in regulating fatty acid composition of pancreatic islet β-cell phospholipids [23].

In view of the involvement of iPLA$_2\beta$ in DHA hydrolysis from phospholipids [1,2] and the reduced plasma DHA incorporation and

Abbreviations: AA, arachidonic acid; ChoGpl, choline glycerophospholipid; COX, cyclooxygenase; cPLA$_2$, cytosolic phospholipase A_2 (Group IVA PLA$_2$); DHA, docosahexaenoic acid; DHA-CoA, docosahexaenoyl-CoA; EtnGpl, ethanolamine glycerophospholipid; FAME, fatty acid methyl ester; GC, gas chromatography; iPLA$_2$, Ca^{2+}-independent phospholipase A_2 (Group VIA PLA$_2$); LOX, lipoxygenase; PUFA, polyunsaturated fatty acid; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; sPLA$_2$, secretory phospholipase A_2; *sn*, stereospecifically numbered; TLC, thin layer chromatography

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signaling in brains of iPLA$_2$β knockout mice [3], it is possible that neuropathology and altered behavior that arise from mutations or deficiencies in iPLA$_2$β are related to disturbed brain DHA metabolism. DHA has been reported to modulate gene transcription and membrane fluidity, to act as a signaling molecule during neurotransmission, to serve as a precursor of antiinflammatory resolvins and neuroprotectins, to influence rodent behavior, to act as an antioxidant, and to alter ion channel activities [1,3,20,24–31].

To further characterize brain DHA metabolism in mice with genetic deficiency of iPLA$_2$β, here we used our *in vivo* kinetic infusion model [32–35] to quantify DHA incorporation and turnover in brain phospholipids and to determine fatty acid concentrations of brain phospholipids and lysophospholipids of iPLA$_2\beta^{-/-}$ and wild type iPLA$_2\beta^{+/+}$ mice at age 4–5 months. We also examined brain expression of enzymes involved in polyunsaturated fatty acid (PUFA) metabolism, including iPLA$_2$β, iPLA$_2$γ, cytosolic cPLA$_2$ (Group IVA PLA$_2$), secretory sPLA$_2$ (Group V PLA$_2$), cyclooxygenase (COX)-1, COX-2, 5-lipoxygenase (LOX), 12-LOX and 15-LOX. Widespread neuropathologic changes develop by age 13 months in iPLA$_2\beta^{-/-}$ mice, so we chose to study younger mice in order to reduce the impact that such neuropathologic abnormalities might have on brain PUFA metabolism, but even at age 4 months, iPLA$_2\beta^{-/-}$ mice exhibit tubulovesicular membranes and small vacuoles with edema in brain [14,20–22].

2. Methods and materials

2.1. Animals

The study was conducted following the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (Publication no. 86-23) and was approved by the Animal Care and Use Committee of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development. Male iPLA$_2\beta^{-/-}$ mice and their littermate iPLA$_2\beta^{+/+}$ controls, derived from a C57BL/6J genetic background [36], were maintained in an animal facility where temperature, humidity, and light cycle were regulated, with free access to water and a diet (Rodent NIH-07) that contained (as percent of total fatty acid concentration), 30.6% saturated, 22.5% monounsaturated, 47.1% linoleic, 4.9% α-linolenic (α-LNA), 0.2% AA, 1.6% eicosapentaenoic (EPA), and 2.2% DHA [3]. Five mice of each genotype underwent surgical procedures, tracer infusion, and microwave fixation for determining brain DHA turnover and concentration. Six mice of each genotype were asphyxiated by CO_2 inhalation and decapitated, and the brains were excised and rapidly frozen in 2-methylbutane with dry ice (at −50 °C) and stored at −80 °C for subsequent analyses.

2.2. Surgical procedures and tracer infusion

At age 4–5 months, mice were anesthetized with 1–3% halothane, and polyethylene catheters were inserted into the femoral artery and vein [33]. Recovery from anesthesia was allowed to occur (3 h, 25 °C) with animal hindquarters loosely wrapped and taped to a wooden block. During recovery, body temperature was maintained at 37 °C with a rectal probe and a heating element (Indicating Temperature Controller; Yellow Springs Instrument, Yellow Springs, OH, USA). After recovery, unanesthetized mice were infused (5 min) intravenously with HEPES buffer (130 μl, pH 7.4) containing fatty acid-free bovine serum albumin (50 mg/ml, Sigma, St. Louis, MO, USA) and [1-^{14}C]DHA (5 μCi, 53 mCi/mmol, 90% pure, Moravek Biochemicals, Brea, CA, USA) at a rate of 0.0223 $(1+e^{-0.032t})$ ml/min, (t = sec), using a computer-controlled infusion pump (No. 22; Harvard Apparatus, South Natick, MA, USA) to achieve steady-state plasma specific activity within 1 min [37]. During infusion, timed arterial blood samples (15 μl) were collected in polyethylene-heparin lithium fluoride-coated Beckman centrifuge tubes at various intervals (0, 0.25, 0.5, 1.0, 1.5, 3.0, and 4.0 min) and a final collection (150 μl) was performed at 4.9 min. Plasma was separated by centrifugation (13,000 rpm, 1 min) and radioactivity determined by liquid scintillation counting. Unlabeled DHA concentrations of the final (4.9 min) sample were measured by gas chromatography (GC). At 5 min, animals were anesthetized (sodium pentobarbital, 50 mg/kg, i.v.) and subjected to head-focused microwave irradiation (5.5 kW, 0.9 s, 75% power output; Cober Electronics, Norwalk, CT, USA) to stop brain lipid metabolism [38,39]. Brains were excised, dissected sagittally, and stored (−80 °C).

2.3. Plasma and brain lipid extraction and separation

Total lipids were extracted from plasma (50 μl) after adding heptadecanoic acid (17:0) as an internal standard, and from one cerebral hemisphere (~0.2 g) as reported [40]. Lipid extracts were separated by thin layer chromatography (TLC) on Silica Gel 60A plates (Whatman, Clifton, NJ, USA) [41]. Neutral lipid subclasses including unesterified fatty acids were separated using a mixture of heptane/diethylether/glacial acetic acid (60/40/3 v/v/v), alongside authentic standard phospholipids, cholesterol, free fatty acids, triacylglycerols, and cholesteryl esters standards to identify the bands. Phospholipid classes (EtnGpl, ethanolamine glycerophospholipid; ChoGpl, choline glycerophospholipid; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine) were separated in chloroform/methanol/H_2O/glacial acetic acid (60/50/4/1 v/v/v) and identified by comparison with standards in separate lanes. Lysophospholipids were analyzed in chloroform/methanol/acetic acid/acetone/water (35/25/4/14/2 v/v/v/v/v). This method achieves separation of lysophosphatidylcholine (lysoPC), and of co-migrating lysophosphatidylinositol (lysoPI) and lysophosphatidylethanolamine (lysoPE). Plates were sprayed with 0.03% (w/v) 6-*p*-toluidine-2-naphthalene sulfonic acid (Acros, Fairlawn, NJ, USA) in 50 mM Tris–HCl buffer (pH 7.4), and the lipid bands were visualized with UV light. Each band was scraped from the plate, and the silica gel containing the target analyte was used to quantify radioactivity of phospholipid classes by liquid scintillation counting, to prepare fatty acid methyl esters (FAMEs) by transmethylation of neutral lipids, phospholipids, and lysophospholipids (see below), to quantify plasmalogen concentrations, and to measure phospholipid and lysophospholipid phosphorous concentrations.

