Brain Uptake and Metabolism of Eicosapentaenoic Acid in Rodents

by

Tzu-Huan Chuck Chen

A thesis submitted in conformity with the requirements for the degree of Doctoral of Philosophy
Department of Nutritional Sciences
University of Toronto

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Abstract

Omega-3 polyunsaturated fatty acids (n-3 PUFA) are currently being investigated as a potential therapy in mood disorders including depressive disorder and bipolar disorder. Clinical trials that used relatively pure EPA were more effective in alleviating depressive symptoms compared to relatively pure DHA supplementations. However, this poses a potential paradox as EPA levels in the brain are 250 to 300-fold lower than DHA, a finding consistent across mammals. The large difference in brain EPA and DHA levels may be explained by either selective differences in their uptake or metabolism upon entry into the brain. Based on the literature, EPA and DHA can passively diffuse across the blood-brain barrier at similar rate suggesting that the uptake of n-3 PUFA into the brain is non-selective. Therefore, I hypothesize that the difference between EPA and DHA concentrations might be explained by metabolic differences which are yet to be elucidated.

First, we characterized the difference in EPA and DHA $\beta$-oxidation via $\textit{in situ}$ cerebral perfusion. We found that EPA was 2.5-fold more readily $\beta$-oxidized than DHA. Upon $\textit{in vivo}$ kinetic modeling in the rat, we observed a 3-fold lower esterification and 154-fold lower recycling of EPA in brain phospholipids as compared to DHA. This corresponded to a 7-fold increase in EPA
loss from brain phospholipids compared to DHA in the rat brain. Moreover, upon inhibiting mitochondrial fatty acid β-oxidation, we observed a further reduction in EPA recycling and increased EPA elongation to n-3 docosapentaenoic acid (n-3 DPA) suggesting multiple redundant pathways are present to maintain low levels of EPA in the brain.

In conclusion, the low levels of EPA in brain phospholipids, as compared to DHA, are maintained by increased β-oxidation, decreased esterification, increased conversion to n-3 DPA, decreased recycling and increased loss.
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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgments</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>xi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xvi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xvii</td>
</tr>
<tr>
<td>List of Equations</td>
<td>xix</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xx</td>
</tr>
<tr>
<td>Chapter 1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1 Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Chapter 2 Literature Review</td>
<td>5</td>
</tr>
<tr>
<td>2 Brain lipids</td>
<td>7</td>
</tr>
<tr>
<td>2.1 Brain fatty acid composition</td>
<td>7</td>
</tr>
<tr>
<td>2.1.1 EPA concentrations in post-mortem brain samples</td>
<td>7</td>
</tr>
<tr>
<td>2.2 Brain fatty acid uptake</td>
<td>9</td>
</tr>
<tr>
<td>2.2.1 Edmond’s theory: receptor-mediated uptake</td>
<td>9</td>
</tr>
<tr>
<td>2.2.2 Hamilton’s theory: albumin-bound unesterified PUFA uptake</td>
<td>10</td>
</tr>
<tr>
<td>2.2.3 Lagarde’s Theory: albumin-bound lysophosphatidylcholine uptake</td>
<td>10</td>
</tr>
<tr>
<td>2.2.4 Eckel’s theory: lipoprotein lipase-mediated uptake</td>
<td>11</td>
</tr>
<tr>
<td>2.3 Brain fatty acid metabolism</td>
<td>13</td>
</tr>
<tr>
<td>2.3.1 Fatty acid esterification</td>
<td>14</td>
</tr>
<tr>
<td>2.3.2 Fatty acid β-oxidation</td>
<td>14</td>
</tr>
<tr>
<td>2.3.3 Fatty acid elongation and desaturation</td>
<td>17</td>
</tr>
<tr>
<td>2.3.4 Lipid mediator synthesis</td>
<td>19</td>
</tr>
<tr>
<td>2.4 EPA and mood disorders</td>
<td>20</td>
</tr>
</tbody>
</table>
2.5 Hypothesis and Objectives ........................................... 21

Chapter 3 Rapid β-oxidation of eicosapentaenoic acid in mouse brain: an *in situ* study ........... 22

3 Objective 1: *in situ* cerebral perfusion ................................................................. 23

3.1 Abstract .................................................................................................................. 23

3.2 Introduction ............................................................................................................. 23

3.3 Materials and Methods ....................................................................................... 25

3.3.1 Animals ............................................................................................................ 25

3.3.2 *In situ* cerebral perfusion ............................................................................... 26

3.3.3 Brain lipid extraction and FAME preparation ................................................. 27

3.3.4 Liquid scintillation counting and volume of distribution ............................. 28

3.3.5 High-performance liquid chromatography (HPLC) ........................................ 28

3.3.6 Gas chromatography-flame ionization detection (GC-FID) ......................... 29

3.3.7 Gas chromatography-mass spectrometry (GC-MS) ...................................... 29

3.3.8 Statistics .......................................................................................................... 29

3.4 Results .................................................................................................................. 30

3.4.1 Brain weight and fatty acid concentration ..................................................... 30

3.4.2 *V*₀ in aqueous, total lipid, neural lipids and phospholipid fractions ............ 30

3.4.3 Radioactivity identification of fatty acids in total phospholipid ...................... 33

3.5 Discussion ............................................................................................................ 35

Chapter 4 The low levels of eicosapentaenoic acid in rat brain phospholipids are maintained via multiple redundant mechanisms ................................................................. 38

4 Objective 2 and 3: *in vivo* intravenous infusion .................................................. 39

4.1 Abstract .................................................................................................................. 39

4.2 Introduction ............................................................................................................ 39

4.3 Methods and Materials ....................................................................................... 42

4.3.1 Animals .......................................................................................................... 42
6.4 Results ............................................................................................................................. 101
  6.4.1 Brain lipidomics of microwave fixed and CO2 asphyxiated rats ....................... 101
  6.4.2 Brain lipidomics of vehicle-injected and MEP-treated rats .............................. 103
6.5 Discussion ....................................................................................................................... 105

Chapter 7 General Discussion .............................................................................................. 107

7 Discussion ............................................................................................................................. 108
  7.1 Overall findings .............................................................................................................. 108
    7.1.1 Biological significance ........................................................................................ 108
    7.1.2 Clinical implication ............................................................................................. 110
  7.2 Limitations ...................................................................................................................... 111
  7.3 Future research ............................................................................................................. 111
  7.4 Conclusion ...................................................................................................................... 112

References ................................................................................................................................... 113
List of Tables

Table 3.4-1. $V_d$ of various fractions of right cerebral hemisphere lipid extract obtained from $^{14}$C-DHA perfused brains and $^{14}$C-EPA perfused brains. ........................................ 32

Table 4.4-1. Brain fatty acid concentrations of unesterified fatty acid (n = 8 per treatment), acyl-CoA (n = 8 per treatment) and total phospholipids (total PL; n = 10-11 per treatment) pools. 55

Table 4.4-2. Brain fatty acid concentrations of four major phospholipid classes (n = 10-11 per treatment). ................................................................................................................ 56

Table 4.4-3. Kinetic parameters in rat brain total phospholipids (total PL) and four major phospholipid classes. ........................................................................................................ 67

Table 5.4-1. Baseline concentration of brain phospholipid fatty acids (n = 7). ......................... 85

Table 5.4-2. Palmitate and EPA kinetic parameters in rat brain total phospholipids and phospholipid fractions. .................................................................................................... 93
List of Figures

Figure 2.2-1. Proposed theory of PUFA uptake................................................................. 12

Figure 2.3-1. Mitochondrial and peroxisomal β-oxidation.............................................. 16

Figure 2.3-2. Conversion of n-6 and n-3 PUFA............................................................. 18

Figure 3.4-1. Volume of distribution (Vd) of aqueous fraction........................................ 31

Figure 3.4-2. HPLC separation and identification (in situ cerebral perfusion)................. 34

Figure 4.3-1. EPA-CoA mass spectra.............................................................................. 47

Figure 4.4-1. HPLC separation and identification (intravenous infusion)........................ 59

Figure 4.4-2. Radioactivity of the aqueous (AQ) and organic fractions including total phospholipids (total PL) and four major phospholipid fractions (n = 3-4) upon HPLC adjustment. ................................................................................................................. 63

Figure 4.4-3. Kinetic summary of palmitate, DHA and EPA in brain total phospholipids for vehicle (black) and MEP-treated (grey) rats.................................................................................................................. 64

Figure 5.4-1. Identification and estimation of baseline unesterified EPA concentration in the brain. ......................................................................................................................................................... 86

Figure 5.4-2. HPLC separation and identification in perfusate and brain total phospholipids (intracerebroventricular infusion). .................................................................................................................. 89

Figure 5.4-3. HPLC separation and identification in four major phospholipid classes (intracerebroventricular infusion). ...................................................................................................................... 90

Figure 5.4-4. Linear regression analysis of log10Radioactivity (nCi/brain) and days post-infusion. ..................................................................................................................................................... 92

Figure 6.4-1. ARA-derived eicosanoid profiles of rat brains fixed with high-energy, head-focused microwave irradiation (white; n = 3) and CO2 asphyxiation (black; n = 3).............. 102
Figure 6.4-2. Eicosanoid and docosanoid profiles with vehicle-injection (white; n = 5) or MEP-treatment (black; n = 6).
List of Equations

**Equation 3-1.** Volume of distribution......................................................................................................................... 28

**Equation 4-1.** Incorporation coefficient of radiotracers. .......................................................................................... 49

**Equation 4-2.** Incorporation of coefficient of elongated/desaturated radiotracers. .................................................. 50

**Equation 4-3.** Rate of incorporation of non-radiolabeled plasma fatty acids........................................................... 50

**Equation 4-4.** Rate of incorporation of elongated/desaturated products. ................................................................. 50

**Equation 4-5.** Dilution factor........................................................................................................................................... 50

**Equation 4-6.** Rate of incorporation of non-radiolabeled brain acyl-CoA................................................................. 51

**Equation 4-7.** Rate of incorporation of elongated/desaturated CoA products............................................................ 51

**Equation 4-8.** Rate of turnover of non-radiolabeled phospholipid fatty acids............................................................ 51

**Equation 4-9.** Rate of turnover of elongated/desaturated phospholipid fatty acids.................................................... 51

**Equation 4-10.** Half-life of non-radiolabeled phospholipid fatty acids................................................................. 52

**Equation 4-11.** Half-life of elongated/desaturated phospholipid fatty acids.............................................................. 52

**Equation 5-1.** Loss half-life of non-radiolabeled phospholipid fatty acids............................................................... 83

**Equation 5-2.** Rate of loss of non-radiolabeled phospholipid fatty acids............................................................... 83

**Equation 5-3.** Fractional loss of non-radiolabeled phospholipid fatty acids............................................................ 83
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACSL</td>
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<td>G-protein coupled receptor</td>
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<td>omega-3 polyunsaturated fatty acids</td>
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<td>PFC</td>
<td>prefrontal cortex</td>
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<tr>
<td>Acronym</td>
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<tr>
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<td>prostaglandin</td>
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<td>very low-density lipoprotein receptor</td>
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Chapter 1
Introduction
1 Introduction

