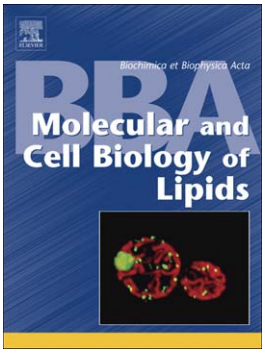

Phospholipid class-specific brain enrichment in response to lysophosphatidylcholine docosahexaenoic acid infusion

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Phospholipid class-specific brain enrichment in response to lysophosphatidylcholine docosahexaenoic acid infusion.

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Abbreviations: AGPATs, 1-acylglycerol-3-phosphate-O-acyltransferases; BBB, blood-brain barrier; CDP, cytidine diphosphate; CerPCho, ceramide phosphocholine or sphingomyelin; DAG, diacylglycerol; DHA, docosahexaenoic acid; CoA, coenzyme A; Ins, inositol; LPLATs, lysophospholipid acyltransferases; LysoPtdCho, lysophosphatidylcholine; LysoPtdCho-^{14}C-DHA, lysoPtdCho-DHA radiolabelled with carbon 14 [1-^{14}C]; LysoPtdEtn, lysophosphatidylethanolamine; LysoPtdIns, lysophophatidylinositol; LysoPtdOH, lysophosphatidic acid; LysoPtdSer, lysophosphatidylserine; Mfsd2a, major facilitator superfamily domain-containing protein 2; NE, non-esterified; NE-^{14}C-DHA, NE-DHA radiolabelled with carbon 14 [1-^{14}C]; PlsCho, plasmenylcholine; PlsEtn, plasmenylethanolamine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; PSS, phosphatidylserine synthase; TAG, triacylglycerol.

Abstract

Recent studies suggest that at least two pools of plasma docosahexaenoic acid (DHA) can supply the brain: non-esterified DHA (NE-DHA) and lysophosphatidylcholine (lysoPtdCho)-DHA. In contrast to NE-DHA, brain uptake of lysoPtdCho-DHA appears to be mediated by a specific transporter, but whether both forms of DHA supply undergo the same metabolic fate, particularly with regards to enrichment of specific phospholipid (PL) subclasses, remains to be determined. This study aimed to evaluate brain uptake of NE-DHA and lysoPtdCho-DHA into brain PL classes. Fifteen-week old rats were infused intravenously with radiolabelled NE-^{14}C-DHA or lysoPtdCho-^{14}C-DHA (n = 4/group) over five mins to achieve a steady-state plasma level. PLs were extracted from the brain and separated by thin layer chromatography and radioactivity was quantified by liquid scintillation counting. The net rate of entry of lysoPtdCho-DHA into the brain was between 62% and 85% lower than the net rate of entry of NE-DHA, depending on the PL class. The proportion of total PL radioactivity in the lysoPtdCho-^{14}C-DHA group compared to the NE-^{14}C-DHA group was significantly higher in choline glycerophospholipids (ChoGpl) (48% vs 28%, respectively) but lower in ethanolamine glycerophospholipids (EtnGpl) (32% vs 46%, respectively). In both groups, radioactivity was disproportionally high in phosphatidylinositol and ChoGpl but low in phosphatidylserine and EtnGpl compared to the corresponding DHA pool size. This suggests that DHA undergoes extensive PL remodeling after entry into the brain.

Keywords: Brain; docosahexaenoic acid; lysophosphatidylcholine; phosphatidylcholine; phosphatidylethanolamine; phosphatidylserine.

1. Introduction

The brain has a unique fatty acid (FA) profile compared to other organs and tissues. It is highly enriched in docosahexaenoic acid (DHA), an omega-3 polyunsaturated FA that cannot be synthesised *de novo* in mammals [1]. DHA is important for brain function including for neurodevelopment, neuronal transmission and integrity, maintaining membrane fluidity and the regulation of neuroinflammation [2-5]. In the human brain, more than 98% of DHA is esterified to phospholipid (PL), with approximately 65% of DHA found in ethanolamine glycerophospholipids (EtnGpl), 27% in phosphatidylserine (PtdSer), and less than 10% in choline glycerophospholipids (ChoGpl) and phosphatidylinositol (PtdIns) [6]. Relative to other FAs, DHA is highly enriched in the PtdSer pool with as much as 60% of PtdSer species containing at least one DHA molecule [7]. DHA esterified to PtdSer is believed to play a crucial role in neuronal growth and survival [7, 8].

While the unique brain PL distribution of DHA has been extensively reported, less is known about how DHA is transported to the brain. There are at least two plasma pools that may supply the brain with DHA: the non-esterified (NE) pool and the lysophosphatidylcholine (lysoPtdCho) pool [9]. Previous studies by our group have shown that the major pool of DHA that supplies the brain is the NE pool with a rate of uptake approximately 10-fold higher than that of the lysoPtdCho pool [9]. However, it is currently unknown whether these two plasma DHA pools enrich the brain PL classes differently and this could have important consequences for targeting specific PL of the brain. Therefore, using an *in vivo* model, the objective of this study was to evaluate

whether plasma lysoPtdCho-DHA preferentially targets specific PL classes of the brain compared to NE-DHA.

2. Methods

This study is a secondary analysis of our previous report [9]. The animal experimental protocol was approved by the Animal Ethics Committee of the University of Toronto. Ten-week old male Sprague Dawley rats were purchased from Charles Rivers (Saint-Constant, Qc, Canada) and were fed *ad libitum* with standard chow (Teklad 2018, Harlan, Madison, WI, USA) for five weeks until the start of the experimental procedure.

2.1. DHA radiotracers

The DHA tracers were radiolabelled on the carboxyl carbon of the DHA molecule [1-^{14}C] (NE-^{14}C-DHA and 1-docosahexaenoyl-2-lysophosphocholine or lysoPtdCho-^{14}C-DHA). NE-^{14}C-DHA was purchased from Moravek Biochemical Inc. (Brea, CA, USA) and lysoPtdCho-^{14}C-DHA was purchased from American Radiolabeled Chemicals Inc. (St-Louis, MO, USA). NE-^{14}C-DHA and lysoPtdCho-^{14}C-DHA specific activity was 58 mCi/mmol and 55 mCi/mmol, respectively. The NE-^{14}C-DHA and lysoPtdCho-^{14}C-DHA perfusates were solubilised in a 5 mM HEPES buffer containing bovine serum albumin to concentrations of 86 µci/ml.