2.4. FAME preparation and GC analysis

After adding appropriate quantities of internal standard (17:0/17:0-PC), FAMEs were formed from brain lipids and plasma esterified lipids in silica gel scraped from TLC plates by acid methanolysis (1% H_2SO_4 in methanol, 70 °C, 3 h). FAMEs were then analyzed by GC (SP™-2330 fused silica capillary column, 30 m × 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA, USA) and detected by flame ionization (Model 6890N detector; Agilent Technologies, Palo Alto, CA, USA). Initial column temperature was 80 °C, followed by a gradient (10 °C/min) to 150 °C and then a gradient (6 °C/min) to 200 °C, where temperature was held for 10 min, and then increased to 240 °C (38 min total run time). Peaks were identified by comparison to the retention times of FAME standards (Nu-Chek-Prep, Elysian, MN, USA). Fatty acid concentrations (nmol/μmol brain total phosphorous or nmol/ml plasma) were calculated by proportional comparison of GC peak areas to that of the 17:0 internal standard.

2.5. Quantification of radioactivity

Samples were placed in scintillation vials and dissolved in liquid scintillation cocktail (ReadySafe™ plus 1% glacial acetic acid), and

their radioactivity was determined by liquid scintillation spectrometry (2200CA, TRI-CARB®; Packard Instruments, Meriden, CT, USA).

2.6. Brain lipid phosphorous and plasmalogens

Phosphorous concentration of brain total lipids and phospholipid classes, separated by TLC, was quantified in phosphorous-free tubes using an assay that measures phosphate concentrations, as previously described [41]. Brain plasmenylethanolamine and plasmenylcholine concentrations were determined in EtnGpl and ChoGpl by an iodine uptake method as reported [41].

2.7. Brain cholesterol

Brain concentration of cholesterol was determined in the total lipid extract by GC as described previously [42]. Total lipids were concentrated to dryness and then subjected to alkaline hydrolysis (1 M KOH in methanol, 1 ml, 1 h, 70 °C). After adding 0.9% saline (1 ml), sterols were extracted twice into hexane (2.5 ml). The extract was dried and derivatized in 0.2 ml trimethylchlorosilane(Thermo Scientific, Rockford, IL, USA; 1 h, 60 °C). The sterol trimethylsilyl ether derivatives were concentrated under nitrogen, reconstituted in hexane (100 μl), and analyzed by GC (SP™-2330 fused silica capillary column, 30 m × 0.25 mm i.d., 0.25 μm film thickness, Supelco, Bellefonte, PA, USA). The temperature program involved an initial temperature of 100 °C (1 min) followed by a gradient (15 °C/min) to 280 °C, where the temperature was maintained (17 min).

2.8. Quantification of labeled and unlabeled acyl-CoA

Acyl-CoA species were extracted from the remaining microwaved half-brain samples using affinity chromatography [43]. After adding internal standard heptadecanoyl-CoA (17:0-CoA, 10 nmol) to weighed brain (~0.2 g), the sample was sonicated (20 s) with a probe sonicator (Model W-225; Misonix, Farmingdale, NY, USA) in 25 mM potassium phosphate (2 ml). Isopropanol (2 ml) was added to the homogenate and it was again sonicated (20 s). Proteins were precipitated by adding saturated ammonium sulfate (0.25 ml), and the sample was mixed by manual shaking. Acetonitrile (4 ml) was added, and the sample was vortex-mixed (30 min) before centrifugation. The supernatant was collected and diluted with 25 mM potassium phosphate (10 ml). Each sample was passed through an activated oligonucleotide purification cartridge (ABI Masterpiece™, OPC®; Applied Biosystems, Foster City, CA, USA) three times, and the cartridge was washed with 25 mM potassium phosphate (10 ml). Acyl-CoA species were eluted with 0.4 ml of isopropanol/1 mM glacial acetic acid (75:25 v/v).

Extracted acyl-CoAs were separated on a reversed phase HPLC column (Symmetry, 5 μm particle size, 4.6 mm × 250 mm, Waters-Millipore, Milford, MA, USA), using a pump coupled with a UV/VIS detector (System Gold, Model 168, Beckman, USA). Chromatography was performed using a linear gradient system (flow rate, 1.0 ml/min) composed of 75 mM potassium phosphate and acetonitrile. At the start, acetonitrile was 44% and held for 1 min, then increased to 49% over 25 min, increased to 68% over 10 min, held at 68% for 4 min, returned to 44% over 6 min, and held for 6 min (52 min total run time). UV absorbance was measured at 260 nm to determine acyl-CoA concentrations and at 280 nm to identify acyl-CoA species (260/280 = 4:1). Acyl-CoA concentrations (nmol/mg brain) were calculated by comparing their peak areas to that of 17:0-CoA and were normalized to brain total lipid phosphorous. The docosahexaenoyl-CoA peak was collected in each sample, and its radioactivity was determined by liquid scintillation counting. These values were used to calculate specific activities of docosahexaenoyl-CoA.

2.9. DHA incorporation rates and turnover

The model for determining kinetics of brain fatty acids in unanesthetized rats is described in detail elsewhere [32]. In this study, we normalized concentrations and kinetic measurements to brain lipid phosphorous rather than to wet weight, because brain edema has been reported in iPLA$_2\beta^{-/-}$ mice at 4–5 months of age [20,21].