For 50 years, it has been recognized that the brain possesses a unique polyunsaturated fatty acid (PUFA) composition where high levels of arachidonic acid (ARA; 20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3) are found in brain phospholipids and low levels of other PUFA (O'Brien et al. 1964; Svennerholm 1964; Svennerholm 1968; Igarashi et al. 2010; Rahman et al. 2010; Chen et al. 2011). One particular striking difference is the levels of eicosapentaenoic acid (EPA; 20:5n-3) and DHA in brain phospholipids. Across mammals, EPA is 250 to 300-fold lower than DHA in brain phospholipids (Chen et al. 2008b; Igarashi et al. 2010; Chen et al. 2011), even though both are commonly consumed marine sources of omega-3 PUFA (n-3 PUFA). There are two theories to explain the difference between DHA and EPA: 1) via selective difference in uptake of n-3 PUFA into the brain and/or 2) via metabolic differences within the brain upon entry. Recently, it was found through an in situ competition assay (Ouellet et al. 2009) and in vivo kinetic modeling of linoleic acid (LA; 18:2n-6) (DeMar et al. 2006), α-linolenic acid (ALA; 18:3n-3) (DeMar et al. 2005), arachidonic acid (20:4n-6) (Washizaki et al. 1994) and DHA (Bazinet et al. 2005; Bazinet et al. 2006a) that the brain takes up albumin-bound unesterified PUFA at similar rates via rapid passive diffusion; thus, it is unlikely that uptake explains the selective difference in the levels of DHA and EPA in brain phospholipid. Hence, the differences in DHA and EPA concentration may be due to metabolic differences. However, to-date, the metabolism of EPA in the brain remains unclear.

The goal of this thesis is to elucidate the metabolism of EPA and to compare various metabolic differences between DHA and EPA including phospholipid esterification, mitochondrial β-oxidation, fatty acid recycling, fatty acid loss from phospholipids and lipid mediator synthesis. Since the concentration of DHA and EPA is similar between human and rodents, kinetic modeling of EPA metabolism in rodent models can provide a better understanding of the unique regulation of PUFA in the brain as well as provide insight to potential targets of EPA that may play a beneficial role in mood disorders.

Chapter 2 is a comprehensive literature review that summarizes the history and the advancements in fatty acid uptake and metabolism in the brain and outlines the rationale for my thesis research. Section 2.1 describes the unique fatty acid composition of brain phospholipids as
well as the general phospholipid distribution in neural membrane. Section 2.2 introduces four theories of fatty acid uptake including: 1) Edmond’s theory on lipoprotein receptor-mediated uptake, 2) Hamilton’s theory on albumin-bound unesterified PUFA uptake, 3) Lagarde’s theory on albumin-bound DHA containing lysophosphatidylcholine uptake and 4) Eckel’s theory on lipoprotein lipase-mediated PUFA uptake. Despite supporting evidence for each theory, previous reports in the literature in addition to studies conducted by our laboratory suggested that Hamilton’s theory of PUFA uptake may be the primary uptake mechanism utilized in the adult brain. Section 2.3 describes various metabolic fates of PUFA upon entry into the brain including fatty acid esterification into brain phospholipid, mitochondrial fatty acid β-oxidation, n-3 PUFA elongation and desaturation and lipid mediator synthesis. The metabolic fate of EPA in each of these processes are investigated in this thesis (Chapter 3-6) as potential explanations to the large differences in brain concentrations of DHA and EPA.

Chapter 3-5 are adapted from published studies and contain all thesis experimentations. Chapter 3 investigated the differences in the fatty acid β-oxidation of DHA and EPA using 14C-radiotracers. Upon 40 seconds of in situ cerebral perfusion, 14C-EPA β-oxidation was found to be 2.5-fold higher as compared to 14C-DHA. While the study concluded that differences in β-oxidation may, in part, explain the difference in brain phospholipid levels, there is a notably big difference in the fold change in β-oxidation and brain phospholipid levels. This may be due to limited oxygen consumption in the in situ system that could result in global down-regulation of fatty acid metabolism. Therefore, free-living, anaesthetic-free, in vivo rodent models were developed to characterize kinetic differences in PUFA metabolism.

Chapter 4 investigated the differences in esterification and recycling of PUFA in vivo. Upon a five-minute 14C-radiotracer intravenous infusion, we found that there was no significant difference in the uptake of DHA and EPA. However, EPA esterification to brain phospholipids was 3-fold lower as compared to DHA. The largest difference in metabolism of PUFA was recycling where EPA recycling was 154-fold lower as compared to DHA. This complements finding from Chapter 5, which investigated loss of PUFA from phospholipid membrane, demonstrating that EPA is de-esterified 7 times faster as compared to phospholipid-bound DHA. In addition to kinetic modeling, Chapter 4 also investigated the necessity of mitochondrial β-oxidation for maintaining low levels of EPA in brain phospholipids. Upon inhibition of β-oxidation via methyl palmoxirate, EPA recycling was significantly reduced while DHA
metabolism was unaffected. Furthermore, elongation of EPA into n-3 docosapentaenoic acid (n-3 DPA; 22:5n-3) significantly increased upon inhibition of β-oxidation. This suggested that the brain possesses other redundant pathways to maintain low levels of EPA in brain phospholipids in case of compromised fatty acid β-oxidation.

Increases in unesterified EPA may be beneficial as it increases available precursors for the synthesis of lipid mediators which may be the key modulators in the alleviation of depressive symptoms. Therefore, Chapter 6, adapted from an accepted study, investigated the modulation of lipid mediator synthesis in rats with reduced brain fatty acid β-oxidation. Contrary to the hypothesis, increased unesterified EPA levels was coupled with reduced levels of EPA-derived eicosanoids. Specifically, the effect was on the production of non-enzymatic auto-oxidative EPA products which implied a reduction in oxidative stress.

Chapter 7 provides an overall summary of the findings in the context of biological significance and clinical implications to prevent repetition of section specific discussions. To conclude, research limitations and future directions are discussed.
Chapter 2
Literature Review
2 Brain lipids

2.1 Brain fatty acid composition

For 50 years, it has been recognized that the brain possess a unique composition of fatty acids that is conserved across mammals (O'Brien et al. 1964; Svennerholm 1964; Svennerholm 1968; Igarashi et al. 2010; Rahman et al. 2010; Chen et al. 2011). In contrast to other tissues, the brain selectively accretes arachidonic acid (ARA; 20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3) while maintaining low levels of other PUFA such as linoleic acid (LA; 18:2n-6), α-linolenic acid (ALA; 18:3n-3) and eicosapentaenoic acid (EPA; 20:5n-3) (Stark et al. 2007). This explains a 250-300-fold difference in EPA and DHA levels in brain phospholipids compared to 4-, 5-, 14- and 86-fold differences of EPA and DHA levels in plasma, erythrocyte, liver and heart, respectively (Kitson et al. 2012). Furthermore, the distribution of ARA and DHA among various phospholipids are also consistent within and between species. A large portion of ARA and DHA are esterified to ethanolamine glycerophospholipids (EtnGpl) where they accounts for 40% of total EtnGpl fatty acids (O'Brien et al. 1964; Svennerholm 1968). In contrast, ARA and DHA in choline glycerophospholipids (ChoGpl) only account for 2-8% of total fatty acids. Selective esterification of ARA and DHA to phospholipids involved in signaling cascades are also apparent. With regards to phosphatidylserine (PtdSer), the predominant esterified PUFA is DHA which accounts for 15-30% of total fatty acids; whereas ARA is the main esterified PUFA in phosphatidylinositol (PtdIns) at approximately 30% of total fatty acids (O'Brien et al. 1964; Svennerholm 1968). Lastly, in all major phospholipid classes, LA and ALA are detected, but in trace quantities while EPA is often non-detectable in the brain (O'Brien et al. 1964; Svennerholm 1968; Chen et al. 2008b).

2.1.1 EPA concentrations in post-mortem brain samples

Similar to control subjects, the level of EPA in brain phospholipids of depressed and bipolar patients are uniformly low in multiple areas of the frontal cortex. In Brodmann’s Area 10 (BA10) of the orbitofrontal cortex, EPA levels were low to undetectable in brain phospholipids (McNamara et al. 2007; McNamara et al. 2008). Furthermore, in BA11 of the orbitofrontal
cortex of patient diagnosed with depressive disorder, EPA composed 0.03% of total phospholipid fatty acid and was not significantly different from control subjects (Lalovic et al. 2007). The finding was reiterated in the ventral prefrontal cortex (BA47) (Lalovic et al. 2007). Similar findings were observed in the BA9 where EPA levels in total and phospholipid classes were not significantly different between bipolar patients (101 nmol/g brain) and control subjects (122 nmol/g brain) (Igarashi et al. 2010).

In the frontal cortex of schizophrenic patients, EPA levels in three major phospholipid classes (EtnGpl, PtdIns and PtdSer) ranged between 316 to 633 nmol/g brain of the respective total phospholipid fatty acids; while EPA in ChoGpl was undetectable (Horrobin et al. 1991). There were no significant differences in the EPA levels of frontal cortex between schizophrenic patients and control subjects. In the prefrontal cortex of the schizophrenic brain, EPA percent composition in all four major phospholipid classes were relatively lower accounting for 0.1% of their respective phospholipid fatty acids at concentrations of 24, 20, 2 and 7 nmol/g brain in EtnGpl, ChoGpl, PtdIns and PtdSer, respectively (Taha et al. 2013). Contrary to Horrobin’s earlier report, the control subjects had variable levels of EPA accounting for 0.2% (36 nmol/g brain), 0.04% (9 nmol/g brain), 0.1% (3 nmol/g brain) and 0.03% (3 nmol/g brain) in EtnGpl, ChoGpl, PtdIns and PtdSer, respectively (Horrobin et al. 1991; Taha et al. 2013). No significant differences were detected between EPA levels in EtnGpl, ChoGpl and PtdIns, but EPA level of PtdSer was 2.3-fold higher (Taha et al. 2013). In the cerebellar cortex of schizophrenic patients, EPA was undetected in EtnGpl and ChoGpl; whereas in the PtdIns and PtdSer, EPA levels were detected at 633 and 1582 nmol/g brain, respectively (Horrobin et al. 1991). The levels of EPA in cerebellar cortex were not significantly different between schizophrenic patients and control subjects.