2.2. Intravenous radiotracer infusion

At the age of 15 weeks, rats were anaesthetised with isoflurane (3% induction and 1-2% maintenance) and underwent surgery to implant catheters in the right carotid artery and right jugular vein as described previously [10]. The catheters were accessible from the back of the rat through a skin buttons sutured subcutaneously at the scapula. Rats were intravenously (iv) infused with 10 µCi of NE-^{14}C-DHA or lysoPtdCho-^{14}C-DHA in the jugular vein over five mins to achieve a plasma steady-state level as described

previously [11]. Thereafter, the rats were euthanized by head-focused high-energy microwave irradiation (13.5kW for 1.6 secs; Cober Electronics Inc., Norwalk, CT, USA). Brains were extracted and stored at -80 °C for biochemical analyses.

2.3. Lipid extraction, PL class separation, and FA quantification

Whole brain total lipids were extracted according to a modified version of the Folch et al. method [12] with the use of chloroform : methanol : 0.88% KCl (2:1:0.75, by volume). Phospholipid class separation was performed using thin layer chromatography (TLC). Briefly, TLC-H plates (Analtech, Newark, DE, USA) were washed in chloroform methanol (2:1, by volume) the day prior to the TLC. From the brain total lipid extract (2000 µl), 250 µl was used for radioactivity counting and 100 µl was used for FA analyses. Samples were loaded on 7 cm wide lanes and migrated to achieve PL class separation with the following migration solvent: chloroform : methanol : 2-propanol : 0.25% KCl : triethylamine (30:9:25:6:18, by volume). PL classes were revealed by spraying 0.1% 8-anilino-1-naphthalene sulfonic acid and visualised under UV light. PL were scraped for radioactivity analyses by liquid scintillation counting or methylated with 14% (v/v) boron trifluoride methanol for 1h at 90ºC for gas chromatography FA analyses. Di-17:0 phosphatidylcholine (PtdCho) (Avanti Polar lipids Inc., Alabaster, AL, USA) was added as an external standard prior to methylation for brain FA quantification. FA methyl esters (FAME) were quantified on a Varian 430 gas chromatograph (Bruker, Billerica, MA, USA) equipped with a SP-2560 biscyanopropyl siloxane, capillary column (100 m length x 0.25 mm diameter x 0.2 µm film thickness; Supelco, Bellefonte, PA, USA). Helium was the carrier gas and 1 µl of samples were injected in splitless injection mode at 250ºC. The gas chromatograph temperature program was: 60ºC for 2 mins followed by

a 10°C/min ramp to 170°C with a 4 min hold, a 6.5°C/min ramp to 175°C, a 2.6°C/min ramp to 185°C, a 1.3°C/min ramp to 190°C and a 8.0°C/min ramp to 240°C with a 11 min hold for a total run time of 42.7 mins. FAME were quantified by flame ionisation detection and the peaks were identified through comparison with an external mixed FAME standard (GLC-569, Nu Chek Prep Inc., Elysian, MN, USA). Plasma NE-DHA and lysoPtdCho-DHA concentrations were quantified by liquid chromatography tandem mass spectrometry and obtained from our previous report [9].

2.4. Plasmalogen separation and DHA quantification

For this procedure, 150 µl of the brain total lipid extract (2000 µl) was used for radioactivity counting and 100 µl was used for DHA quantification. Phospholipid classes were separated on a TLC-H plate and revealed by UV light as described in section 2.3. Plasmalogens were separated from the ChoGpl and EtnGpl fractions by acidic hydrolysis as described previously [13, 14]. Briefly, the bands corresponding to ChoGpl and EtnGpl were scraped and phospholipids were extracted from the silica using 3 ml of Hexane : isopropanol (3:2, by volume) containing 5.5 % water. Phospholipids were dried down under nitrogen and thereafter exposed to concentrated HCL vapors for 15 mins to hydrolyze the vinyl ether linkage of the plasmalogens [14]. The lipid mixture was then reconstituted in 100 µl of $ChCl_3$ and separated on a second TLC-H plate using the same parameters as in section 2.3. The bands corresponding to plasmenylcholine (PlsCho), PtdCho, plasmenylethanolamine (PlsEtn), and PtdEtn were scraped for radioactivity analyses by liquid scintillation counting or extracted from the silica using 3 ml of Hexane : isopropanol (3:2, by volume) containing 5.5 % water. The extracted lipids were thereafter hydrolysed to remove the carrier group from the FAs, dried down under a

stream of nitrogen and derivatized to pentafluorobenzyl (PFB) esters, as previously described [15, 16]. FA PFB esters were quantified on an Agilent 5977A quadrupole mass spectrometer coupled to an Agilent 7890B gas chromatographer (Agilent Technologies, Mississauga, ON, Canada) in negative chemical ionization mode. Graded concentrations of Di-17:0 PtdCho were hydrolysed and derivatized to FA PFB esters in parallel to produce the standard curve for DHA quantification. FA PFB esters were injected via an Agilent 7693 autosampler into a DB-FFAP 30 m x 0.25 mm i.d. x 25 µm film thickness capillary column (J&W Scientific from Agilent Technologies, Mississauga, ON, Canada) interfaced into the ion source with helium as the carrier gas.

2.5. Liquid scintillation counting and kinetics calculations

Brain PL classes and plasma NE-FA and lysoPtdCho-FA radioactivity was quantified by a Packard TRI-CARB2900TR liquid scintillation analyzer (Packard, Meriden, CT, USA). Radioactivity, expressed in counts per mins, was converted to disintegration per mins and then to nCi (1 nCi corresponds to 37 Becquerel). Brain PL class radioactivity (in nCi/g of brain), the area under the curve of plasma radioactivity (in µCi/ml*s), and plasma DHA concentration (in nmol/ml) were then used to calculate a brain uptake coefficient of DHA (k^*, in µl x s^{-1} x g of $brain^{-1}$) and a rate of DHA entry (J_{in}, in nmol x g of $brain^{-1}$ x d^{-1}) into each PL class as described previously [9]. Briefly, k^* for each PL class was calculated with the following equation:

$$k^* = \frac{C^*_{brain}(T)}{\int_0^T C^*_{plasma}\, dt}$$

where $C^*_{brain}\ (T)$ is the radioactivity of the brain PL class at the time of sacrifice and $\int_0^T C^*_{plasma}\, dt$ is the integral (area under the curve) of plasma total radioactivity over the course of the five mins infusion. The J_{in}, was calculated by the following equation:

$$J_{in} = k^* \times C^*_{plasma}$$

Where C^*_{plasma} is the concentration of DHA in the NE plasma pool (for the NE-^{14}C-DHA group) or the lysoPtdCho plasma pool (for the lysoPtdCho-^{14}C-DHA group). For calculation of relative radioactivity, brain radioactivity within a specific PL class was compared against the brain radioactivity of total PLs and reported as percent of total PL radioactivity. Radioactivity controlled for the brain DHA pool or the total FA pool was calculated by dividing brain radioactivity within each PL class separately (nCi/g of brain) against DHA or total FA concentrations (μmol/g of brain), respectively, in the corresponding PL pools.