Unidirectional incorporation coefficients, $k^*_{i(DHA)}$ (ml / μmol phosphorous / s) of DHA, representing incorporation of unesterified DHA from plasma into brain lipid *i*, were calculated as follows:

$$k^*_{i(DHA)} = \frac{c^*_{brain,i(DHA)}(T)}{\int_0^T c^*_{plasma(DHA)}dt} \quad (1)$$

$c^{**}_{brain,i(DHA)}$(nCi / μmol phosphorous) is radioactivity of brain lipid *i* at time $T = 5$ min (time of termination of experiment); *t* is time after starting infusion; and $c^{**}_{plasma(DHA)}$ (nCi/ ml) is plasma concentration of labeled unesterified DHA during infusion. Integrals of plasma radioactivity were determined by trapezoidal integration. Net rates of incorporation of unlabeled unesterified DHA from plasma into brain lipid *i*, $J_{in,i(DHA)}$, and from the brain docosahexaenoyl-CoA precursor pool, $J_{FA,i(DHA)}$, were calculated as follows:

$$J_{in,i(DHA)} = k^*_{i(DHA)} c_{plasma(DHA)} \quad (2)$$

$$J_{FA,i(DHA)} = J_{in,i(DHA)}/\lambda_{DHA-CoA} \quad (3)$$

$c_{plasma(DHA)}$ (nmol/ ml) is the concentration of unlabeled unesterified DHA in plasma. A "dilution factor" λ is defined as the steady-state ratio during [1-^{14}C]DHA infusion of specific activity of brain docosahexaenoyl-CoA to specific activity of plasma unesterified DHA:

$$\lambda_{DHA-CoA} = \frac{c^*_{brain_{(DHA-CoA)}}/c_{brain_{(DHA-CoA)}}}{c^*_{plasma(DHA)}/c_{plasma(DHA)}}. \quad (4)$$

A steady state is reached within 1 min after infusion starts [37]. The fractional turnover of DHA within phospholipid *i*, $F_{FA,i(DHA)}$ (% / h), is defined as:

$$F_{FA,i(DHA)} = \frac{J_{FA,i(DHA)}}{c_{brain,i(DHA)}}. \quad (5)$$

2.10. Preparation of cytoplasmic extracts

Brain tissue was homogenized in buffer (3 vol, 10 mM HEPES, pH 7.5, with 1 mM EDTA, 0.34 M sucrose, and protease inhibitor cocktail (Roche, Indianapolis, IN, USA)) in a glass apparatus. The homogenized sample was centrifuged (100,000 *g*, 1 h, 4 °C), and the supernatant was used for PLA_2 enzyme activity measurements and Western blotting. Supernatants were stored at −80 °C until use. Protein content was determined by the Bradford assay (Bio-Rad) [44].

2.11. Western blotting

Proteins from the cytoplasmic extracts (50 μg) were analyzed on 4–20% SDS-polyacrylamide gels (PAGE) (Bio-Rad). Following SDS-PAGE, proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Bio-Rad). Protein blots were incubated (overnight, 4 °C) in Tris-buffered saline containing 5% nonfat dried milk and 0.1% Tween-20 with specific primary antibodies (1:1000 dilution) directed against cPLA$_2$-IVA, sPLA$_2$-V, iPLA$_2\beta$, COX-1, COX-2, 5-LOX, 12-LOX and 15-LOX (Santa Cruz Biotech, Santa Cruz, CA, USA). Protein blots were incubated with appropriate HRP-conjugated secondary antibodies (Cell Signaling Beverly, MA, USA) and visualized

by chemiluminescence (Pierce, Rockford, IL, USA) using BioMax X-ray film (Eastman Kodak, Rochester, NY, USA). Optical densities of immunoblot bands were measured with Alpha Innotech Software (Alpha Innotech, San Leandro, CA, USA) and were normalized to the optical density of β-actin (Sigma-Aldrich, St. Louis, MO, USA) to correct for unequal loading. All experiments were performed with 6 independent samples per group. Values are expressed as percent of control.

2.12. RNA isolation and real time RT-PCR

Total RNA was isolated from brain using commercial kits (RNeasy Lipid Tissue Kit; Qiagen, Valencia, CA, USA). cDNA was prepared from total RNA using a high-capacity cDNA Archive Kit (Qiagen). Taqman® gene expression master mix and specific primers for real time RT-PCR were purchased from Applied Biosystems (Foster City, CA, USA). Levels of mRNA for cPLA$_2$-IVA, sPLA$_2$-V, iPLA$_2$β, iPLA$_2$γ, COX-1 and COX-2 were measured by real time quantitative RT-PCR using the ABI PRISM 7000 sequence detection system (Applied Biosystems). The fold-change in gene expression was determined by the $\Delta\Delta C_T$ method [45]. Data are expressed as the relative level of the target gene in the iPLA$_2\beta^{-/-}$ group normalized to the endogenous control (β-globulin) and relative to the level in the iPLA$_2\beta^{+/+}$ group. All experiments were carried out in triplicate with 6 independent samples per group.

2.13. Phospholipase A$_2$ activities

A radioisotopic method was used to measure cPLA$_2$ type IV and iPLA$_2$ type VI activities in cytoplasmic extracts (0.3 mg protein per assay) as described elsewhere [6,46]. The activity of sPLA$_2$ was measured using an sPLA$_2$ assay kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.14. Statistical analyses

Data were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and are presented as mean ± SEM of 5–6 independent measurements per group. Brain fatty acid concentrations and rates of DHA incorporation and turnover are expressed per μmol lipid phosphorous [20,21]. Breeding limitations and surgical losses limited sample size and precluded establishing normality of distribution criteria. The probability of Type II errors was mitigated by using Cohen's *d* test as a measure of effect size [47], which permits qualitative interpretations of differences between means. An effect size corresponding to Cohen's *d* of 0.3 is considered small, of 0.5 medium, and of 0.8 large [47]. We considered effect sizes greater than 0.5 to be significant.

3. Results

3.1. Plasma radioactivity and unesterified fatty acid concentrations

Steady-state plasma radioactivity was achieved within 1 min after initiating [1-^{14}C]DHA infusion (Fig. 1). The integral of plasma radioactivity (denominator of Eq. (1)) during the 5-min infusion was 72,419 ± 13,121 nCi s/ml for iPLA$_2\beta^{+/+}$ mice and 73,203 ± 19,411 nCi s/ml for iPLA$_2\beta^{-/-}$ mice ($d = 0.05$), which indicates no significant difference.

Table 1 indicates that mean plasma concentrations of unesterified palmitate (16:0), palmitoleate (16:1n−7), stearate (18:0), oleate (18:1n−9), linoleate (18:2n−6) and α-linolenate (18:3n−3) were significantly ($d > 0.8$) higher by 18–57% in the iPLA$_2\beta^{-/-}$ compared to iPLA$_2\beta^{+/+}$ mice. Concentrations of n−3 fatty acids, including eicosapentaenoic acid (20:5n−3), docosapentaenoic acid (22:5n−3) and DHA (22:6n−3), were about 20% lower in the iPLA$_2\beta^{-/-}$ mice, with medium-to-large effect sizes ($d = 0.59$, 0.59, and 0.64, respectively).