To-date, no studies have quantified the levels of EPA in brain phospholipids of patients with Alzheimer’s disease and only two reported the levels of EPA in the neocortex of patients with Parkinson’s disease (PD). In the temporal cortex of PD patient, EPA accounted for 0.09% and was not significantly different from control subjects (Julien et al. 2006). While EPA level were measured in the lipid rafts of frontal cortex from patients with PD and incidental PD, it was reported as negligible (Fabelo et al. 2011).
2.2 Brain fatty acid uptake

Since the *de novo* synthesis of PUFA is very low within the brain (DeMar et al. 2005; DeMar et al. 2006), the blood must supply PUFA to the brain either from exogenous PUFA obtained through diet or from endogenous liver synthesis of PUFA from dietary precursors. However, the mechanism by which PUFA enter the brain remains controversial (Figure 2.2-1).

2.2.1 Edmond’s theory: receptor-mediated uptake

In 2000, John Edmond proposed that lipoprotein receptors transport PUFA to the brain (Edmond 2001). Edmond based his theory on the selective differences in brain PUFA levels and the expression of lipoprotein receptors on endothelial cells of the blood-brain barrier (Meresse et al. 1989; Osono et al. 1995; Turley et al. 1996; Dehouck et al. 1997; Stockinger et al. 1998). The model hypothesized that the binding of circulating lipoprotein particles, most likely low-density lipoprotein (LDL), to the appropriate lipoprotein receptors, LDLr, expressed on the luminal membrane will initiate the endocytosis of a lipoprotein-lipoprotein receptor complex which is hydrolyzed to release PUFA. Subsequently, unesterified PUFA are bound by fatty acid transport proteins (FATP) or the monocarboxylic acid transporter (MCT) for transport across the abluminal membrane into the brain (Edmond 2001) (Figure 2.2-1A).

Therefore, to investigate Edmond’s theory on the importance of LDLr for PUFA uptake, we compared the brain concentration of wild-type mice to that of LDLr knockout (−/−) mice. Since there are regional differences in LDLr expression, cortex (low expression) and brainstem (high expression) were isolated for separate analysis (Hofmann et al. 1987; Lein et al. 2007). No significant differences in the concentrations of all saturated, monounsaturated and PUFA between wild-type and knockout were observed in either region suggesting that LDLr was not necessary in maintaining brain PUFA levels (Chen et al. 2008b). A similar study was repeated with very low-density lipoprotein receptor (VLDLr) knockout mice, and again no significant differences were found in PUFA levels implying that VLDLr was also not necessary for maintaining brain PUFA levels (Rahman et al. 2010).
2.2.2 Hamilton’s theory: albumin-bound unesterified PUFA uptake

In 2001, Hamilton proposed that albumin-bound unesterified PUFA may be the primary source of PUFA uptake by the brain (Hamilton et al. 2001). His model is based on his previous observation that albumin-bound unesterified PUFA may rapidly “flip-flop” across artificial, protein-free, model membranes (Kamp and Hamilton 1992). In other words, he concluded that PUFA can passively diffuse through biological membrane without the active use of transporters. However, *in vivo*, albumin-bound unesterified PUFA may also utilize active transporters such as fatty acid translocase, CD36, to enter the brain (Hamilton and Brunaldi 2007) (Figure 2.2-1C).

Therefore, to test Hamilton’s theory in animal models, two studies were conducted: 1) gene ablation of CD36 and 2) *in situ* uptake to radiolabeled PUFA. Firstly, to identify the importance of CD36 on PUFA uptake, brain PUFA concentrations of wild-type mice were compared against CD36-/- mice. No significant differences were detected in PUFA suggesting that CD36 is not necessary for the maintenance of PUFA levels in brain phospholipids (Song et al. 2010). However, it is possible that the uptake of PUFA may be mediated through unidentified transporters. Hence, to investigate the importance of transporters in the uptake of PUFA, a competition assay in an *in situ* mouse model of uptake was conducted. Mouse brain was co-perfused simultaneously with either 5, 10 or 100 μM of non-radiolabeled DHA/EPA and 0.3 μCi/ml of radiolabeled DHA/EPA for 40 seconds. The study found that increasing the concentrations of non-radiolabeled PUFA did not significantly alter the rate of uptake of radiolabeled PUFA. Furthermore, the rate of uptake between DHA and EPA into the brain were similar implying that PUFA uptake is rapid and most likely via a passive diffusion model as the competition assay exhibited no saturation in the rate of uptake of unesterified PUFA (Ouellet et al. 2009).

2.2.3 Lagarde’s Theory: albumin-bound lysophosphatidylcholine uptake

In the same year as Hamilton, Lagarde also proposed that the brain uses albumin-bound PUFA as the primary source (Lagarde et al. 2001). However, in contrast to Hamilton, Lagarde hypothesized that the brain preferentially takes up an esterified form of PUFA in lysophosphatidylcholine (LPC) rather than an unesterified form (Lagarde et al. 2001) (Figure
In human and rat plasma, 55% of albumin-bound DHA and more than 80% of albumin-bound ARA are esterified in LPC (Croset et al. 2000). The basis of the theory stems from evidence in the developing rat and *in vitro* artificial blood-brain barrier models. In developing rats, Thiès *et al* found that with increasing unsaturation of fatty acids and esterification to LPC, the uptake efficiency of PUFA increased in the brain. Furthermore, in contrast to the liver, kidney and heart which preferred unesterified PUFA, the preferential uptake of LPC-bound PUFA was specific to the brain (Thies *et al* 1992; Thies *et al* 1994). Similar preferential uptake of LPC-bound PUFA was observed in a co-cultured model of blood-brain barrier endothelial cells and astrocytes (Bernoud *et al* 1999). However, this was not elucidated in adult rats, so it is unclear if there are switches in the source of PUFA uptake post development.

**2.2.4 Eckel’s theory: lipoprotein lipase-mediated uptake**

In 2008, Goldberg, Eckel and Abumrad proposed an alternative theory of PUFA uptake that encompasses different aspects of Hamilton’s and Edmond’s theory. The model hypothesized that lipoprotein lipase (LPL) hydrolyzes triacylglycerides from circulating lipoprotein; thereby releasing unesterified PUFA that are internalized by cells via CD36 (Goldberg *et al* 2009) (*Figure 2.2-1B*). As stated previously, CD36 was not necessary for the maintenance of PUFA concentration in the brain; thus the uptake of released PUFA may be via simple passive diffusion (Song *et al* 2010). Recently, this hypothesis was tested via tail vein injection of radiolabeled triolein-containing chylomicrons by Eckel’s group. Wang *et al* observed a selective reduction in the uptake of radiolabeled oleate in the hypothalamus of LPL-/- mice as compared to wild-type mice (Wang *et al* 2011). This suggested that the mechanism of uptake may be region specific. In other words, while the uptake in hypothalamus was LPL-dependent, other brain regions including hippocampus, hindbrain and forebrain may rely on other fatty acid uptake mechanisms.
(A) Edmond’s theory of lipoprotein receptor-mediated PUFA uptake. The binding of low density lipoprotein (LDL) particles to LDL receptor (LDLr) induces endocytosis of LDL particles into the blood-brain barrier where it is hydrolyzed and releases unesterified PUFA. (B) Eckel’s theory of lipoprotein lipase-mediated PUFA uptake. Circulating LDL particles binds to lipoprotein lipase (LPL) where PUFA are de-esterified and taken up into the blood-brain barrier via passive diffusion or transporter, CD36. (C) Hamilton’s theory of albumin-bound unesterified PUFA uptake. Albumin-bound unesterified PUFA passively diffuse across into the blood-brain barrier via a “flip-flop” mechanism or transporter, CD36. (D) Lagarde’s theory of albumin-bound lysophosphatidylcholine (LPC) uptake. Albumin-bound LPC containing PUFA are taken up into blood-brain barrier by passive diffusion. Upon entry into the endothelial cells of blood-brain barrier, unesterified PUFA are bound to fatty acid binding protein (FABP) and shuttled to the brain where fatty acid transport protein (FATP) or monocarboxylic acid transporter (MCT) transport PUFA into the brain. PUFA are then converted to CoA thioesters by acyl-CoA synthetase (ACSL/ACSVL), FATP or bubblegum (BG) and targeted for esterification in brain phospholipids or β-oxidation in peroxisomes/mitochondria.
2.3 Brain fatty acid metabolism

Based on the most current literature, it appears that the uptake of PUFA is likely mediated by simple passive diffusion which, by definition, is non-selective. Since, the 250 to 300-fold differences in brain phospholipid levels of DHA and EPA are not explained by uptake, one potential explanation for this observation may be differences in the metabolism of DHA and EPA upon entry into the brain. Fatty acid binding proteins (FABP) may differentially shuttle PUFA for conversion to CoA thioesters which activates fatty acids for downstream anabolic and catabolic processes (Figure 2.2-1). There are five identified long chain acyl-CoA synthetases (ACSL), but only four, ACSL1-4, are expressed in rat brains (Suzuki et al. 1990; Fujino and Yamamoto 1992; Fujino et al. 1996; Kang et al. 1997). Among the four, ACSL1 has the lowest expression in the brain and is mostly found in liver, heart and adipose tissue (Suzuki et al. 1990). On the other hand, ACSL2 and ACSL3 are predominantly expressed in rat brains (Fujino and Yamamoto 1992; Fujino et al. 1996) and have high affinity for ARA and EPA. However, ACSL2 has a high affinity for DHA as opposed to ACSL3 (Fujino et al. 1996; Iijima et al. 1996). ACSL4 is expressed in the brain and it is also found in the adrenal gland, lung, liver, testes, ovary and epididymis and it exhibits a similar affinity profile for PUFA as ACSL3 (Kang et al. 1997).

Additional to ACSL, fatty acid transport proteins (FATP), which are commonly found in complex with ACSL, have also been found to possess ACSL activity for very long chain fatty acids (VLCFA; greater than C22) including DHA (Coe et al. 1999; Steinberg et al. 1999a; Steinberg et al. 1999b; Herrmann et al. 2001; Pei et al. 2004) (Figure 2.2-1). To date, five murine isoforms and six human isoforms of FATP have been characterized (Schaffer and Lodish 1994; Schaap et al. 1997; Berger et al. 1998; Fitscher et al. 1998; Hirsch et al. 1998; Stuhlsatz-Krouper et al. 1998; Stahl et al. 1999; Watkins et al. 1999). However, only FATP1 and FATP4 are expressed in the brain (Schaffer and Lodish 1994; Fitscher et al. 1998; Hirsch et al. 1998; Stahl et al. 1999). Furthermore, other families of proteins like bubblegum (BG), an AMP binding protein, also have ACSL activity which, in contrast to FATP, have higher affinity for long chain fatty acids (LCFA; C14-C20) including EPA as opposed to very long chain fatty acids (Pei et al. 2003; Fraisl et al. 2004). However, the human ortholog, hsBG, also has high affinity for VLCFA in addition to LCFA (Steinberg et al. 2000).
Furthermore, the subcellular localization of ACSL, FATP and BG also differs (Figure 2.2-1). ACSL2 is localized to the plasma membrane (Iijima et al. 1996); whereas ACSL3 is found in the endoplasmic reticulum (ER) and mitochondria (Pei et al. 2004; Poppelreuther et al. 2012). In contrast to ACSL2 and 3, ACSL4 is the only identified ACSL isoform present in peroxisomes as well as mitochondria (Lewin et al. 2002). While FATP1 is localized to the plasma membrane and vesicles distributed across the cytoplasm (Schaffer and Lodish 1994), FATP4 is found on the apical membrane of ER as well as vesicles located in the proximity of apical membrane (Stahl et al. 1999; Milger et al. 2006). Like FATP, BG is also found in vesicles that are in close proximity to mitochondria (Pei et al. 2003). The subcellular localization of ACSL has implications in the downstream metabolic processing of bound acyl-CoA including esterification in phospholipid membrane, degradation by mitochondrial β-oxidation, elongation/desaturation by ER and peroxisome or synthesis of lipid mediators.