2.6. Statistical analyses

Radioactivity and kinetics results are expressed as mean ± SEM. The differences between the results of the lysoPtdCho-^{14}C-DHA group (n = 4) and the NE-^{14}C-DHA group (n = 4) for radioactivity and kinetics results were assessed by nonparametric Mann-Whitney tests (SPSS statistics 20, IBM Corp., NY, USA). Brain total FA concentrations and brain DHA concentrations are reported for the DHA groups together (n = 8). No statistical test was performed on these results. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Radioactivity is higher in brain PtdIns and ChoGpl of lysoPtdCho-*^{14}C-DHA infused rats than in NE-^{14}C-DHA infused rats.*

In the lysoPtdCho-^{14}C-DHA infused rats, the ChoGpl pool contained the most radioactivity (in nCi/g of brain) followed by the EtnGpl pool whereas in NE-^{14}C-DHA infused rats, radioactivity was higher in the EtnGpl than in the ChoGpl pool (Fig. 1a). There was a significant difference in radioactivity between lysoPtdCho-^{14}C-DHA infused rats and NE-^{14}C-DHA infused rats in ChoGpl (1.75 ± 0.11 nCi/g vs 0.53 ± 0.08 nCi/g, respectively, $p = 0.03$) and in PtdIns (0.43 ± 0.03 nCi/g vs 0.26 ± 0.05 nCi/g, respectively, $p = 0.03$) (Fig. 1a). The radioactivity difference between the two groups within the ChoGpl pool was driven by the PtdCho pool (1.51 ± 0.10 nCi/g in the lysoPtdCho-^{14}C-DHA group vs 0.45 ± 0.06 nCi/g in the NE-^{14}C-DHA, $p = 0.03$). There was no difference between the two groups regarding radioactivity in the PlsCho pool (Fig. 1a).

3.2. Relative to other brain PLs, lysoPtdCho-*DHA enriches more of the ChoGpl pool and less of the EtnGpl pool compared to NE-DHA.*

The proportion of total PL radioactivity in the lysoPtdCho-^{14}C-DHA group compared to the NE-^{14}C-DHA group was significantly higher in ChoGpl (48 ± 2% vs 28 ± 3%, respectively, $p = 0.03$, Fig. 1b) but lower in EtnGpl (32 ± 1 % vs 46 ± 4%, respectively, $p = 0.03$, Fig. 1b).

Within the ChoGpl pool, the proportion of total PL radioactivity in PtdCho was 44 ± 2 % in the lysoPtdCho-^{14}C-DHA group compared to 24 ± 3 % in the NE-^{14}C-DHA group ($p = 0.03$, Fig 1b). Within the EtnGpl pool, the proportion of total PL radioactivity

in PtdEtn was 26 ± 1 % in the lysoPtdCho-^{14}C-DHA group compared to 36 ± 2 % in the NE-^{14}C-DHA group (p = 0.03, Fig 1b). There was no difference between the DHA groups regarding the proportion of total PL radioactivity in the PlsCho and PlsEtn pools (Fig 1b).

3.3. *The net rate of DHA entry into brain PL classes is lower from the plasma* lysoPtdCho *pool than from the NE pool.*

Brain DHA exposure to the infusate was 2.8 times higher in the lysoPtdCho-^{14}C-DHA group than in the NE-^{14}C-DHA group, as suggested by plasma radioactivity area under the curve (35.6 ± 1.5 µCi/ml*s vs 13.3 ± 2.8 µCi/ml*s, respectively, p = 0.03, Fig. 2a). Compared to the NE-^{14}C-DHA group, the k* in the lysoPtdCho-^{14}C-DHA group was 50-51% lower for EtnGpl, PtdEtn and PlsEtn (p = 0.03 for all), but similar for PtdIns, PtdSer, ChoGpl, PtdCho, PlsCho and ceramide phosphocholine (CerPCho or sphingomyelin) (Fig. 2b).

In the plasma lysoPtdCho pool of the lysoPtdCho-^{14}C-DHA group, DHA concentration was 0.92 ± 0.11 nmol/ml whereas it was 2.88 ± 0.32 nmol/ml in the plasma NEFA pool of the NE-^{14}C-DHA group. This information was previously reported in [9] and was used to calculate J_{in} (in nmol x g of brain^{-1} x d^{-1}, Fig. 2c). In the lysoPtdCho-^{14}C-DHA group as compared to the NE-^{14}C-DHA group, J_{in} was 85% lower into EtnGpl (2.62 ± 0.32 vs 16.94 ± 3.22, respectively, p = 0.03), 84% lower into PtdEtn (2.09 ± 0.24 vs 13.41 ± 2.35, respectively, p = 0.03), 85% lower into PlsEtn (0.53 ± 0.10 vs 3.53 ± 1.15, respectively, p = 0.03), 83% lower into PtdIns (0.96 ± 0.13 vs 5.50 ± 1.47, respectively, p = 0.03), 86% lower into PtdSer (0.27 ± 0.07 vs 1.95 ± 0.36, respectively, p = 0.03), 62% lower into ChoGpl (3.85 ± 0.28 vs 10.15 ± 1.60, respectively, p = 0.03),

59% lower into PtdCho (3.55 ± 0.27 vs 8.73 ± 1.28, respectively, $p = 0.03$), 79% lower into PlsCho (0.30 ± 0.01 vs 1.42 ± 0.37, respectively, $p = 0.03$), and 84% lower into CerPCho (0.48 ± 0.22 vs 2.96 ± 1.78, respectively, $p = 0.03$).

3.4. LysoPtdCho-*DHA enriches more of the ChoGpl pool than NE-DHA after controlling for the DHA concentrations within this pool.*

Similar to previously published manuscripts in rats [10, 17], total FA concentrations (in µmol/g of brain) were the highest in ChoGpl (49.26 ± 1.92), followed by EtnGpl (32.47 ± 1.44), PtdSer (14.56 ± 0.67), PtdIns (5.05 ± 0.19) and CerPCho (2.57 ± 0.14) (Fig. 3a). DHA concentrations (in µmol/g of brain), however, were the highest in EtnGpl (5.30 ± 0.20), followed by PtdSer (2.29 ± 0.11), ChoGpl (1.26 ± 0.07), PtdIns (0.20 ± 0.01) and CerGpl (0.02 ± 0.01) (Fig. 3b). Within the EtnGpl pool, approximately 57 % of DHA was esterified to PtdEtn compared to 43 % esterified to PlsEtn whereas in the ChoGpl pool, approximately 92 % of DHA was esterified to PtdCho compared to 8 % esterified to PlsCho (Fig 3c).

Radioactivity was approximately two-fold higher in the ChoGpl and PtdCho of the lysoPtdCho-^{14}C-DHA group compared to the NE-^{14}C-DHA group when controlling for DHA concentrations in ChoGpl and PtdCho, respectively ($p = 0.03$ for both, Fig 4a). When controlling for total FA concentrations, radioactivity was also two-fold higher in the ChoGpl of the lysoPtdCho-^{14}C-DHA group compared to the NE-^{14}C-DHA group ($p = 0.03$, Fig 4b).