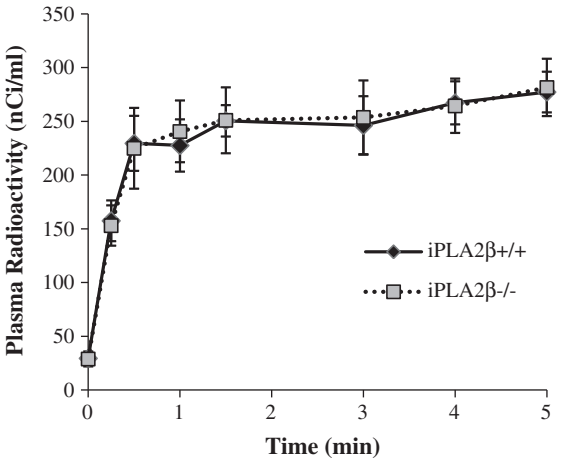

Fig. 1. Time course of arterial plasma [^{14}C] radioactivity (nCi/ml) in iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice during intravenous infusion of 5 μCi/mouse of [1-^{14}C]docosahexaenoic acid. Values are mean ± SEM (n = 5/group).

The concentration of palmitoleate (16:1n−7) esterified in plasma triglycerides and phospholipids was 26–49% higher for iPLA$_2\beta^{-/-}$ than for iPLA$_2\beta^{+/+}$ mice ($d > 0.5$), but the concentration of esterified AA was 8–17% lower. The concentrations of stearate (18:0) and linoleate (18:2n−6) esterified in triglycerides and cholesteryl esters were 24% and 13% lower, respectively, for iPLA$_2\beta^{-/-}$ than for iPLA$_2\beta^{+/+}$ mice ($d > 0.5$) (Table 1). Other differences included a 21% lower concentration of eicosapentaenoic acid (20:5n−3) esterified in triglycerides and a 20–25% higher concentration of 20:5n−3 esterified in phospholipids and cholesteryl esters for iPLA$_2\beta^{-/-}$ mice ($d > 0.5$). The concentration of DHA esterified in phospholipids and cholesteryl esters was 14–20% lower for iPLA$_2\beta^{-/-}$ mice ($d > 0.5$).

Brain total lipid phosphorous concentration [(μmol P)/(g brain wet weight)] was significantly lower for iPLA$_2\beta^{-/-}$ than for iPLA$_2\beta^{+/+}$ mice (59.17 ± 2.74 vs. 65.68 ± 1.20 μmol/g, $d > 0.8$), likely reflecting brain edema [20,21]. Accordingly, we normalized lipid concentrations and kinetic measurements to brain total lipid phosphorous. Table 2 summarizes the fractional concentration of individual phospholipid classes and plasmalogen species [in units of (μmol phosphorus of an individual phospholipid class)/(μmol total lipid phosphorous)] and cholesterol concentration [(μmol cholesterol)/(μmol total lipid phosphorous)] in brains from the two genotypes. Concentrations of EtnGpl and PtdIns were higher and that of lysoPC was lower for iPLA$_2\beta^{-/-}$ than for iPLA$_2\beta^{+/+}$ mice ($d > 0.5$). Plasmenylethanolamine was increased in iPLA$_2\beta^{-/-}$ mice ($d = 0.5$). No significant difference was seen in the plasmenylcholine concentration of ChoGpl between genotypes.

3.2. Concentration of esterified fatty acids in brain phospholipids

Table 3 summarizes mean esterified fatty acid concentrations in iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ brains (nmol fatty acid in an individual phospholipid class per μmol total lipid phosphorous) for EtnGpl, ChoGpl, PtdIns, PtdSer, and total phospholipids. The total fatty acid content of PtdIns was higher by 6.5% in iPLA$_2\beta^{-/-}$ mice ($d = 0.66$), and this reflects increased concentrations of stearate, linoleate, arachidonate and DHA. In contrast, the total fatty acid concentration in EtnGpl, ChoGpl, PtdSer, and total phospholipid was lower in iPLA$_2\beta^{-/-}$ mice by 5% ($d = 0.96$), 3% ($d = 0.78$), 6% ($d = 0.86$), and 4% ($d = 0.85$), respectively, and this reflected decreased concentrations of saturated and monounsaturated fatty acids in ChoGpl and PtdSer and decreased concentrations of PUFAs in EtnGpl, ChoGpl

Table 1
Plasma lipid concentrations in iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice.

Fatty acid	Unesterified fatty acids		Triglycerides		Phospholipids		Cholesteryl ester	
	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$
	nmol/ml plasma		nmol/ml plasma		nmol/ml plasma		nmol/ml plasma	
16:0	167.5 ± 15.8	222.8 ± 15.4**	63.7 ± 8.9	69.4 ± 13.2	759.6 ± 26.6	730.0 ± 69.1	37.2 ± 1.8	38.3 ± 5.3
16:1n−7	23.7 ± 3.0	37.3 ± 5.2**	4.9 ± 0.9	7.3 ± 1.7**	13.6 ± 1.7	17.1 ± 1.9**	15.2 ± 2.1	18.3 ± 4
18:0	43.0 ± 4.7	50.8 ± 4.3*	33.9 ± 7.5	25.7 ± 2.9*	359.9 ± 9.3	347.3 ± 19.5	16.0 ± 5.6	13.0 ± 1.3
18:1 n−9	162.0 ± 17.7	198.2 ± 14.4**	70.5 ± 8.6	73.7 ± 12.3	160.6 ± 4.7	164.6 ± 15.2	47.6 ± 2.3	46.2 ± 4.1
18:2 n−6	224.6 ± 24.6	271.9 ± 18.5**	100.9 ± 17.2	103.3 ± 18.8	631.5 ± 1.3	621.5 ± 2.1	359.7 ± 14	313.7 ± 42.8*
18:3 n−3	18.7 ± 2.0	24.0 ± 2.5**	4.0 ± 0.6	4.6 ± 0.8	4.9 ± 16.6	4.7 ± 54.5	3.7 ± 0.5	3.7 ± 0.3
20:4 n−6	6.8 ± 0.9	6.4 ± 0.8	12.7 ± 1.3	10.5 ± 1.7*	162.7 ± 0.2	149.3 ± 0.4**	118.8 ± 24.2	113.3 ± 10.5
20:5 n−3	9.0 ± 1.9	7.0 ± 0.9*	26.1 ± 1.7	20.5 ± 2.3**	26.1 ± 3.9	31.3 ± 6.6**	29.2 ± 4.5	36.5 ± 7.3*
22:5 n−3	6.1 ± 1.0	4.9 ± 0.7*	ND	ND	9.7 ± 2.4	9.6 ± 3.3	ND	ND
22:6 n−3	40.9 ± 6.7	33.0 ± 4.0*	52.5 ± 4.2	44.9 ± 5.8*	215 ± 2.1	200.1 ± 2.4	57.5 ± 2.1	45.9 ± 7.8**