2.3.1 Fatty acid esterification

Phospholipids are essential components to the stability, fluidity, permeability and functioning of neural membranes; hence, phospholipid remodeling and fatty acid recycling are dynamic yet organized (Farooqui et al. 2000). This results in continuous shuttling of fatty acids between phospholipid classes which is crucial for the esterification of PUFA into the sn-2 position of available lysophospholipid glycerol backbone (Sun and MacQuarrie 1989). The cycle of deacylation-reacylation was first described by Lands in 1960 (Lands 1960). This process of deacylation involves the hydrolysis of fatty acid from the sn-2 position of a glycerophospholipid via phospholipase A2 activity. The reacylation of lysophospholipid with fatty acids from acyl-CoA is achieved by acyl-CoA: lysophospholipid acyltransferase (Yamashita et al. 1997). The rapid recycling of fatty acids maintains and regulates the unique brain fatty acid composition.

2.3.2 Fatty acid β-oxidation

Fatty acid β-oxidation can occur in both mitochondria and peroxisomes via four common steps: oxidation, hydration, oxidation and thiolysis (Figure 2.3-1). In the mitochondria, acyl-CoA is
converted to acylcarnitine by carnitine palmitoyltransferase I (CPTI), located in the outer membrane, for uptake. Upon entry to the inner membrane, fatty acids are β-oxidized, sequentially, by very long chain acyl-CoA dehydrogenase and trifunctional protein complex involving enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase (Eaton et al. 1996; Reddy and Hashimoto 2001). In contrast, the peroxisome uptake of fatty acid for β-oxidation is independent of CPTI and degraded by different enzymes including fatty acyl-CoA oxidase, bifunctional protein of enoyl-CoA hydratase and l-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase (Hashimoto 1999; Reddy and Hashimoto 2001). In regards to PUFA β-oxidation, both mitochondria and peroxisomes require 2,4-dienoyl-CoA reductase. However, different isoforms of enoyl-CoA isomerases are required to complete the processing of conjugated double bonds (Hiltunen et al. 1996; Liang et al. 1999; Reddy and Hashimoto 2001).

Although the basic mechanisms are similar between mitochondrial and peroxisomal β-oxidation, in many aspects, the process greatly differs between the two organelles. First, even though both the mitochondria and peroxisome can oxidize LCFA, VLCFA is largely, if not exclusively, β-oxidized by the latter (Lageweg et al. 1991). The absence of VLCFA β-oxidation by mitochondria is likely the result of: 1) low rate of activation of VLCFA due to lack of very long chain ACSL and 2) low rate of acylcarnitine synthesis of VLCFA by CPTI which have higher affinity for ALA, EPA and palmitate than ARA and DHA (Gavino and Gavino 1991; van den Bosch et al. 1992; Reddy and Mannaerts 1994; Uchiyama et al. 1996). Secondly, carnitine is differentially utilized by the organelles. While carnitine is used for the uptake of fatty acid across mitochondrial membrane for degradation, it is solely used by the peroxisomes to export chain-shortened fatty acids to the mitochondria for complete degradation or to the ER microsome for phospholipid synthesis (Reddy and Hashimoto 2001). Lastly, peroxisomes do not possess the machinery necessary to degrade fatty acids into acetyl-CoA and acetyl-CoA into carbon dioxide and water. Therefore, with the lack of TCA cycle, the peroxisome is only capable of partial degradation of fatty acids or chain-shortening; whereas mitochondria can perform complete β-oxidation of fatty acids (Vanhove et al. 1993; Verhoeven et al. 1998).
Mitochondrial and peroxisomal β-oxidation involve four basic steps: oxidation, hydration, oxidation and thiolysis. However, separate enzymatic machineries are utilized where mitochondria completely break down fatty acids into acetyl-CoA and peroxisomes shortens the fatty acids down to eight carbon before shuttling fatty acid to mitochondria for complete β-oxidation. ACSL, long chain acyl-CoA synthetase; ACSVL, very long chain acyl-CoA synthetase; CPT, carnitine palmitoyltransferase; CAT, carnitine acyltransferase.
2.3.3 Fatty acid elongation and desaturation

*De novo* fatty acid synthesis can actively produce saturated fatty acids (SFA) from sequential additions of acetyl-CoA to malonyl-CoA which will terminate at palmitate (16:0) (Smedley and Lubrzynska 1913; Rittenberg and Bloch 1945; Ponticorvo et al. 1949; Williamson and Wakil 1966; Brophy and Vance 1975). While SFA can be further elongated and desaturated into longer chain SFA and monounsaturated fatty acids (MUFA), mammalian tissues cannot synthesize PUFA from SFA or MUFA. However, ARA and DHA can be synthesized separately from C18 PUFA precursors, LA and ALA, respectively (Brenna et al. 2009) (*Figure 2.3-2*). Despite the expression of elongase and Δ6/Δ5-desaturase in the liver, heart and brain (Cho et al. 1999a; Cho et al. 1999b; Leonard et al. 2002), the liver is the primary tissue involved in endogenous ARA and DHA synthesis (Igarashi et al. 2007c). In contrast to the liver, the synthesis of ARA and DHA in rat brains is negligible because upon entry, 67% of LA and 59% of ALA is β-oxidized (DeMar et al. 2005; DeMar et al. 2006).

The first step in ARA and DHA synthesis is the desaturation into γ-linolenic acid (18:3n-6) and stearidonic acid (18:4n-3) by Δ6-desaturase (Voss et al. 1991; Sprecher 2000; Astudillo et al. 2012). Subsequently, these PUFA are elongated to dihomo-γ-linolenic acid (20:3n-6) and eicosatrienoic acid (20:4n-3) which are desaturated by Δ5-desaturase to ARA and EPA, respectively (Sprecher 2000; Astudillo et al. 2012). Following two more elongations, ARA is converted to adrenic acid (22:4n-6) and tetracosatetraenoic acid (24:4n-6); whereas DHA is converted to n-3 DPA and tetracosaapentaenoic acid (24:5n-3) (Sprecher 2000; Astudillo et al. 2012). After the final desaturation by Δ6-desaturase, n-6 tetracopentaenoic acid (24:5n-6) and tetracosahexaenoic acid (24:6n-3) are transferred from ER to peroxisome where a single cycle of β-oxidation will produce n-6 DPA and DHA (Voss et al. 1991). Alternatively, it has been proposed that n-3 DPA can convert directly into DHA via Δ4-desaturase (Brenna 2002). DHA is then preferentially shuttled to ER for esterification. Under n-3 PUFA deprivation, n-6 DPA esterification in phospholipid membrane increases (Orr et al. 2010). Since both desaturases utilizes both n-6 and n-3 PUFA as substrates in addition to multiple substrates within each synthetic step, more EPA is synthesized from ALA than n-3 DPA which exceeds DHA synthesis due to competition (Sprecher 2000).
Figure 2.3-2. Conversion of n-6 and n-3 PUFA.

Linoleic acid and α-linolenic acid can be converted into ARA and DHA via the same pathway. 18-carbon PUFA are converted into longer chain PUFA using Δ6-desaturase, elongase and Δ5-desaturase. Δ6-desaturase and elongase have multiple substrate that can compete for the enzyme (denoted in same colour). The synthesis of 22-carbon PUFA requires one cycle of fatty acid β-oxidation in the peroxisomes.
2.3.4 Lipid mediator synthesis

After deacylation from phospholipids, unesterified ARA, EPA and DHA may be synthesized into eicosanoids (ARA and EPA derived) and docosanoids (DHA derived) which are bioactive lipid mediators that can regulate multiple cellular functions from inter- and intracellular communications to inflammation and resolution (Serhan et al. 2000a; Harizi et al. 2008; Serhan 2010; Orr et al. 2013; Shahabi et al. 2013). There are three major pathways for the synthesis of eicosanoids and docosanoids (Bannenberg and Serhan 2010): 1) cyclooxygenase (COX), 2) lipoxygenase (LOX) and 3) cytochrome P450. There are two COX isozymes, a constitutive form (COX-1) and an inducible form (COX-2) (Rouzer and Marnett 2003; Rouzer and Marnett 2009). Moreover, there are three isozymes of LOX, 5-, 12- and 15-LOX which differs in their positional affinity for double bonds (Dobrian et al. 2011; Haeggstrom and Funk 2011). Lastly, there are several isozymes of cytochrome P450 epoxygenase that can synthesize all regioisomers and enantiomers of ARA and EPA (Spector 2009; Arnold et al. 2010a; Arnold et al. 2010b).

Since ARA and EPA are structurally similar, these pathways can produce eicosanoids with different properties depending on the substrate. Firstly, when ARA undergoes metabolism via COX pathways, classical series 2 prostaglandins, prostacyclin and thromboxane are synthesized in conjunction with appropriate synthases. These COX mediated eicosanoids are largely inflammatory and thrombotic. However, when EPA outcompetes ARA, COX can produce series 3 prostaglandins, prostacyclins and thromboxane which are relatively anti-inflammatory and anti-thrombotic. Secondly, under the LOX-mediated pathway, ARA is converted to hydroperoxyeicosatetraenoic acid (HPETE) which is then converted to hydroxyeicosatetraenoic acid (HETE) and leukotrienes. When EPA is converted by LOX, hydroxyeicosapentaenoic acid (HEPE) are formed. Furthermore, LOX can also convert DHA to hydroxydocosahexaenoic acid (HDHE) that can be a precursor to a group of specialized pro-resolving mediators known as protectins, D-series resolvins and maresins. Lastly, ARA is converted to epoxytrieneoic acid (EET) via cytochrome P450 epoxygenase; while EPA is converted to epoxyeicosatetraenoic acid (EpETE).

In the recent decade, EPA and DHA have also been demonstrated to undergo aspirin-triggered COX metabolism where E-series and D-series resolvins are produced via a series of enzymatic
reactions (Bannenberg and Serhan 2010). Furthermore, in absence of aspirin, DHA can be converted into D-series resolvins via the 15-LOX pathway. Specifically, 17(S)-hydroxydocosahexaenoic acid (HDHA) is converted into protectin D1, which is a potent anti-inflammatory mediator found to be elevated post-neuroinflammation, via 15-LOX. Finally, 12-LOX has been shown to synthesize a distinctive pro-resolving mediator, maresin 1, from 14(S)-HDHA.