4. Discussion

The objective of this study was to evaluate whether plasma lysoPtdCho-DHA preferentially targets specific PL of the brain compared to NE-DHA. Our primary finding is that lysoPtdCho-^{14}C-DHA was relatively more incorporated into PtdCho within the ChoGpl pool and relatively less into PtdEtn within the EtnGpl pool compared to NE-^{14}C-DHA. These results support the hypothesis that after crossing the blood-brain barrier (BBB), a significant portion of lysoPtdCho-DHA undergoes rapid acylation with another FA to form PtdCho. NE-DHA, on the other hand, is mostly esterified to ethanolamine to form PtdEtn. It is important to note, however, that after controlling for plasma DHA concentration and exposure, the net rate of DHA entry into the brain was significantly lower in the lysoPtdCho-^{14}C-DHA group compared to the NE-^{14}C-DHA group for every PL class. This confirms findings from our previous report [9] and suggests that the NE-DHA plasma pool is a quantitatively larger contributor to brain DHA uptake than the lysoPtdCho-DHA pool.

De novo PL biosynthesis within the brain is a tightly regulated process. PL synthesis occurs in the endoplasmic reticulum with the two most abundant PL classes in the brain being PtdCho and PtdEtn. The main synthesis pathway of PtdCho and PtdEtn is believed to be through diacylglycerol association with cytidine diphosphate choline or cytidine diphosphate ethanolamine, respectively [18, 19] (Fig. 3). This pathway is known as the Kennedy pathway [20]. Another contributor to PtdCho and PtdEtn synthesis is through acylation of lysoPtdCho and lysophosphatidylethanolamine (lysoPtdEtn), respectively (Fig. 3). PL synthesis from the lyso pool is catalysed by a family of enzyme called 1-acylglycerol-3-phosphate-O-acyltransferases [21] (Fig. 3), but the contribution

of these pools to the synthesis of PtdCho and PtdEtn *in vivo* in the brain is not clear. Our results suggest that lysoPtdCho-DHA tends to be preferentially reacylated to form PtdCho with less being deacylated to form NE-DHA which could then enter the Kennedy pathway. It also supports the hypothesis that a significant portion of lysoPtdCho-DHA might be transported intact across the BBB and that this passage likely would require a transporter such as Mfsd2a as suggested by other investigators [22, 23] (Fig. 3). However, we detected radioactivity in the brain diacylglycerol, NE-FA and triacylglycerol combined pool of the lysoPtdCho-^{14}C-DHA infused rats suggesting that some lysoPtdCho-DHA is deacylated within five mins (data not shown). On the other hand, NE-^{14}C-DHA mostly went through the Kennedy pathway or was associated with lysoPtdCho or lysoPtdEtn to form PtdCho and PtdEtn (Fig. 3), but the relative contribution of either of these pathways cannot be evaluated here.

The results reported here for brain radioactivity after infusion of NE-^{14}C-DHA are similar to previous reports [11, 24], but there are some differences with regards to the results for lysoPtdCho-^{14}C-DHA [24]. Thies et al. reported that 2.5 mins after a single bolus iv infusion of lysoPtdCho-^{14}C-DHA in rats, radioactivity was similar between the EtnGpl pool and the ChoGpl pool, but that EtnGpl was the main radioactive pool 30 mins after the infusion [24]. These results support our hypothesis that lysoPtdCho-DHA preferentially incorporates into PtdCho when compared to NE-DHA, but that reacylation from PtdCho to PtdEtn might be a significant contributor to PtdCho turnover. However, in our previous report related to this study, radioactivity in the lysoPtdCho pool represented only 31% of plasma total lipid radioactivity 30 mins after iv bolus infusion of lysoPtdCho-^{14}C-DHA [9]. This suggests that at that time point, a significant proportion of

brain exposure to plasma DHA is not from the lysoPtdCho-DHA pool and thus comparisons between iv bolus and the five mins iv protocol performed here are difficult to make.

The transporter-mediated passage of lysoPtdCho-DHA across the BBB might be an important contributor to brain choline enrichment. Most choline transport across the BBB is believed to be through facilitated diffusion of free choline although specific transporters with various affinities have been identified [25-27]. The J_{in} of free choline has been estimated by at least two methods; *in situ* rat brain perfusions [27] or the Braun method *in vivo* [28-31]. From these studies, we estimate that under normal physiological plasma concentrations (10 μM), the free choline J_{in} would be 300-600 nmol/g of brain/d [27, 29-31]. Here, we observed that the J_{in} for lysoPtdCho-DHA into brain total PLs is about 8 nmol/g of brain/d, but only approximately 1% of plasma lysoPtdCho is acylated with DHA [32, 33] and thus uptake of total lysoPtdCho could be a significant contributor to brain choline enrichment. This is based on the assumption that the vast majority of choline within lysoPtdCho enters the brain with lysoPtdCho and this is supported by a previous study [24], but also that all lysoPtdCho species enter the brain. However, it has been reported that lysoPtdCho acylated with monounsaturates and polyunsaturates are more efficient for enriching the brain than lysoPtdCho-palmitate [34] and we believe that this could be due to a higher affinity of Mfsd2a for mono- and polyunsaturates than for saturates [35]. Moreover, we need to be cautious with the comparison between the lysoPtdCho-DHA J_{in} reported here vs the choline J_{in} reported by the other investigators [27, 29, 31] since the methods used to quantify J_{in} are not the same between the studies. Nonetheless, the question of whether the lysoPtdCho pool is a significant contributor to

brain choline uptake merits further investigation because this could be important for developing therapies for patients with *MFSD2A* mutations [35, 36].

Both DHA forms were found to significantly enrich the brain PtdIns pool; radioactivty in the PtdIns represented approximately 13% of total PL radioactivity in both groups. PtdIns is synthesised with diacylglycerol as a substrate in a one-step process [37] (Fig. 5). Interestingly, PtdIns is the smallest pool of brain PLs, representing only 5% of total PL FA concentrations and <3% of DHA concentration and this is in accordance to previously published reports in both humans [6] and rats [10, 17]. PtdIns is an important signaling molecule as it serves, notably, as a substrate for phophatidylinositol bis- and trisphosphates. These molecules are crucial in the regulation of cell growth and survival through, in part, the PI3K-PKB/Akt pathway [38]. Therefore, PtdIns turnover might be more rapid than other phospholipids and most DHA-containing PtdIns detected after the five mins iv infusion could serve as substrates to enter the PI3K-PKB/Akt pathway. A previous study reported that the DHA half-life in the PtdIns and ChoGpl pools is 27.3% and 30.3% shorter, respectively, than in the total PL pool [39]. This could explain the apparent discrepancy between the results we report here for radioactivity into the PtdIns and ChoGpl pools vs the DHA concentration within these pools. Moreover, previous studies report similar results to what is reported here with regards to the relative distribution of k^* and DHA in the PtdIns and ChoGpl pools compared to the other PL pools five mins after iv infusion with NE-^{14}C-DHA [17, 40].