Values are means ± SEM (n = 5).
ND, not detected
** $d \geq 0.8$.
* $0.5 \leq d < 0.8$.

and PtdSer. Similar differences were also observed for total phospholipids. The concentration of stearate in ChoGpl and PtdSer was 4.7–7.5% lower for iPLA$_2\beta^{-/-}$ mice, but that for PtdIns was higher ($d > 0.5$). The concentration of several monounsaturated (*e.g.*, oleate) fatty acids in EtnGpl, ChoGpl and PtdSer was 14–20% lower for iPLA$_2\beta^{-/-}$ than for iPLA$_2\beta^{+/+}$ mice, but that for PtdIns was higher ($d > 0.5$). The esterified concentrations of several PUFAs (*e.g.*, AA, DHA, and 22:5n−3) in brain PtdSer and EtnGpl were up to 30% lower ($d > 0.5$). The concentrations of esterified oleate (18:1n−9), 20:1n−9, 22:4n−6, 22:5n−3, and DHA in total brain phospholipid were also 5–17% lower for iPLA$_2\beta^{-/-}$ mice ($d > 0.5$).

3.3. Esterified fatty acid concentrations of brain lysophospholipids

Since esterified fatty acid concentration of brain phospholipids relative to total lipid phosphorus was reduced in iPLA$_2\beta^{-/-}$ mice, we tested whether phosphorus-containing lipids with a relatively low fatty acid content, such as lysophospholipids, might be more abundant in iPLA$_2\beta^{-/-}$ than in iPLA$_2\beta^{+/+}$ mice. Lysophospholipids have free hydroxyl groups at the *sn*-1 or *sn*-2 position and contain a single fatty acid per phosphorus atom, whereas diacyl-phospholipids have two fatty acids. The fatty acid concentrations of 18:1n-9, 18:1n-7, AA, 22:4n-6 and DHA in lysoPC (nmol fatty acid per μmol total lipid phosphorous) were increased in brains of iPLA$_2\beta^{-/-}$ mice ($d > 0.5$; Table 4). The concentrations of 16:0, 18:2n−6, 20:1n−9 and 22:4n−6 in lysoPI plus lysoPE were higher in iPLA$_2\beta^{-/-}$ mice ($d > 0.5$; Table 4). Total esterified fatty acid concentration in the combined lysoPI and lysoPE fraction did not differ significantly between genotypes.

Table 2
Brain phosphorous and cholesterol concentrations, per μmol phosphorous (P) of brain total lipids, in iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice.

	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$
	μmol/μmol P of brain total lipids	
Total phospholipid		
EtnGpl	0.30 ± 0.03	0.36 ± 0.04*
ChoGpl	0.38 ± 0.021	0.37 ± 0.010
PtdIns	0.06 ± 0.005	0.07 ± 0.003**
PdtSer	0.13 ± 0.006	0.13 ± 0.004
Lyso PC	0.010 ± 0.003	0.006 ± 0.001*
LysoPE + LysoPI	0.022 ± 0.002	0.020 ± 0.002
Cholesterol	0.28 ± 0.004	0.27 ± 0.009
Plasmalogen		
Plasmenylethanolamine	0.14 ± 0.003	0.15 ± 0.003*
Plasmenylcholine	0.008 ± 0.002	0.006 ± 0.001

Values are means ± SEM (n = 5).
* $0.5 \leq d < 0.8$.
** $d \geq 0.8$.

3.4. Brain acyl-CoA concentrations and specific activities

Table 5 summarizes the mean brain concentrations of long chain fatty acyl-CoA species, specific activity of docosahexaenoyl-CoA (DHA-CoA) and mean values for λ (dilution coefficient, Eq. 4). Brain concentrations of palmitoyl-CoA, oleaoyl-CoA, linoleoyl-CoA, arachidonoyl-CoA and DHA-CoA were higher for iPLA$_2\beta^{-/-}$ than for iPLA$_2\beta^{+/+}$ mice ($d > 0.5$). The specific activity of DHA-CoA also was higher ($d = 0.76$), but λ did not differ between genotypes ($d < 0.5$).

3.5. DHA incorporation into brain phospholipids

Incorporation of unesterified plasma DHA into brain lipids is characterized by an incorporation coefficient (k^*) and rate (J_{in}) (Eqs. 1 and 2), and the mean values of these parameters for various lipid classes are summarized in Table 6. k^* for DHA incorporation into PtdSer was 41% higher for iPLA$_2\beta^{-/-}$ than for iPLA$_2\beta^{+/+}$mice ($d = 1.84$), but k^* for total phospholipid or other phospholipid classes did not differ significantly between genotypes. $J_{in,i}$, the product of k^* and plasma unesterified unlabeled DHA concentration, was decreased for iPLA$_2\beta^{-/-}$ mice by 17% for EtnGpl ($d = 0.51$) and by 18% for PtdIns ($d = 0.53$).

3.6. DHA turnover in brain phospholipids

Table 6 summarizes DHA incorporation rates from the brain precursor DHA-CoA pool (J_{FA}) and turnover (F_{FA}) of DHA in total phospholipid and in individual brain phospholipid classes (Eqs. 3 and 5). J_{FA} for total phospholipids did not differ between genotypes, but J_{FA} for brain PtdSer was increased by 33% ($d = 0.87$) in iPLA$_2\beta^{-/-}$ mice. DHA turnover in brain PtdSer was 52% higher ($d = 1.04$) for iPLA$_2\beta^{-/-}$ mice, but did not differ between genotypes for any other phospholipid class or for total phospholipid.

Table 3
Esterified fatty acid concentrations in brain phospholipids of iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice.