2.4 EPA and mood disorders

Mood disorders, including depressive disorder, bipolar disorder, postpartum depression and seasonal affective disorder, are an emerging pandemic (WHO 2012). The World Health Organization predicts that by 2020, depressive disorder will be the leading cause of disability worldwide and the primary contributor to the global burden of disease (WHO 2012). Currently, 79% of the all diagnosed mental illnesses are anxiety and depression (MDSC 2009). In Canada, one in five adults will suffer from the debilitating effects of mood disorders (MDSC 2009). This is alarming as 15% of patients with diagnosed mood disorder commit suicide (MDSC 2009; WHO 2012). While drug therapies are available, more than two-thirds of Canadians with significant depression do not seek proper medical attention due to the stigma of mental illness (MDSC 2009). Therefore, alternative therapies to drugs for the prevention and treatment of mood disorders are often preferred by patients. One of the common alternative nutritional therapies currently being investigated is the consumption of fish or more specifically fish oil containing n-3 PUFA, EPA and DHA.

Even though, epidemiology studies have shown a consistent strong negative correlation between fish/seafood consumption and mood disorders (Hibbeln 1998; Tanskanen et al. 2001; Hibbeln 2002; Noaghiul and Hibbeln 2003; Hakkarainen et al. 2004; Timonen et al. 2004), interventions with fish oil supplementation have yielded inconsistent results (Fenton et al. 2001; Nemets et al. 2002; Peet and Horrobin 2002; Llorente et al. 2003; Marangell et al. 2003; Su et al. 2003; Silvers et al. 2005; Frangou et al. 2006; Keck et al. 2006; Nemets et al. 2006; Grenyer et al. 2007; da Silva et al. 2008; Freeman et al. 2008; Jazayeri et al. 2008; Rees et al. 2008; Rogers et al. 2008; Su et al. 2008; Carney et al. 2009; Doornbos et al. 2009; Mischoulon et al. 2009). One large
difference among randomized controlled trials is the type of intervention where some studies use pure DHA or EPA while others use fish oil containing different ratios of n-3 PUFA. Upon further investigation using meta-analysis, it was repeatedly shown that when trials were stratified by type of intervention (EPA, mainly EPA, DHA and mainly DHA), supplementation with EPA and mainly EPA resulted in significant reductions of depressive symptoms, whereas DHA did not alleviate depressive symptoms in depressive and bipolar disorders (Martins 2009; Sublette et al. 2011; Martins et al. 2012).

Despite the therapeutic effect of EPA for mood disorders, in the brain EPA levels are the lowest among various n-3 PUFA and often hardly detected in phospholipid membranes. On the other hand, DHA is highly accreted in brain phospholipids (O'Brien et al. 1964; Svennerholm 1964; Svennerholm 1968). This 250 to 300-fold difference in brain phospholipid levels of DHA and EPA was observed across species (Igarashi et al. 2007a; Chen et al. 2008b; Igarashi et al. 2010; Chen et al. 2011). Therefore, prior to understanding the therapeutic mechanism of EPA, we must first understand the regulation behind relatively low levels of EPA in the brain.

### 2.5 Hypothesis and Objectives

#### Overall Hypothesis

The large differences in brain phospholipid concentrations of DHA and EPA may be explained by multiple differences in their metabolism upon entry to the brain.

#### Specific Objectives

1. To determine if EPA $\beta$-oxidation in the brain is higher than DHA.
2. To determine if EPA esterification and recycling in brain phospholipids is lower than DHA.
3. To determine if mitochondrial $\beta$-oxidation is necessary for maintaining low levels of EPA.
4. To determine if EPA loss from brain phospholipids is higher than DHA.
5. To determine if increasing unesterified EPA will modulate eicosanoid synthesis in the brain.
Chapter 3
Rapid β-oxidation of eicosapentaenoic acid in mouse brain: an in situ study


Contribution: As the first author, I performed all the experimental procedures with exception of the in situ cerebral perfusion which was conducted by Melissa Ouellet at Laval University. All data analysis and drafting of the published manuscript was done by me. My overall contribution to the publication is 90%.
3 Objective 1: *in situ* cerebral perfusion

3.1 Abstract

Analyses of brain phospholipid fatty acid profiles reveal a selective deficiency and enrichment in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively. In order to account for this difference in brain fatty acid levels, we hypothesized that EPA is more rapidly β-oxidized upon its transport into the brain. Wild-type C57BL/6 mice were perfused with either $^{14}$C-EPA or $^{14}$C-DHA via *in situ* cerebral perfusion for 40 sec, followed by a bicarbonate buffer to wash out residual radiolabeled PUFA in the capillaries. $^{14}$C-PUFA-perfused brains were extracted for chemical analyses of neutral lipid and phospholipid fatty acids. Based on the radioactivity in aqueous, total lipid, neutral lipid and phospholipid fractions, volume of distribution ($V_D$, μl/g) was calculated. The $V_D$ between $^{14}$C-EPA- and $^{14}$C-DHA-perfused samples was not statistically different for total lipid, neutral lipids or total phospholipids. However, the $V_D$ of $^{14}$C-EPA in the aqueous fraction was 2.5 times higher than that of $^{14}$C-DHA ($p = 0.025$), suggesting more extensive β-oxidation than DHA. Furthermore, radiolabeled palmitoleic acid, a fatty acid that can be synthesized de novo, was detected in brain phospholipids from $^{14}$C-EPA but not $^{14}$C-DHA-perfused mice suggesting that β-oxidation products of EPA were recycled into endogenous fatty acid biosynthetic pathways. These findings suggest that the low levels of EPA in brain phospholipids compared to DHA may be the result of its rapid β-oxidation upon uptake by the brain.

3.2 Introduction

Eicosapentaenoic acid (EPA, 20:5n-3) is commonly considered an essential n-3 polyunsaturated fatty acid (PUFA), because it must be consumed in the diet or synthesized from its 18 carbon precursor, α-linolenic acid (ALA, 18:3n-3) (Scott and Bazan 1989; Voss et al. 1991; Cunnane 2003). EPA has several known metabolic products including group 3 prostanoids and E-series resolvins (Serhan et al. 2000a; Serhan et al. 2000b; Serhan et al. 2002). In addition, EPA competitively inhibits cyclooxygenase-2 (Black et al. 1984a), consequently decreasing the
synthesis of eicosanoids derived from arachidonic acid (ARA, 20:4n-6). Collectively, these metabolites are considered important for the maintenance of several homeostatic functions including immunomodulation (Peterson et al. 1998; Thies et al. 2001; Kew et al. 2003; Miles et al. 2004; Song et al. 2004; Song et al. 2008), platelet aggregation (Ahmed and Holub 1984; Turini et al. 1994; Nieuwenhuys and Hornstra 1998; Adan et al. 1999; Park and Harris 2002) and blood flow (Black et al. 1984a; Black et al. 1984b; Katayama et al. 1997; Katsumata et al. 1999). The brain, however, is enriched with the n-3 PUFA docosahexaenoic acid (DHA, 22:6n-3) while levels of EPA are low to non-detectable (Philbrick et al. 1987; Chen et al. 2008b; DeMar et al. 2008).

The mechanism by which PUFA enter the brain from the plasma is not agreed upon. Two general models involving different pools of fatty acids have been proposed. The first model involves the binding of plasma lipoproteins (LDL, VLDL, HDL) to their appropriate receptors (LDLr, VLDLr, HDLr) (Edmond 2001; Spector 2001). This leads to the endocytosis of PUFA containing lipoprotein particles across endothelial cells where they can be hydrolyzed to release free PUFA (Edmond 2001). The second model involves the transport of unesterified PUFA by passive diffusion or by active transporters that may selectively facilitate the transport of PUFA across endothelial cells (Hamilton et al. 2001; Rapoport et al. 2001; Spector 2001; Hamilton and Brunaldi 2007).

Several indirect observations including selective esterification of orally administered radiolabeled PUFA but not saturated fatty acids in brain phospholipids (Marbois et al. 1992; Edmond et al. 1998; Innis et al. 1999), selective enrichment of DHA but not EPA in brain phospholipids (Chen et al. 2008b; DeMar et al. 2008), and the presence of lipoprotein receptors (Hofmann et al. 1987; Meresse et al. 1989; Hanaka et al. 2000) and CD36 (Ricciarelli et al. 2004; Lein et al. 2007) on the brain endothelium, collectively, have been used to corroborate the receptor-mediated transport hypothesis. On the other hand, studies examining the transport of unesterified fatty acids in artificial membranes support a passive diffusion model (Kamp and Hamilton 1992; Kamp et al. 1995). Furthermore, Ouellet et al., demonstrated that EPA and DHA entry into the brain, in situ, is not a saturable process up to 100 μM, well above their physiological concentration in plasma; further supporting a passive diffusion model (Ouellet et al. 2009).
An assumption of passive diffusion is that there is little selectivity for fatty acids that cross the brain endothelium. However, this does not explain why the brain is highly enriched in DHA but low in EPA. Similar to EPA, other PUFA such as ALA and linoleic acid (LA, 18:2n-6) are present at considerably low concentrations in brain phospholipids (Igarashi et al. 2007a; Chen et al. 2008b). By means of in vivo tracer infusion in awake rats, DeMar et al., have demonstrated that upon entry into the brain, ALA (DeMar et al. 2005) and LA (DeMar et al. 2006) are readily β-oxidized (67% and 59% of radioactivity detected as aqueous oxidation products, respectively); consequently minimizing the incorporation of these PUFA into brain phospholipids. Similarly, Golovko and Murphy (Golovko and Murphy 2006) demonstrated that erucic acid (22:1n-9) is also heavily β-oxidized (60% of radioactivity detected as aqueous oxidation products) in the brain of awake rats. Therefore, we hypothesized that the low level of EPA (<1% of total) detected in brain phospholipids (Philbrick et al. 1987; Chen et al. 2008b; DeMar et al. 2008) may be a consequence of EPA’s rapid β-oxidation.

With the adaptation of in situ cerebral perfusion for mice (Takasato et al. 1984; Dagenais et al. 2000; Smith and Nagura 2001), it is possible to trace the metabolism of 14C-EPA in the mouse brain. Adult (12-16 week-old) mice were perfused with either radiolabeled 14C-DHA or 14C-EPA and the perfused cerebral hemisphere was then washed prior to analysis.Upon finding that EPA was more readily β-oxidized than DHA, we further analyzed brain phospholipids for evidence of fatty acid recycling.