In this study, the smallest PL pool of radioactivity was PtdSer (3-5% of total PL radioactivity) whereas this pool was the second most enriched with DHA. DHA-containing PtdSer are believed to be critical for cell survival and differentiation in

addition to regulating the PI3K-PKB/Akt pathway [7, 8]. However, in humans and animals, PtdSer cannot be synthesised *de novo*; it is formed by base exchange with either PtdEtn or PtdCho [41]. Therefore, the turnover of DHA in the PtdSer pool might be longer than in other PL pools as previously suggested [11, 39] and this would explain why low levels of radioactivity were detected in PtdSer relative to EtnGpl, ChoGpl and PtdIns after five mins iv infusion of DHA.

In support of the primary study related to this manuscript [9], we report that the lysoPtdCho-^{14}C-DHA infused animals had significantly more brain radioactivity than the NE-^{14}C-DHA infused animals, but this was specific to the PtdIns and ChoGpl pools. These results are mostly attributable to a higher DHA plasma exposure in the lysoPtdCho-^{14}C-DHA group compared to the NE-^{14}C-DHA group. This is attributed to the lysoPtdCho-DHA having a approximately 20-fold longer plasma half-life compared to NE-DHA [9].

In summary, we report that after a five mins iv infusion, lysoPtdCho-^{14}C-DHA preferentially incorporates into ChoGpl and less into EtnGpl compared to NE-^{14}C -DHA. These findings suggest that lysoPtdCho-DHA might selectively enrich certain types of PL within the ChoGpl pool, contributing to brain choline homeostasis. Both forms of DHA were found to significantly enrich the PtdIns pool which was surprising considering the relatively low DHA concentrations within this pool. In the future, it will be relevant to assess whether chronic feeding with PC-DHA could result in similar PL class-specific brain enrichment to what is reported here for lysoPtdCho-DHA.

Conflicts of interest

Dr. R.P. Bazinet holds a Canada Research Chair in Brain Lipid Metabolism and he has received research grants from Bunge Ltd., Arctic Nutrition, the Dairy Farmers of Canada and Nestle Inc., as well as travel support from Mead Johnson and mass spectrometry equipment and support from Sciex. In addition, R.P. Bazinet is on the executive of the International Society for the Study of Fatty Acids and Lipids and held a meeting on behalf of Fatty Acids and Cell Signaling, both of which rely on corporate sponsorship. R.P. Bazinet has given expert testimony in relation to supplements and the brain. R.P. Bazinet also provides complimentary fatty acid analysis for farmers, food producers and others involved in the food industry, some of whom provide free food samples. There are no other conflicts of interest.

Acknowledgments

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Fig. legends

Fig. 1: (a) Whole brain and (b) relative radioactivity in the EtnGpl, PtdIns, PtdSer, CholGpl and CerPCho pools of rats iv infused over five mins with either NE-^{14}C-DHA or lysoPtdCho-^{14}C-DHA (n = 4 per group). Differences between the DHA groups' results were assessed by nonparametric Mann-Whitney tests. * $p < 0.05$. CerPCho, ceramide phosphocholine or sphingomyelin; ChoGpl, choline glycerophospholipid; EtnGpl, ethanolamine glycerophospholipid; lysoPtdCho-^{14}C-DHA, lysophosphatidylcholine docosahexaenoic acid radiolabelled with carbon 14 [1-^{14}C]; NE-^{14}C-DHA, non-esterified docosahexaenoic acid radiolabelled with carbon 14 [1-^{14}C]; PlsCho, plasmenylcholine; PlsEtn, plasmenylethanolamine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phophatidylserine.

Fig. 2: (a) Total plasma radioactivity area under the curve over five mins, (b) DHA uptake coefficient (k^*), and (c) net rate of DHA entry (J_{in}) in the EtnGpl, PtdIns, PtdSer, CholGpl and CerPCho pools of rats iv infused over five mins with either NE-^{14}C-DHA or lysoPtdCho-^{14}C-DHA (n = 4 per group). Differences between the DHA groups' results were assessed by nonparametric Mann-Whitney tests. * $p < 0.05$. CerPCho, ceramide phosphocholine or sphingomyelin; ChoGpl, choline glycerophospholipid; EtnGpl, ethanolamine glycerophospholipid; lysoPtdCho-^{14}C-DHA, lysophosphatidylcholine docosahexaenoic acid radiolabelled with carbon 14 [1-^{14}C]; NE-^{14}C-DHA, non-esterified docosahexaenoic acid radiolabelled with carbon 14 [1-^{14}C]; PlsCho, plasmenylcholine; PlsEtn, plasmenylethanolamine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phophatidylserine.

Fig. 3: Whole brain (a) total FA concentrations and (b) DHA concentrations in the EtnGpl, PtdIns, PtdSer, CholGpl and CerPCho pools of rats iv infused over five mins with either NE-^{14}C-DHA or lysoPtdCho-^{14}C-DHA (n = 4 per group). DHA plasmalogen repartition within the EtnGpl pool and the ChoGpl pool is shown in (c). CerPCho, ceramide phosphocholine or sphingomyelin; ChoGpl, choline glycerophospholipid; EtnGpl, ethanolamine glycerophospholipid; FA, fatty acid; lysoPtdCho-^{14}C-DHA, lysophosphatidylcholine docosahexaenoic acid radiolabelled with carbon 14 [1-^{14}C]; NE-^{14}C-DHA, non-esterified docosahexaenoic acid radiolabelled with carbon 14 [1-^{14}C]; PlsCho, plasmenylcholine; PlsEtn, plasmenylethanolamine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phophatidylserine.

Fig 4: Whole brain radioactivity controlled for (a) DHA concentrations and (b) total FA concentrations in the EtnGpl, PtdIns, PtdSer, CholGpl and CerPCho pools of rats iv infused over five mins with either NE-^{14}C-DHA or LPC-^{14}C-DHA (n = 4 per group). Differences between the DHA groups' results were assessed by nonparametric Mann-Whitney tests. * $p < 0.05$. CerPCho, ceramide phosphocholine or sphingomyelin; ChoGpl, choline glycerophospholipid; EtnGpl, ethanolamine glycerophospholipid; FA, fatty acid; LPC-^{14}C-DHA, lysophosphatidylcholine docosahexaenoic acid radiolabelled with carbon 14 [1-^{14}C]; NE-^{14}C-DHA, non-esterified docosahexaenoic acid radiolabelled with carbon 14 [1-^{14}C]; PlsCho, plasmenylcholine; PlsEtn, plasmenylethanolamine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phophatidylserine.