Fatty acid	*EtnGpl*		*ChoGpl*		*PtdIns*		*PtdSer*		*Total phospholipids*	
	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$
	nmol/μmol P of brain total lipids		nmol/μmol P of brain total lipids		nmol/μmol P of brain total lipids		nmol/μmol P of brain total lipids		nmol/μmol P of brain total lipids	
16:0	36.7 ± 0.6	36.3 ± 0.8	297.2 ± 4.5	291.2 ± 10.2	8.9 ± 0.4	8.0 ± 1.2	3.7 ± 0.2	4.2 ± 0.7	348.9 ± 5.6	342.9 ± 10.5
18:0	124.5 ± 2.6	126.0 ± 6.2	96.1 ± 1.5	91.6 ± 2.2**	29.2 ± 1.3	31.4 ± 1.9*	101.6 ± 2.8	93.9 ± 1.7**	367.0 ± 7.8	357.9 ± 8.7
18:1 n−9	91.7 ± 1.3	87.9 ± 4.8	151.2 ± 2.4	143.3 ± 2.8**	14.6 ± 1.1	14.8 ± 0.8	43.5 ± 0.8	41.4 ± 2.8	310.8 ± 5.0	296.4 ± 5.8**
18:1 n−7	18.5 ± 0.6	21.3 ± 3.4*	41.2 ± 0.5	39.7 ± 1.2*	3.5 ± 0.2	3.5 ± 0.1	ND	ND	63.3 ± 0.8	64.7 ± 4.6
18:2 n−6	4.6 ± 0.3	4.6 ± 0.4	5.5 ± 0.2	5.5 ± 0.3	0.9 ± 0.1	1.4 ± 0.4**	0.6 ± 0.0	0.6 ± 0.1*	11.6 ± 0.4	12.3 ± 0.9
20:1n−9	21.4 ± 1.2	19.0 ± 1.4**	7.8 ± 0.2	7.1 ± 0.4**	1.9 ± 0.2	1.8 ± 0.0	3.7 ± 0.2	3.1 ± 0.3**	35.6 ± 1.6	32.2 ± 2.2*
20:4 n−6	72.4 ± 1.5	67.8 ± 2.1**	31.4 ± 0.6	29.9 ± 1.8	27.2 ± 1.2	30.2 ± 2.7*	5.6 ± 0.1	5.2 ± 0.2*	136.7 ± 2.9	133.5 ± 6.2
22:4 n−6	25.2 ± 0.8	21.5 ± 0.7**	ND	ND	ND	ND	5.6 ± 0.3	5.4 ± 0.2	31.5 ± 0.8	27.8 ± 0.8**
22:5 n−3	2.4 ± 0.0	2.0 ± 0.1**	ND	ND	ND	ND	0.7 ± 0.0	0.6 ± 0.0**	3.2 ± 0.1	2.6 ± 0.1**
22:6 n−3	148.1 ± 4.2	132.7 ± 3.6**	31.3 ± 0.7	29.6 ± 1.6*	3.1 ± 0.1	3.8 ± 0.3**	65.5 ± 2.3	62.2 ± 1.6*	249.4 ± 4.8	230.0 ± 5.5**
Total	549.1 ± 10.4	522.5 ± 16.2**	665.5 ± 9.7	642.0 ± 17.4*	89.2 ± 4.1	95.0 ± 3.7*	230.5 ± 5.4	216.7 ± 5.4**	1570.0 ± 26.6	1510.5 ± 33.2**

Values are means ± SEM (n = 5).
** $d \geq 0.8$.
* $0.5 \leq d < 0.8$.

3.7. Brain enzymatic activity and levels of mRNA and protein for sPLA$_2$, cPLA$_2$ and iPLA$_2$

Compared to wild type mice, brains of iPLA$_2\beta^{-/-}$ mice had much reduced iPLA$_2\beta$ mRNA (93%) and protein (99.9%), and total brain iPLA$_2$ activity was also reduced, as expected ($d > 0.8$; Table 7). Measured residual brain iPLA$_2$ activity in the iPLA$_2\beta^{-/-}$ mouse is attributable to iPLA$_2\gamma$ [48]; iPLA$_2\gamma$ mRNA was detected in iPLA$_2\beta^{-/-}$ and iPLA$_2\beta^{+/+}$ mice, but was 40% less abundant in the knockout mice ($d > 0.8$; Table 7). We did not measure iPLA$_2\gamma$ protein because a suitable antibody was not available.

Brain cPLA$_2$-IVA mRNA was 50% higher for iPLA$_2\beta^{-/-}$ than for iPLA$_2\beta^{+/+}$ mice ($d = 1.05$), but a significant difference between genotypes was not found for brain cPLA$_2$-IVA protein or activity (Table 7). Levels of mRNA, protein, and enzymatic activity for sPLA$_2$-V were higher in iPLA$_2\beta^{-/-}$ than iPLA$_2\beta^{+/+}$ mice by 50% ($d = 1.32$), 25% ($d = 0.57$), and 11% ($d = 0.79$), respectively (Table 7).

3.8. COX and LOX mRNA and protein

There was no significant difference in brain COX-1 mRNA between genotypes (Table 7). Brain COX-1 protein was 21% lower ($d = 0.68$) and COX-2 protein was 54% higher ($d = 0.97$), however (Table 7). COX-2 mRNA also was higher by 17% in iPLA$_2\beta^{-/-}$ mice ($d = 0.52$). No significant difference between genotypes was observed for 5-LOX, 12-LOX or15-LOX protein (Table 7).

4. Discussion

Values for wild type mice observed here for DHA kinetic parameters and levels of lipids, including unesterified fatty acids, in plasma and in brain, are similar to published values without normalization for brain lipid phosphorous content [33,41]. Compared with iPLA$_2\beta^{+/+}$ controls, iPLA$_2\beta^{-/-}$ mice at 4-5 months of age exhibited reduced brain consumption of DHA, reflected by reduced incorporation rates (J_{in}) of unesterified DHA from plasma into several phospholipid classes; in agreement with quantitative autoradiographic observations [3]. DHA in brain cannot be synthesized *de novo*, and is insignificantly (<0.5%) converted from its α-LNA precursor or from eicosapentaenoic acid in brain [49,50]. iPLA$_2\beta^{-/-}$ mice also exhibited altered brain expression of other brain phospholipases, COX-1 and COX-2, but not of LOX isozymes. Altered fatty acid concentrations in various phospholipid classes were also observed, but DHA turnover in brain phospholipids did not differ between genotypes (Table 6), whereas J_{in} for brain EtnGpl and PtdIns was lower for iPLA$_2\beta^{-/-}$ mice. Because J_{in} was reduced proportionately to esterified DHA and because λ did not differ between genotypes, calculated DHA turnover in brain phospholipids (Eq. (5)) also did not differ.

Table 4
Esterified fatty acid concentrations in brain lysoPC and combined lysoPE and lysoPI fractions in iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice.