### 3.3 Materials and Methods

#### 3.3.1 Animals

The use of animals was approved by the Laval University Animal Research Committee in accordance with the standards of the Canadian Council on Animal Care. Twelve wild-type C57BL/6 mice between the age of three and four months were purchased from Charles River Laboratories (Saint-Constant, Québec, Canada). All mice were housed in an animal facility with a 12-h light-dark cycle maintained at 22°C. All mice had ad libitum access to laboratory rodent chow (Teklad 2018, Harlan, Madison, WI) and water. The 18:2n-6 and 18:3n-3 fatty acid percent
composition of the diet (measured by gas chromatography) was 55.1% and 5.4%, respectively, and the longer chain PUFAs (20:3n-3, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-6, 22:5n-3 and 22:6n-3) were all below 0.2%. Mice were initially randomized into four groups and received radiolabeled PUFA via in situ perfusion of the right cerebral hemisphere. Two groups were subjected to intracarotid perfusion of \(^{14}\text{C}\)-DHA ([\(1-^{14}\text{C}\)]-DHA, specific activity: 52 mCi/mmol, Moravek, Brea, CA) followed by a 1- or 5-minute bicarbonate buffer post-wash. The other two groups were subjected to intracarotid perfusion of \(^{14}\text{C}\)-EPA ([\(1-^{14}\text{C}\)]-EPA, specific activity: 54 mCi/mmol, Moravek, Brea, CA) also followed by a 1- or 5-minute bicarbonate buffer post-wash. The \(^{14}\text{C}\)-DHA perfusion served as a positive control because DHA uptake and metabolism by the brain is better characterized than EPA (Chen et al. 2008a; Green et al. 2008). Since there was no effect of post-wash time after analysis by two-way ANOVA, we combined the 1- and 5-minute post-wash groups of the \(^{14}\text{C}\)-DHA and \(^{14}\text{C}\)-EPA perfused brain samples.

### 3.3.2 In situ cerebral perfusion

Mice were anesthetised with ketamine (140 mg/kg) and xylazine (8 mg/kg) intraperitoneally prior to surgery and the body temperature was maintained at 37°C. When mice were anesthetised, the salivary glands located at the neck were removed to isolate the right common carotid artery which was subsequently bonded to the right external carotid artery. Then, a catheter containing heparin (25 UI) was introduced into the common carotid artery to guide the perfusate solutions. Prior to the perfusion, the thorax was opened and the heart was cut open to evacuate the perfusate solutions in order to avert cerebral suppression and recirculation of \(^{14}\text{C}\)-EPA or \(^{14}\text{C}\)-DHA. After the opening of the heart, mouse brains were perfused with a bicarbonate buffer. The buffer included 128 mM NaCl, 24 mM NaHCO\(_3\), 4.2 mM KCl, 2.4 mM NaH\(_2\)PO\(_4\), 1.5 mM CaCl\(_2\), 0.9 mM MgSO\(_4\) and 9 mM D-glucose and was adjusted to pH 7.4 with 95% O\(_2\) and 5% CO\(_2\) at 37°C. \(^{14}\text{C}\)-EPA or \(^{14}\text{C}\)-DHA was perfused into the mouse brains at a concentration of 0.15 nCi/\(\mu\)l (2.8 \(\mu\)M) or 0.15 nCi/\(\mu\)l (2.9 \(\mu\)M), respectively, for 40 seconds and at a constant speed of 2.5 ml/min. Finally, the capillaries were washed clean with physiological bicarbonate buffer for one to five minutes. After the perfusion, mouse brain samples were collected, froze in isopentane on dry ice and stored at -80°C for further analyses.
3.3.3 Brain lipid extraction and FAME preparation

Total lipids were extracted from the right hemisphere according to the method of Folch et al. (Folch et al. 1957). The aqueous fraction, containing water and methanol, was washed three times with chloroform.

Isolation of various lipids classes from the total lipid extract was achieved by thin-layer chromatography (TLC). All TLC plates were washed in chloroform and methanol (2:1) and activated by heating at 100°C for 1 hour prior to use. Neutral lipids were separated with TLC G-plates (EMD Chemical, Gibbstown, NJ) along with authentic standards (Avanti, Alabaster, AL) in petroleum ether: diethyl ether: glacial acetic acid (80:20:1 by volume). Cardiolipin was separated along with an authentic standard (Avanti, Alabaster, AL) by means of two-phase TLC with G-plates. First the G-plates were ran in acetone:petroleum ether (30:90 by volume) then in chloroform:methanol:acetic acid:distilled water (80:13:8:0.3 by volume). TLC H-plates (Analtech, Newark, DE) were used to separate phospholipid fractions along with authentic standards in chloroform: methanol: 2-propanol: KCl (0.25% w/v): triethylamine (30:9:25:6:18 by volume). Bands corresponding to authentic standards for total phospholipids, cholesterol, free fatty acids, triglycerides, cholesteryl esters, cardiolipin, ceramide phosphocholine (CerPCho), choline glycerophospholipids (ChoGpl), phosphatidylserine (PtdSer), phosphatidylinositol (PtdIns) and ethanolamine glycerophospholipids (EtnGpl) were visualized under UV light after spraying with 8-anilino-1-naphthalene sulfonic acid (0.1% w/v).

Total phospholipid bands, as separated by TLC, were collected into a test tube containing a known amount of heptadecanoic acid (17:0) then converted to fatty acid methyl esters (FAMEs) with 14% boron trifluoride-methanol at 100°C for an hour. FAMEs were then analysed by HPLC and GC-FID as described below.
3.3.4 Liquid scintillation counting and volume of distribution

Lipid class and phospholipid fraction bands were scraped into scintillation vials with 5 ml of scintillation cocktail (ASC; GE Healthcare Bio-sciences Corp., Piscataway, NJ). Radioactivity of all collected bands was quantified by a Packard TRI-CARB2900TR liquid scintillation analyzer (Packard, Meriden, CT) with a detector efficiency of 48.8%. Radioactivity expressed in units of decays per minute (dpm); then converted to measurements of volume of distribution ($V_D$) calculated using the following equation,

**Equation 3-1.** Volume of distribution.

\[
V_D = \frac{X_{PUFA}}{C_{PUFA} \text{ perfusate}}
\]

where $X_{PUFA}$ is the amount of detected radioactivity per gram of brain wet weight and $C_{PUFA \text{ perfusate}}$ is the concentration of radiolabeled PUFA perfused into the mouse brain. Lipid class bands were measured in duplicates while phospholipid fractions were measured in triplicates.

3.3.5 High-performance liquid chromatography (HPLC)

Radiotracer separation and identification were performed on FAMEs by HPLC (Waters 2690, Boston, USA) with a Luna C18 reverse column (4.6 x 250 mm, 100 Å; Phenomenex, Torrance, CA) equipped with an in-line UV photodiode array detector (Waters 996, Boston, USA) which monitors wavelength of 242 nm. Initial conditions were set at 1 ml/min gradient system consisting of (A) 100% H$_2$O and (B) 100% acetonitrile. The gradient commenced with 85% (B) for 30 minutes, then increased to 100% (B) over a ten-minute period where it was maintained for 20 minutes before returning to 85% (B) over a five-minute period. All fractions were collected at 1 min intervals for a total of 55 min and then analyzed by liquid scintillation counting.
3.3.6  Gas chromatography-flame ionization detection (GC-FID)

FAMEs from brain total phospholipids, rodent chow total lipids and the unknown radioactive peak detected by HPLC at approximately 38 min were analyzed using a Varian-430 gas chromatograph (Varian, Lake Forest, CA, USA) equipped with a Varian FactorFour capillary column (VF-23ms; 30 m x 0.25 mm i.d. x 0.25 μm film thickness) and an FID. Samples were injected in splitless mode. The injector and detector ports were set at 250°C. FAMEs were eluted using a temperature program set initially at 50°C for 2 min, increased at 20°C/min and held at 170°C for 1 min, then at 3°C/min and held at 212°C for 5 min to complete the run at 28 min. The carrier gas was helium, set to a constant flow rate of 0.7 ml/min. Peaks were identified by retention times of FAME standards (Nu-Chek-Prep, Elysian, MN).

3.3.7  Gas chromatography-mass spectrometry (GC-MS)

Identification of the unknown radioactive peak detected by HPLC at approximately 38 minutes was also separated with an Agilent 6890 series gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a HP-5 ms capillary column (Agilent; 30 m x 0.25 mm i.d. x 0.25 μm film thickness) and an Agilent 5973 Network Mass Selective Detector (Agilent Technologies, Wilmington, DE, USA). The sample was injected in splitless mode. The injector port, ion source, and interface were 250, 230 and 280°C, respectively. FAMEs were eluted using a temperature program initially set at 50°C for 1 min, increased at 15°C/min to 240°C for 5 min and then at 15°C/min to 280°C for 8 min to complete the run of 29.33 min. The carrier gas was helium, set to a 1 ml/min constant flow. A mass range from 50 to 700 amu was scanned using electron ionization energy of 70 eV. Peaks were identified on basis of selected mass fragments.

3.3.8  Statistics

There was no effect of post-wash time after analysis by two-way ANOVA; thus the 1- and 5-minute groups of the 14C-DHA and 14C-EPA perfused brain samples were combined. Data are expressed as mean ± SEM (n = 6 independent samples per group). Statistical comparisons
between means were performed using unpaired, two-tailed Student’s $t$-test. Differences were considered statistically significant when $p \leq 0.05$.

3.4 Results

3.4.1 Brain weight and fatty acid concentration

The weights of the right cerebral hemisphere of the $^{14}$C-EPA and $^{14}$C-DHA perfused brains were not statistically significantly different (0.20 ± 0.03 and 0.18 ± 0.02 g, respectively). Similar to previous reports, brain EPA and DHA concentrations were 37 ± 14 and 10196 ± 872 nmol/g brain, respectively (Philbrick et al. 1987; Igarashi et al. 2007a; Chen et al. 2008b; DeMar et al. 2008).