Fig. 5: Schematic representation of glycerophospholipid biosynthesis within the brain. The proposed lysoPtdCho-DHA pathways follow the red arrows whereas the proposed NE-DHA pathways follow the green arrows. After being transported across the BBB through the Mfsd2a transporter, our results suggest that most of lysoPtdCho-DHA is rapidly incorporated into the PtdCho pool, supporting the hypothesis that a portion of lysoPtdCho-DHA crosses the BBB intact. DHA can be released from lysoPtdCho or from PtdCho to be converted to DHA-CoA and esterified to other PLs, notably to PtdEtn via the Kennedy pathway or to PtdIns via a one-step reaction involving CDP-DGA. NE-DHA is believed to cross the BBB through passive diffusion before being converted to DHA-CoA. Some DHA-CoA is used for DAG formation which is then esterified to either PtdEtn or PtdChol via the Kennedy pathway. The synthesis of PtdSer is performed by base exchange with either PtdEtn or PtdChol via PSS2 or PSS1, respectively. It is important to note that some DHA-CoA can also contribute to the formation of plasmalogens (gold arrow) which represent a significant proportion of brain choline and ethanolamine glycerophospholipids. Moreover, there are quantifiable concentrations of lysoPtdOH, lysoPtdCho, lysoPtdEtn, lysoPtdIns and lysoPtdSer inside the brain which can serve as substrates for the synthesis of PtdOH, PtdCho, PtdEtn, PtdIns and PtdSer, respectively (blue arrows). These reactions are catalysed by a family of enzymes called AGPATs but the contribution of these to brain PL synthesis is unknown. AGPATs, 1-acylglycerol-3-phosphate-O-acyltransferases; BBB, blood-brain barrier; CDP, cytidine diphosphate; CerPCho, ceramide phosphocholine or sphingomyelin; CoA, coenzyme A; DAG, diacylglycerol; DHA, docosahexaenoic acid; Ins, inositol; LPLATs, lysophospholipid acyltransferases; LysoPtdCho, lysophosphatidylcholine; LysoPtdEtn,

lysophosphatidylethanolamine; LysoPtdIns, lysophophatidylinositol; LysoPtdOH, lysophosphatidic acid; LysoPtdSer, lysophosphatidylserine; Mfsd2a, major facilitator superfamily domain-containing protein 2; NE, non-esterified; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; PSS, phosphatidylserine synthase; TAG, triacylglycerol.