Fatty acid	LysoPC		LysoPE and LysoPI	
	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$
	nmol/μmol P of brain total lipids		nmol/μmol P of brain total lipids	
16:0	1.03 ± 0.06	1.06 ± 0.07	1.32 ± 0.18	2.09 ± 0.57**
18:0	2.89 ± 0.34	2.63 ± 0.15	12.80 ± 2.00	12.36 ± 2.69
18:1 n−9	0.35 ± 0.03	0.40 ± 0.03*	9.41 ± 1.20	8.50 ± 1.56
18:1 n−7	0.10 ± 0.01	0.13 ± 0.01*	ND	ND
18:2 n−6	ND	ND	0.08 ± 0.01	0.10 ± 0.02*
20:1 n−9	0.23 ± 0.09	0.29 ± 0.10	0.55 ± 0.17	0.96 ± 0.34**
20:4 n−6	0.06 ± 0.01	0.09 ± 0.01**	0.14 ± 0.02	0.16 ± 0.03
22:4 n−6	0.24 ± 0.02	0.34 ± 0.05**	0.46 ± 0.08	0.56 ± 0.07*
22:6 n−3	0.02 ± 0.00	0.03 ± 0.01**	1.32 ± 0.60	1.62 ± 0.69
total	4.94 ± 0.47	4.97 ± 0.20	26.13 ± 4.12	26.42 ± 5.91

Values are means ± SEM (n = 5).
** $d \geq 0.8$.
* $0.5 \leq d < 0.8$.

Table 5
Brain acyl-CoA concentrations in total lipids of iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice.

Acyl-CoA		iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$
nmol/μmol P of brain total lipids			
Mystearoyl-CoA	14:0	0.009 ± 0.002	0.011 ± 0.003
Palmitoyl-CoA	16:0	0.089 ± 0.010	0.119 ± 0.014**
Stearoyl-CoA	18:0	0.084 ± 0.015	0.094 ± 0.018
Oleaoyl-CoA	18:1	0.093 ± 0.011	0.121 ± 0.013**
Linoleoyl-CoA	18:2 n6	0.015 ± 0.004	0.023 ± 0.006*
Arachidonoyl-CoA	20:4 n6	0.015 ± 0.003	0.020 ± 0.005*
Docosahexaenoyl-CoA	22:6 n3	0.015 ± 0.002	0.018 ± 0.004*
nCi/μmol P of brain total lipids			
Docosahexaenoyl-CoA		0.011 ± 0.001	0.014 ± 0.002*
Lambda (λ)		0.129 ± 0.036	0.103 ± 0.026

P, phosphorous. Values are means ± SEM (n = 5).
** $d \geq 0.8$
* $0.5 \leq d < 0.8$.

Table 6
Brain incorporation coefficients (k^*), incorporation rates (J_{in}) of unesterified DHA from plasma, net incorporation rates from brain docosahexaenoyl-CoA (J_{FA}) and turnover of DHA (F_{FA}) in brain phospholipids of iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice.

	k^*		J_{in}		J_{FA}		F_{FA}	
	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$
	ml/μmol P/s × 10^{-5}		nmol/μmol P/s × 10^{-4}		nmol/μmol P/s × 10^{-2}		%/h	
Total phospholipids	0.371 ± 0.027	0.389 ± 0.020	1.51 ± 0.25	1.30 ± 0.18	0.144 ± 0.030	0.146 ± 0.021	2.08 ± 0.42	2.31 ± 0.37
EtnGpl	0.150 ± 0.010	0.152 ± 0.007	0.61 ± 0.11	0.51 ± 0.07*	0.058 ± 0.012	0.057 ± 0.009	1.43 ± 0.30	1.57 ± 0.27
ChoGpl	0.130 ± 0.008	0.139 ± 0.010	0.52 ± 0.08	0.46 ± 0.06	0.051 ± 0.010	0.053 ± 0.008	5.76 ± 1.14	6.41 ± 1.06
PtdIns	0.075 ± 0.009	0.075 ± 0.004	0.31 ± 0.05	0.25 ± 0.04*	0.029 ± 0.006	0.028 ± 0.003	33.20 ± 7.12	27.38 ± 4.46
PtdSer	0.017 ± 0.001	0.024 ± 0.002**	0.07 ± 0.01	0.08 ± 0.01	0.006 ± 0.001	0.009 ± 0.002**	0.35 ± 0.06	0.53 ± 0.09**

Values are means ± SEM (n = 5).
* $0.5 \le d < 0.8$.
** $d \ge 0.8$.

Significant reductions in net k^* for DHA have been demonstrated with quantitative autoradiography in 70 of 81 brain regions examined in unanesthetized iPLA$_2\beta^{-/-}$ mice compared to wild type controls [3]. Here we demonstrate by direct chemical analyses that reduced incorporation of unesterified plasma DHA into brain EtnGpl and PtdIns in iPLA$_2\beta^{-/-}$ mice accounts for most of the reduction of net incorporation into total phospholipids (Table 6). Although J_{in} for brain PtdSer is increased, this is a minor contributor to the net change.

Changes in the brain of iPLA$_2\beta^{-/-}$ mice include increases in cPLA$_2$-IVA mRNA; in sPLA$_2$-V mRNA, protein and activity; and in COX-2 mRNA and protein (Table 7), but a reduction in COX-1 protein. These changes reflect a profound reorganization of brain lipid metabolism and lack of enzyme redundancy. AA can be released from phospholipids by each of the three PLA$_2$ enzymes, and COX and LOX can convert released AA to bioactive oxygenated metabolites, including prostaglandins, thromboxanes, and leukotrienes [48]. The increase in COX-2 protein but reduction in COX-1 protein in brains of iPLA$_2\beta^{-/-}$ mice might reflect coupling of COX-1 to iPLA$_2$ and of COX-2 to cPLA$_2$ [51–53]. The reduced brain AA concentration in EtnGpl and PtdSer in iPLA$_2\beta^{-/-}$ mice and the increased AA concentration of PtdIns may result from the enzymatic changes.

Brains of iPLA$_2\beta^{-/-}$ mice exhibit reduced concentrations of several fatty acid substituents esterified in EtnGpl, ChoGpl and PtdSer, which contain two fatty acids for each phosphorus atom. In contrast, brains of iPLA$_2\beta^{-/-}$ mice exhibit increased concentrations of several fatty acids esterified in lysophospholipids and of acyl-CoA species. Lysophospholipids contain a single fatty acid residue per phosphorus atom, and acyl-CoAs contains three phosphorus atoms for each fatty acid. The brain plasmenylethanolamine content is also increased for iPLA$_2\beta^{-/-}$ mice, and these ether lipids also have a single mole of saponifiable fatty acid per mole of phosphate and also exhibit a lower fatty acid to phosphorus ratio than do diacyl phospholipids. Ether linked lysophospholipids contain no saponifiable fatty acid residues and thus contribute no signal to the fatty acid content of lysophospholipid classes.