3.4.2 $V_D$ in aqueous, total lipid, neural lipids and phospholipid fractions

The $V_D$ of the aqueous fraction was 2.5 fold higher in $^{14}$C-EPA-perfused brain samples (253 ± 77 µl/g) as compared to the $^{14}$C-DHA-perfused samples (103 ± 23 µl/g) ($p = 0.025$) (Figure 3.4-1). No statistically significant differences in the $V_D$ were observed between $^{14}$C-EPA- and $^{14}$C-DHA-perfused brains in regards to the total lipid and total phospholipids fractions (Table 3.4-1). $^{14}$C-DHA-perfused brains had a significantly higher $V_D$ for triglyceride (230%, $p = 0.04$) fractions, as compared to $^{14}$C-EPA-perfusion. Whereas, $^{14}$C-EPA-perfused samples had a significantly higher $V_D$ in the cholesteryl ester (180%, $p = 0.05$) fraction as compared to $^{14}$C-DHA-perfused brains. However, collectively these fractions contributed less than 5% of the total measured $V_D$. The majority of $V_D$ from $^{14}$C-EPA and $^{14}$C-DHA perfused samples was detected in phospholipid classes. No statistically significant differences were detected in any of the phospholipid classes except for PtdIns which was 3.1 fold higher in mice perfused with $^{14}$C-EPA (260 ± 58 µl/g) compared to $^{14}$C-DHA (85 ± 12 µl/g) ($p = 0.002$) and cardiolipin which was 5.5 fold lower in mice perfused with $^{14}$C-EPA (2.2 ± 1.3 µl/g) as compared to $^{14}$C-DHA (12 ± 1.9 µl/g) ($p = 0.0001$) (Table 3.4-1).
**Figure 3.4-1.** Volume of distribution ($V_D$) of aqueous fraction.

$$V_D = \frac{X_{PUFA}}{C_{PUFA\,Perfusate}};$$ where $X_{PUFA}$ is the measured radioactivity (nCi/g) and $C_{PUFA\,Perfusate}$ is the concentration of $^{14}$C-DHA/EPA perfused (0.15 nCi/μl) for 40 sec. Data are mean ± SEM (n = 6). Significantly different from DHA perfusion; * $p = 0.025$ (unpaired Student’s $t$-test).
Table 3.4-1. VD of various fractions of right cerebral hemisphere lipid extract obtained from $^{14}$C-DHA perfused brains and $^{14}$C-EPA perfused brains.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>DHA Perfusion (n = 6)</th>
<th>EPA Perfusion (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Lipid</td>
<td>1162 ± 145</td>
<td>998 ± 66</td>
</tr>
<tr>
<td>Total Phospholipid</td>
<td>477 ± 98</td>
<td>537 ± 107</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>28 ± 9.1</td>
<td>12 ± 2.4 *</td>
</tr>
<tr>
<td>Cholesteryl Esters</td>
<td>3.6 ± 0.8</td>
<td>6.3 ± 1.6 *</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>12 ± 1.9</td>
<td>2.2 ± 1.3 **</td>
</tr>
<tr>
<td>CerPCho</td>
<td>1.3 ± 1.2</td>
<td>2.4 ± 1.3</td>
</tr>
<tr>
<td>ChoGpl</td>
<td>190 ± 39</td>
<td>163 ± 29</td>
</tr>
<tr>
<td>PtdSer</td>
<td>14 ± 2.1</td>
<td>13 ± 3.4</td>
</tr>
<tr>
<td>PtdIns</td>
<td>85 ± 12</td>
<td>260 ± 58 **</td>
</tr>
<tr>
<td>EtnGpl</td>
<td>146 ± 46</td>
<td>80 ± 16</td>
</tr>
</tbody>
</table>

Data shown are $\mu$l/g and are mean ± SEM. $VD = X_{PUFA}/C_{PUFA \text{ Perfusate}}$; where $X_{PUFA}$ is the measured radioactivity (nCi/g) and $C_{PUFA \text{ Perfusate}}$ is the concentration of $^{14}$C-DHA/EPA perfused (0.15 nCi/$\mu$l) for 40 sec. Significantly different from DHA perfusion; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (unpaired Student’s $t$-test). CerPCho, ceramide 1-phosphorylcholine; ChoGpl, choline glycerophospholipid; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; EtnGpl, ethanolamine glycerophospholipid.
3.4.3 Radioactivity identification of fatty acids in total phospholipid

As determined by HPLC-scintillation counting, the majority of radioactivity detected in the total phospholipid fractions of \textsuperscript{14}C-DHA-perfused brains corresponded to DHA (Figure 3.4-2A). However, in all six \textsuperscript{14}C-EPA-perfused brain samples, one other radiolabeled peak at 38 minutes (Figure 3.4-2B) was also detected then subsequently identified as palmitoleic acid (16:1n-7) via GC-FID and GC-MS (Figure 3.4-2C).
Figure 3.4-2. HPLC separation and identification *(in situ* cerebral perfusion).

HPLC separation and identification of fatty acid methyl esters (FAME) radioactivity of perfusates and total phospholipid fractions from (A) ^14^C-DHA and (B) ^14^C-EPA-perfused brain samples. (C) Mass spectrum of peak isolated at 38 minutes of HPLC run.
3.5 Discussion

The low level of EPA in brain phospholipids may be due to either selective transport or subsequent metabolism including rapid β-oxidation, upon its entry into the brain. Similar to previous reports, in the current study, the concentration of DHA (10196 ± 872 nmol/g brain) in mouse brain total phospholipids was 274 fold higher than that of EPA (37 ± 14 nmol/g brain) (Philbrick et al. 1987; Igarashi et al. 2007a; Chen et al. 2008b; DeMar et al. 2008). Similar to what DeMar et al. (DeMar et al. 2005; DeMar et al. 2006) proposed for ALA and LA, we hypothesized that the low level of EPA in brain phospholipids may be due to its rapid β-oxidation. The radioactivity in the aqueous fraction of 14C-EPA and 14C-DHA perfused brains was analyzed because it contains water soluble products of β-oxidation (Miller et al. 1987; Golovko and Murphy 2006). Through in situ perfusion of 14C-DHA or 14C-EPA to mouse brain, we found that EPA was indeed rapidly β-oxidized as 20% of the radioactivity was present as aqueous oxidation products, upon the 40 sec perfusion. Furthermore, 2.5 fold more radioactivity was detected as water soluble β-oxidation products in 14C-EPA perfused brains as compared to 14C-DHA perfused brains. The observed 2.5 fold increase in EPA β-oxidation, relative to DHA, does not appear to explain why brain phospholipid EPA concentrations are 274 fold lower than DHA. In our study, EPA and DHA were perfused at similar concentrations, whereas, in vivo, plasma unesterified EPA concentrations are several fold lower than DHA (Igarashi et al. 2007b; Sublette et al. 2007). Also, it is possible that upon being released from phospholipids by phospholipase A₂, EPA continues to be more readily β-oxidized than DHA. Furthermore, it is possible that EPA β-oxidation may be attenuated in situ, relative to in vivo.

The observed extensive β-oxidation of EPA as compared to DHA agrees with Gavino and Gavino who demonstrated that carnitine palmitoyltransferase (CPT), an enzyme involved in the partitioning of long chain fatty acids to mitochondria for β-oxidation, had a higher affinity for EPA compared to DHA (Gavino and Gavino 1991). Therefore, the extensive β-oxidation of EPA may be the result of its higher uptake by peroxisomes and/or by CPT into the mitochondria. Furthermore, the BBB is highly enriched with mitochondria (Oldendorf and Brown 1975; Oldendorf et al. 1976; Oldendorf et al. 1977) but whether CPT isoforms in the BBB also have a higher affinity for EPA is not known. In contrast to the β-oxidation of ALA (67%) and LA
(59%) determined by DeMar et al. (DeMar et al. 2005; DeMar et al. 2006), the extent of EPA β-oxidation (20%) in our study is lower (DeMar et al. 2005; DeMar et al. 2006). This difference in β-oxidation may be attributed to two factors: the length of PUFA perfusion time (40 sec vs. 5 min) and the experimental system (in situ vs. in vivo). However, similar to in vivo studies examining DHA metabolism we also find that DHA is predominantly esterified to EtnGpl and ChoGpl (Bazinet et al. 2005; Lee et al. 2005; Bazinet et al. 2006a).

When total phospholipids were analyzed no differences in the $V_D$ were observed between the two groups. However, exclusively, in all the $^{14}$C-EPA-perfused samples, an additional radioactive peak, subsequently identified by GC-MS and GC-FID as palmitoleic acid, was detected by HPLC at 38 minutes. Palmitoleic acid is a monounsaturated fatty acid that can be synthesized from its precursor, palmitic acid (16:0), which can be synthesized de novo via fatty acid synthetase and Δ9-desaturase. It is possible that the unknown peak may also be sapienic acid (16:1n-10) which can be synthesized de novo from palmitic acid via Δ6-desaturase (Ge et al. 2003). However, Δ6-desaturase activity is low in adult mouse brain (Bourre et al. 1990; Bourre and Piciotti 1992). The detection of radioactive palmitoleic acid implied that the β-oxidation product of EPA, acetyl-CoA, were recycled into the palmitic acid biosynthesis pathway, ultimately resulting in the production of radiolabeled palmitoleic acid, similar to what has been reported by others for ALA, LA and erucic acid (Menard et al. 1998; Cunnane et al. 2003; DeMar et al. 2005; Cunnane et al. 2006; DeMar et al. 2006; Golovko and Murphy 2006; Taha et al. 2006; Murphy et al. 2008).

Because the total phospholipid fraction was the major contributor to the total lipid $V_D$, further characterization of radioactivity in various phospholipid classes were performed. Although no differences were observed in four (CerPCho, ChoGpl, PtdSer, and EtnGpl) of the six phospholipid fractions, a significant difference was detected in the PtdIns and the cardiolipin fraction. The observation of a significantly higher $V_D$ in the PtdIns fraction of $^{14}$C-EPA perfused brains suggests that EPA was more readily incorporated into PtdIns, a phospholipid involved in several cell signaling cascades including phosphatidylinositol 3-kinase (PI3K), G-protein coupled receptor (GPCR), and receptor tyrosine kinase (RTK) pathways (Berridge et al. 1989). This is of further interest as PtdIns is a potential target of drugs used to manage bipolar disorder (Jope et al. 1996; O'Donnell et al. 2000; Silverstone et al. 2002; Ding and Greenberg 2003), for
which EPA is potentially therapeutic (Frangou et al. 2006; Frangou et al. 2007). Brain PtdIns usually incorporates 20-carbon PUFA, as shown by its enrichment with arachidonic acid (ARA; 20:4n-6) at the stereospecifically numbered-2 (sn-2) position, suggesting that EPA may have been esterified to PtdIns because the lysophosphatidylinositol acyltransferase involved in sn-2 esterification is 20-carbon selective (Lee et al. 2008). Similarly, cholesteryl ester is often enriched with arachidonic acid, thus a brain acyl-CoA:cholesterol acyltransferase may also be 20-carbon selective (Rumsey et al. 1995; Bazinet et al. 2003). Although the enzyme isoforms involved in the remodeling of brain cardiolipin have not been identified, our finding of relatively higher esterification with DHA as compared to EPA suggests these enzymes may be selective for DHA (Chicco and Sparagna 2007).

In conclusion, similar to ALA, LA and erucic acid, upon EPA’s entry into the brain, it is rapidly β-oxidized and a portion of the non-β-oxidized EPA is esterified to PtdIns, a candidate target for drugs used in the treatment of bipolar disorder. The rapid β-oxidation of EPA, compared to DHA, by the brain may explain its relatively low concentration within brain phospholipids.
Chapter 4
The low levels of eicosapentaenoic acid in rat brain phospholipids are maintained via multiple redundant mechanisms

Chen CT, Domeniciello AF, Trépanier MO, Liu Z, Masoodi M, Bazinet RP. The low levels of Eicosapentaenoic acid in rat brain phospholipids are maintained via multiple redundant mechanisms. J Lipid Res. 54(9):2410-22.