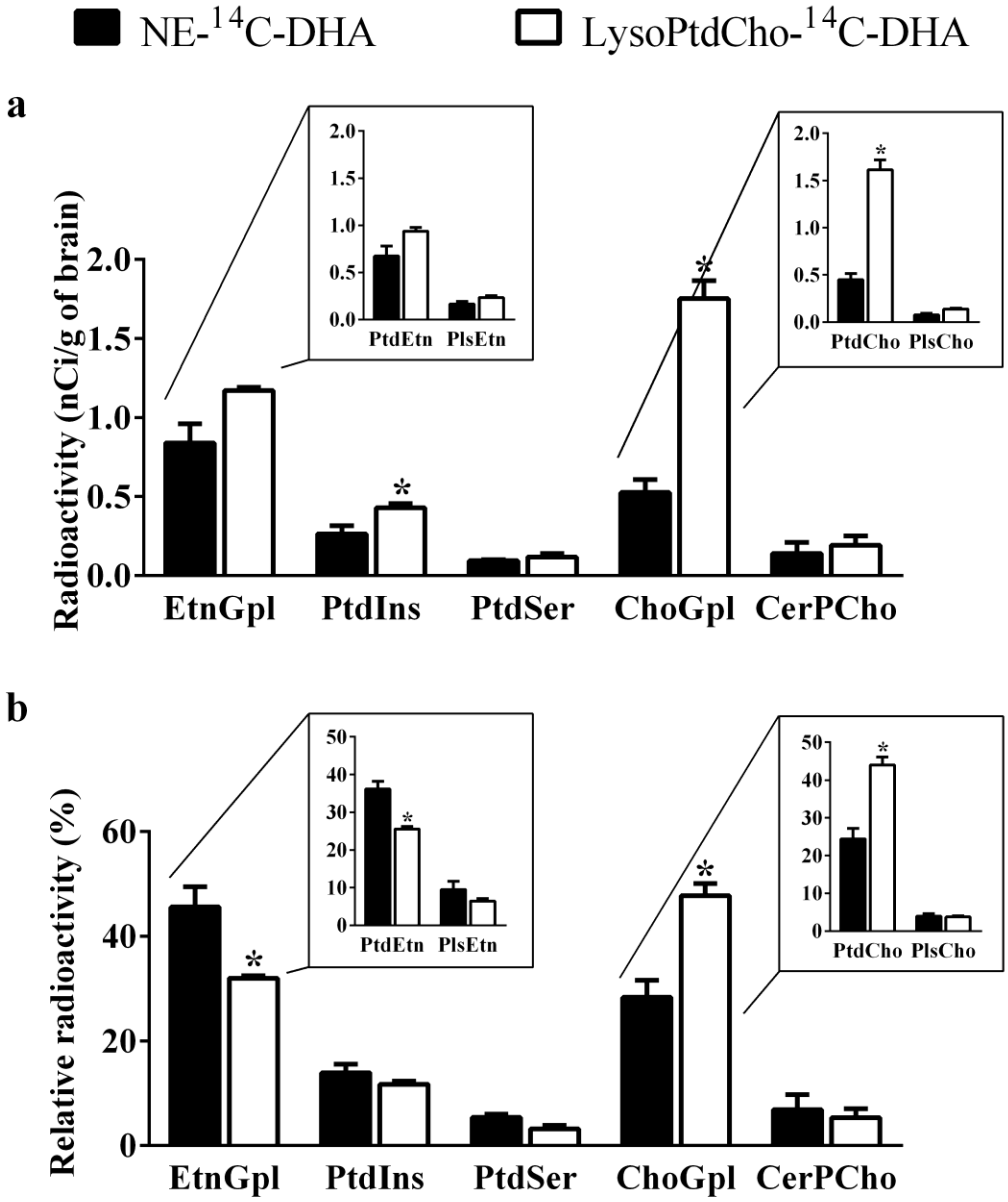

Figure 1

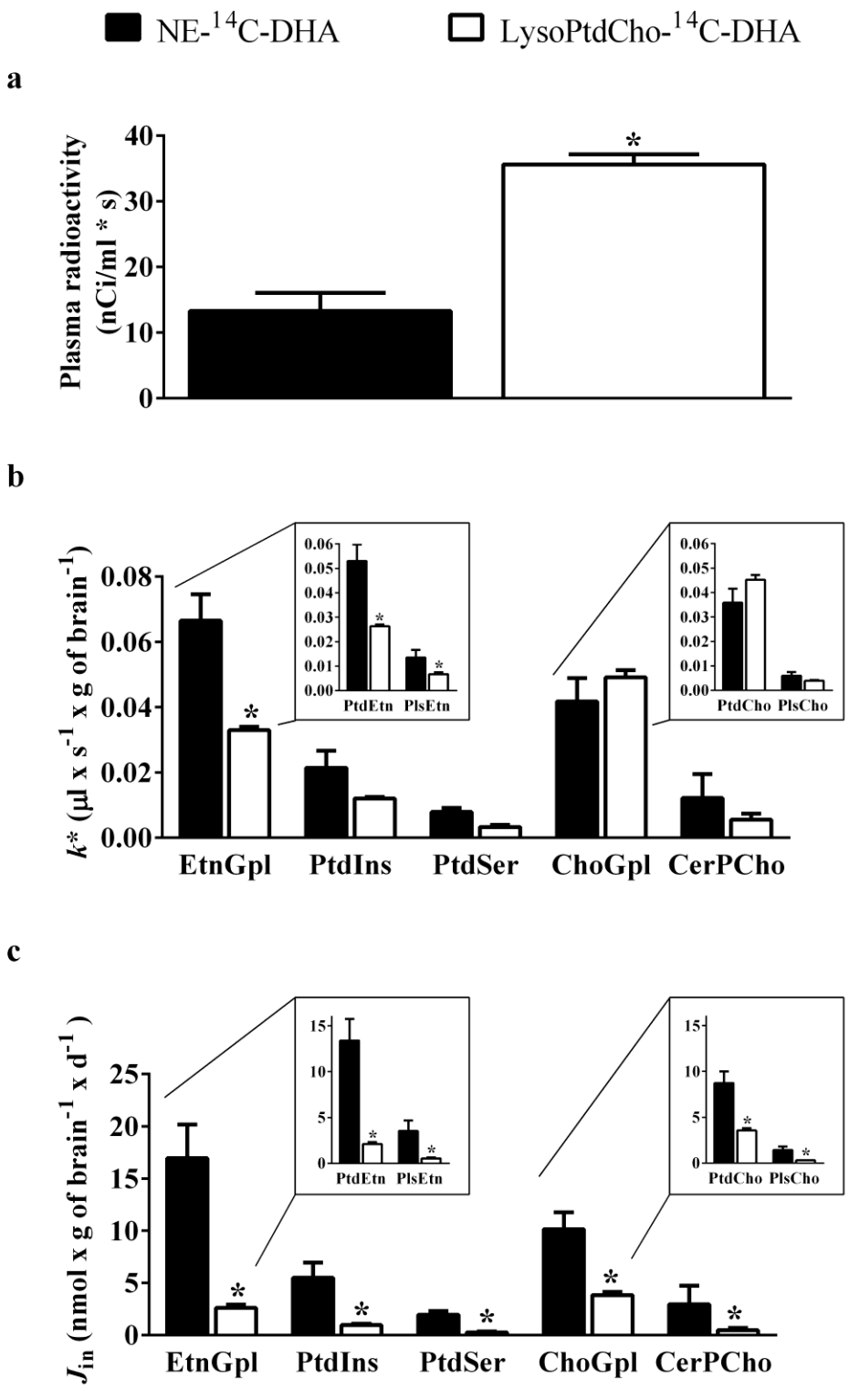

Figure 2

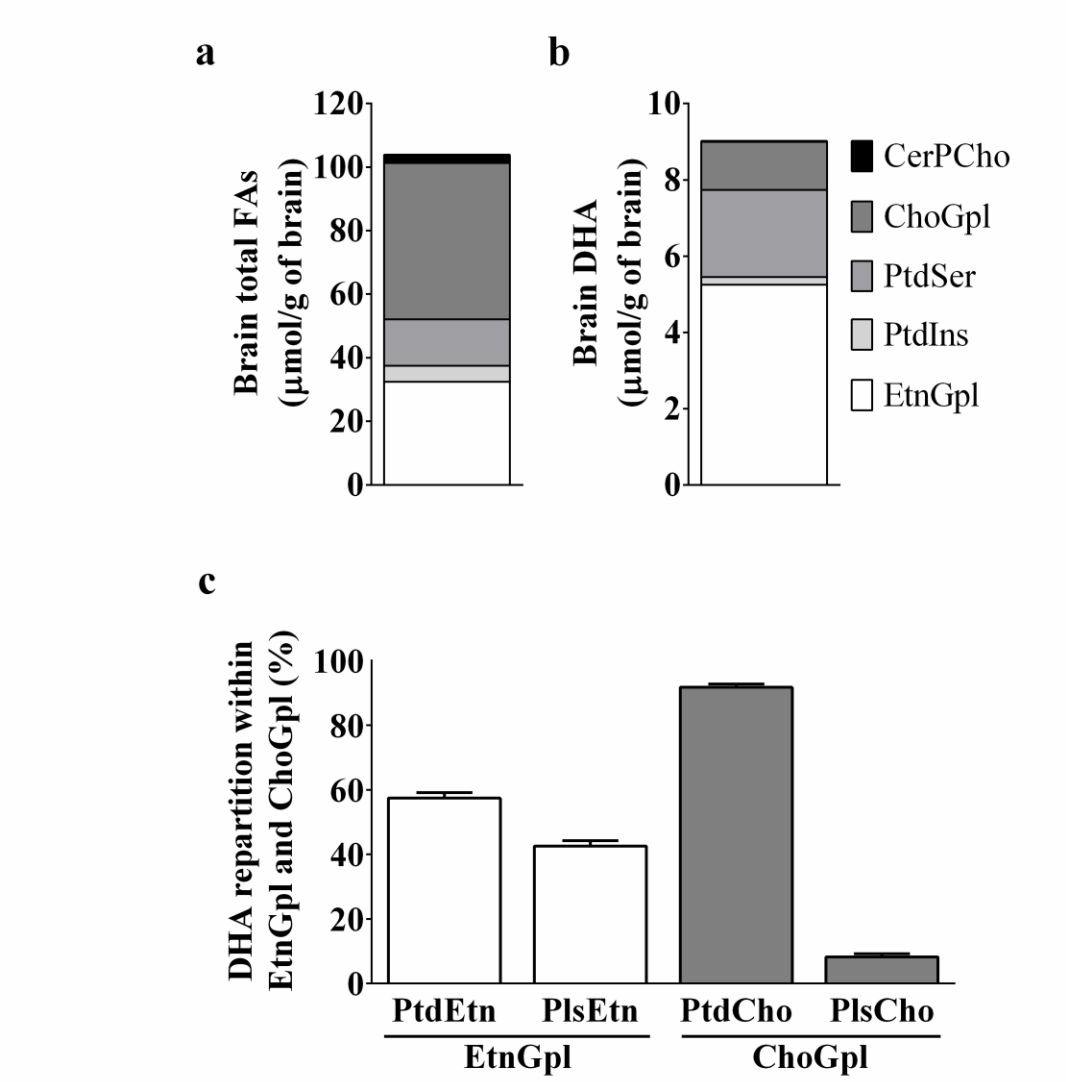

Figure 3

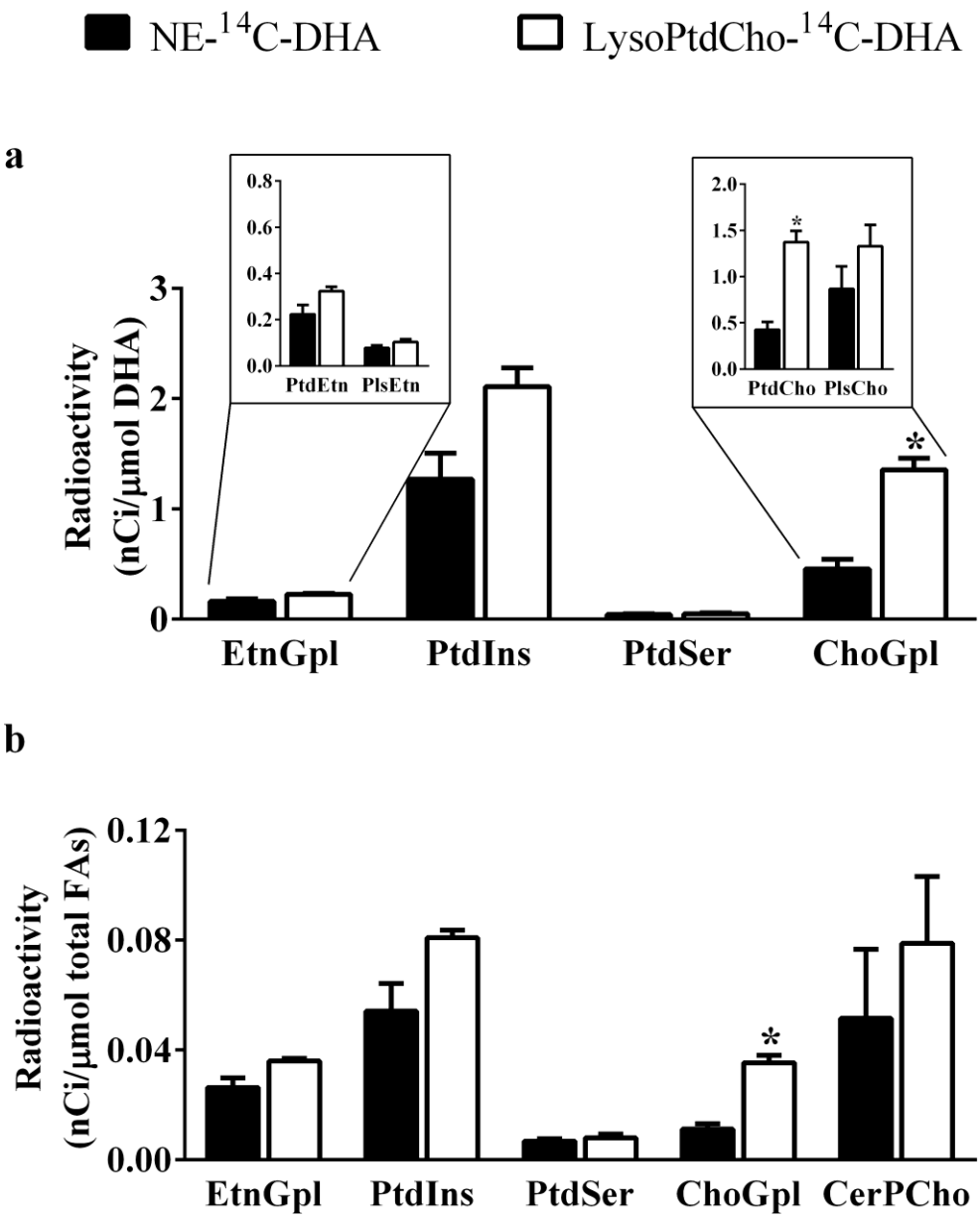

Figure 4

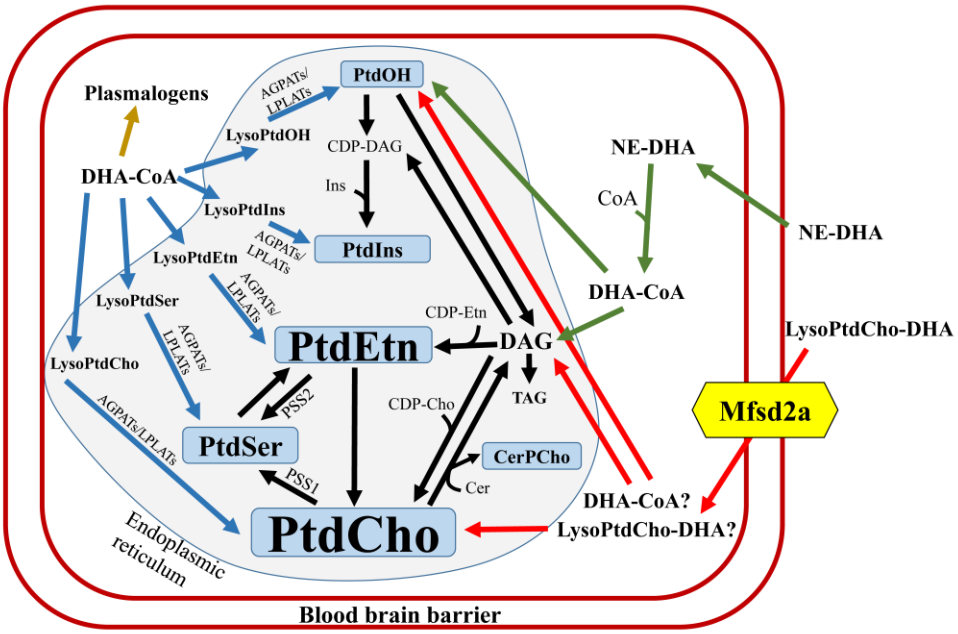

Figure 5

Highlights

- LysoPtdCho-DHA selectively enriches brain cholineglycerophospholipids.
- Uptake of lysoPtdCho-DHA into the brain might contribute to brain choline enrichment.
- The net rate of DHA entry into the brain from the plasma lysoPtdCho pool is lower than from the NE pool.
- Brain radioactivity from lysoPtdCho-^{14}C-DHA infused rats is higher than from NE-^{14}C-DHA infused rats.