Table 7
Enzymatic activity, protein expression and mRNA levels in the brains of iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice.

	iPLA$_2\beta^{+/+}$	iPLA$_2\beta^{-/-}$
Activity	*pmol/mg protein/min*	
cPLA$_2$	6.6 ± 0.1	6.8 ± 0.4
sPLA$_2$	7.6 ± 0.5	8.4 ± 0.3*
iPLA$_2$ (β + γ)	11.9 ± 1.4	2.0 ± 0.3**
Protein	*% expression*	
cPLA$_2$-IVA	100 ± 20	117 ± 16
sPLA$_2$-V	100 ± 25	129 ± 17*
iPLA$_2\beta$	100 ± 13	7 ± 0.3**
COX-1	100 ± 16	79 ± 6*
COX-2	100 ± 28	154 ± 16**
5-LOX	100 ± 16	111 ± 10
12-LOX	100 ± 20	120 ± 24
15-LOX	100 ± 11	115 ± 18
mRNA	*Relative fold change*	
cPLA$_2$-IVA	1.0 ± 0.1	1.4 ± 0.2**
sPLA$_2$-V	1.0 ± 0.1	1.5 ± 0.2**
iPLA$_2\beta$	1.0 ± 0.03	0.01 ± 0.003**
iPLA$_2\gamma$	1.0 ± 0.1	0.6 ± 0.1**
COX-1	1.0 ± 0.1	1.1 ± 0.1
COX-2	1.0 ± 0.1	1.2 ± 0.2*

Values are means ± SEM (n = 6).
* $0.5 \le d < 0.8$.
** $d \ge 0.8$.

The altered brain phospholipid concentrations in iPLA$_2\beta^{-/-}$ mice may reflect disturbed membrane remodeling as a consequence of iPLA$_2\beta$ deficiency and compensatory changes in the expression of other PLA$_2$ enzymes. Tubulovesicular membranes and small vacuoles and edema are observed in the brain of iPLA$_2\beta^{-/-}$ mice at age 4 months, but more dramatic neuropathologic abnormalities are manifest by 13 months [20,21]. We confirmed the presence of edema at 4 months by demonstrating reduced total lipid phosphorus concentration per gram brain wet weight. Developmental abnormalities in fatty acid and phospholipid metabolism may contribute to early changes and to more significant neurodegenerative and behavioral abnormalities in older mice [14,20–22].

Brains of 4 month-old iPLA$_2\beta^{-/-}$ mice exhibited lower iPLA$_2\gamma$ transcript levels than did brains of wild type mice, and iPLA$_2\beta^{-/-}$ brain EtnGpl and PtdSer phospholipid exhibited a lower esterified DHA concentration than in wild type mice. This was associated with reduced incorporation of unesterified DHA from plasma into these phospholipid classes. Both iPLA$_2\beta$ and iPLA$_2\gamma$ can hydrolyze DHA from the *sn*-2 position of phospholipids [1,2]. DHA is a precursor of anti-inflammatory neuroprotectins and resolvins [26], and the reduced brain DHA concentration associated with iPLA$_2\beta$ deficiency may increase vulnerability to neuroinflammatory processes and other insults. Enzymes not measured in this study that may influence DHA metabolism include plasmalogen-selective PLA$_2$, which has not yet been cloned to our knowledge [54]. Net iPLA$_2$ activity and iPLA$_2\beta$ mRNA and protein also have been reported to be reduced in brains of rats deprived of dietary n−3 PUFA [52], and these animals also exhibit reduced brain DHA consumption and concentration and increased sensitivity to neuroinflammatory stress [55,56].

Mutations in the *PLA2G6* gene encoding iPLA$_2\beta$ have been reported in humans with infantile neuroaxonal dystrophy, idiopathic neurodegeneration with brain iron accumulation, dystonia-parkinsonism, and cerebellar cortical atrophy with gliosis [16–19]. These conditions are characterized by motor and often cognitive impairments. iPLA$_2\beta$ or iPLA$_2\gamma$ knockout mice show significant motor and cognitive deficits by 13 months of age, associated with synaptic loss and α-synuclein accumulation in brain [20,21]. α-Synuclein and DHA strongly interact in a manner that affects both the structure of the protein and the physical state of the lipid [57,58]. Similar but less severe motor and cognitive behavioral abnormalities have been

reported in rats that have been deprived of dietary n−3 PUFA and exhibit reduced brain DHA concentration [7,50,55,56], and this is also associated with altered expression of AA and DHA metabolizing enzymes [20,28,52,59].

The changes in brain DHA metabolism and concentration and in lipid metabolizing enzymes in iPLA$_2\beta^{-/-}$ mice occurred despite the high (2.2%) dietary DHA concentration (Rodent NIH-07) (see Methods and materials). This may have slowed the evolution of neuropathology [60,61], which was described in mice fed a diet that contained only 0.9% DHA (PicoLabA 5053, LabDiet, Purina Mills International, St. Louis, MO) [20]. Dietary deficiency of DHA or its precursors (α-LNA and EPA) could exacerbate the effects of iPLA$_2\beta$ deficiency by further reducing plasma DHA incorporation into brain, but this remains to be tested.

This study underscores the importance of iPLA$_2\beta$ in brain lipid metabolism [2,46,48,62], and highlights the lack of redundancy with regard to iPLA$_2\beta$ function in brain, in view of the many changes in expression of other enzymes and in lipid composition in iPLA$_2\beta$ knockout mice. Lack of redundancy also has been reported for mice with a genetic deficiency of cPLA$_2$ IVA, COX-2 and COX-1 [33,41,63–66]. In this regard, even heterozygous iPLA$_2\beta^{+/-}$ mice exhibit reduced plasma DHA incorporation into brain and altered DHA signaling in response to cholinergic muscarinic receptor activation [3]. Brain AA metabolism and signaling may also be disturbed in iPLA$_2\beta^{-/-}$ mice in view of our findings that these mice exhibit increased brain activity of sPLA$_2$ and cPLA$_2$-IV mRNA, and the fact that both enzymes can release AA from membrane phospholipid. The esterified AA in phospholipids is reduced in iPLA$_2\beta^{-/-}$ mice (Table 3).

In summary, iPLA$_2\beta^{-/-}$ mice at age 4–5 months exhibit disturbances in whole brain lipid composition and metabolism and in expression of enzymes involved in metabolism. These abnormalities are associated with reduced incorporation of unesterified DHA from plasma into brain lipids and reduced esterified DHA concentrations in various lipid classes, and may contribute to neuropathological and behavioral abnormalities. Our observations may be relevant to human clinical syndromes (*e.g.*, dystonia-parkinsonism and infantile neuroaxonal dystrophy) associated with *PLA2G6* mutations. In such conditions, DHA incorporation into brain could be imaged directly with positron emission tomography [67], and dietary n−3 PUFA supplementation and/or n−6 PUFA deprivation might be considered for therapeutic trials [51,60,61]. Future characterization of brain lipid changes in iPLA$_2\beta$-deficient mice of different ages could elucidate mechanisms for neuropathology in patients with *PLA2G6* mutations and provide guidance for therapeutic intervention.

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