Contribution: As the first author, I performed all experimental procedures, data analysis and drafting of the published manuscript. The second and third authors helped in conducting the infusion of radiotracers. My overall contribution to the publication is 90%.
4 Objective 2 and 3: *in vivo* intravenous infusion

4.1 Abstract

Brain eicosapentaenoic acid (EPA) levels are 250-300 fold lower than docosahexaenoic acid (DHA), at least partly, because EPA is rapidly $\beta$-oxidized and lost from brain phospholipids. Therefore, we examined if $\beta$-oxidation was necessary for maintaining low EPA levels by inhibiting $\beta$-oxidation with methyl paloxirate (MEP). Furthermore, because other metabolic differences between DHA and EPA may also contribute to their vastly different levels, this study aimed to quantify the incorporation and turnover of DHA and EPA into brain phospholipids. Fifteen-week-old rats were subjected to vehicle or MEP prior to a 5-minute intravenous infusion of $^{14}$C-palmitate, $^{14}$C-DHA or $^{14}$C-EPA. MEP reduced the radioactivity of brain aqueous fractions for $^{14}$C-palmitate, $^{14}$C-EPA and $^{14}$C-DHA-infused rats by 74%, 54% and 23%, respectively; while it increased the net rate of incorporation of plasma unesterified palmitate into PtdIns and EPA into EtnGpl and PtdSer. MEP also increased the synthesis of n-3 docosapentaenoic acid (n-3 DPA) from EPA. Moreover, the recycling of EPA into brain phospholipids was 154 fold lower than DHA. Therefore, the low levels of EPA in the brain are maintained by multiple redundant pathways including $\beta$-oxidation, decreased incorporation from plasma unesterified fatty acid pool, elongation/desaturation to n-3 DPA and lower recycling within brain phospholipids.

4.2 Introduction

The brain has a unique fatty acid composition with high levels of arachidonic acid (ARA; 20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3), but low levels of other polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA; 20:5n-3) (Svennerholm 1964; Crawford and Sinclair 1971; Diau et al. 2005; Brenna and Diau 2007). The maintenance of the unique PUFA composition in brain phospholipids is hypothesized to be the result of differences in uptake and/or metabolism upon fatty acid entry into the brain (Chen et al. 2009; Chen et al. 2011). Although brain EPA levels are low, several recent meta-analyses suggest that EPA is more
effective than DHA for alleviating depressive symptoms (Martins 2009; Sublette et al. 2011; Martins et al. 2012). This poses a potential paradox as it is unclear how EPA, which is low within brain phospholipids, would be therapeutic (Sublette et al. 2011).

In regards to receptor-mediated uptake, we established that the LDLr (Chen et al. 2008b), VLDLr (Rahman et al. 2010) or CD36 (Song et al. 2010) are not necessary for maintaining brain PUFA concentrations. Thus, to examine the uptake of PUFA via simple passive diffusion, an in situ cerebral perfusion study was conducted. In addition to rapid passive diffusion, the rate of DHA and EPA uptake into the brain was similar (Chen et al. 2009; Ouellet et al. 2009).

Collectively, results from these studies suggested that the observed 250-300 fold differences between brain DHA and EPA concentrations are unlikely due to differences in uptake (Chen et al. 2008a).

Previously, via in situ cerebral perfusion and in vivo intracerebroventricular infusion in rodents, we demonstrated that EPA was rapidly (Chen et al. 2009) and extensively (Chen et al. 2011) β-oxidized by the brain. Furthermore, we also observed that esterified EPA was rapidly lost from brain phospholipids to cellular metabolism at a rate of 14% per day (Chen et al. 2011) as compared to DHA (DeMar et al. 2004), arachidonic acid (Green et al. 2010) and palmitate (Chen et al. 2011) all at 2% per day. However, there are other potential differences in metabolism between DHA and EPA that could contribute to their large difference in brain phospholipid levels. Therefore, one of the objectives of this study was to investigate further differences in the metabolism of DHA and EPA upon entry into the brain.

In addition to quantifying loss kinetics of EPA from brain phospholipids, the kinetics of EPA uptake, incorporation and turnover can also be quantified via in vivo intravenous radiotracer infusion in rodents as described by Rapoport and others (Robinson et al. 1992; Rapoport 1999; Golovko et al. 2005). The method of Rapoport allows for the calculation of brain kinetic parameters upon intravenous infusion of a high specific activity radiotracer at steady-state into a plasma pool available to the brain. Upon oral administration of a radiotracer, it appears in multiple pools and is not always at steady-state making the calculation of the brain kinetic parameters difficult, if not impossible (Purdon et al. 1997). In this report, we quantified and compared four key kinetic parameters of palmitate, DHA and EPA including $k^*$, $J_{in}$, $J_{FA}$ and $F_{FA}$. The incorporation coefficient ($k^*$) describes the proportional uptake of radiolabeled fatty acid
from the plasma unesterified fatty acid pool into stable brain phospholipids (Robinson et al. 1992; Rapoport et al. 1997; Rapoport 1999; Contreras et al. 2000; DeMar et al. 2005; Golovko et al. 2005; DeMar et al. 2006). The \( J_{\text{in}} \) describes the net rate of plasma unesterified fatty acid incorporation into brain phospholipids whereas the \( J_{\text{FA}} \) describes the net rate of brain acyl-CoA incorporation into brain phospholipids (Grange et al. 1995). Lastly, the rate of turnover \( (F_{\text{FA}}) \) describes the turnover of de-esterified fatty acid from phospholipids that are re-esterified into brain phospholipid via Land’s recycling (Grange et al. 1995).

Since the synthesis of PUFA within the brain is negligible relative to brain uptake (DeMar et al. 2005; DeMar et al. 2006), the mathematical model can predict the relative contribution of plasma pools from which fatty acids enter the brain via comparison of the \( J_{\text{in}} \) to the net rate of loss from brain phospholipids, \( J_{\text{out}} \) (Duncan and Bazinet 2010). If the plasma unesterified PUFA pool is the major plasma contributor to brain phospholipid PUFA, then the \( J_{\text{in}} \) should approximate the \( J_{\text{out}} \). However, if the \( J_{\text{out}} \) exceeds the \( J_{\text{in}} \), then there may be additional plasma fatty acid pools contributing to PUFA uptake into brain phospholipids. In contrast, if the \( J_{\text{in}} \) exceeds the \( J_{\text{out}} \), then it would suggest that either the known kinetic parameters of PUFA are overestimations or modifications to the current model may be necessary.

Because \( \beta \)-oxidation appears to be a major contributor to the observed difference in brain PUFA concentrations (Chen et al. 2011), another objective of this study was to examine if mitochondrial fatty acid \( \beta \)-oxidation is necessary for maintaining low levels of EPA in brain phospholipids by irreversibly inhibiting carnitine palmitoyltransferase Ia (CPT-Ia), the rate-limiting enzyme in mitochondrial fatty acid \( \beta \)-oxidation that catalyzes the formation of acyl-carnitine from acyl-CoA, with methyl palmostirate (MEP; methyl-2-tetradecylglycide) (Chang et al. 1994; Freed et al. 1994; Chang et al. 1997). Although CPT-Ic is predominantly expressed in the brain (Price et al. 2002), it is localized to the endoplasmic reticulum where it mediates food intake via endocannabinoids and ghrelin without modulating brain fatty acid \( \beta \)-oxidation (Wolfgang et al. 2006; Sierra et al. 2008; Wolfgang et al. 2008; Lee and Wolfgang 2012; Ramirez et al. 2013). Therefore, the inhibitory effects of MEP would likely be through CPT-Ia, the other isoform of CPT-I that is localized to the mitochondria in the brain (Brown et al. 1997). Previously, Freed et al reported that MEP-treatment reduced the \( \beta \)-oxidation of \(^{14}\text{C}\)-palmitate and \(^{14}\text{C}\)-arachidonate (Freed et al. 1994). However, MEP-treatment only increased the esterification of \(^{14}\text{C}\)-palmitate into brain total lipids and triacylglycerol. Overall, we found that MEP-treatment
led to significant increases in the $J_{in}$ of EPA into EtnGpl and PtdSer. Interestingly, there were also significant increases in n-3 docosapentaenoic acid (n-3 DPA; 22:5n-3) within ChoGpl and EtnGpl. Therefore, β-oxidation is involved, but not necessary for maintaining low EPA levels in brain phospholipids and elongation/desaturation of EPA into longer n-3 PUFA species may compensate for decreased β-oxidation in order to maintain low levels of EPA in brain phospholipids.

4.3 Methods and Materials

4.3.1 Animals

All procedures were performed in accordance with the policies set out by the Canadian Council on Animal Care and were approved by the Animal Ethics Committee at the University of Toronto. Male Sprague Dawley rats were purchased from Charles Rivers (Saint-Constant, QC, Canada) at 12 weeks of age and kept at the animal facility with automated 12 hour light-dark cycle and a constant temperature of 22°C for three weeks. The rats received ad libitum access to water and standard chow (Teklad 2018, Harlan, Madison, WI, USA) which was composed of 54% of linoleate (18:2n-6), 5% of α-linolenate (18:3n-3) and < 0.3% of longer chained PUFA (20:2n-6, 20:3n-3, 20:4n-6, EPA, 22:4n-6, 22:5n-6, 22:5n-3 and DHA), as measured by gas chromatography-flame ionization detection (GC-FID).

4.3.2 Radiotracer perfusate and methyl palmoxirate preparation

Radiolabeled $^{14}$C-palmitate ($[1-^{14}$C]-palmitate, specific activity: 55 mCi/mmol, Moravek Biochemical Inc, Brea, CA, USA), $^{14}$C-DHA ($[1-^{14}$C]-DHA, specific activity: 53 mCi/mmol, Moravek Biochemical Inc, Brea, CA, USA) and $^{14}$C-EPA ($[1-^{14}$C]-EPA, specific activity: 54 mCi/mmol, Moravek Biochemical Inc, Brea, CA, USA) were dissolved in 5 mM HEPES buffer (pH 7.4) containing 50 mg/ml fatty acid-free bovine serum albumin to a perfusate concentration of 66 μCi/ml. The purity of $^{14}$C-radiotracers was > 99.9% as confirmed by high performance liquid chromatography (HPLC) and liquid scintillation counting (LSC) (Figure 4.4-1A). MEP
(donated by Dr. S.I. Rapoport) was solubilized overnight in Tween 80 and diluted to 10 mg/ml in carboxymethylcellulose (0.1% in saline) (Freed et al. 1994).

### 4.3.3 Surgery

Fifteen week-old rats were anesthetised with isofluorane inhalation (3% induction, 1-2% maintenance). Rats were given a lower back subcutaneous injection of 100 mg/kg of Ketoprofen (MERIAL Canada, Inc., Baie d’Urfé, QC, Canada). Polyethylene catheters (PE 50, Intramedic™, Becton Dickinson, Franklin Lakes, NJ, USA) with silicone tubing (Silicone tubing 0.020 in (I.D.) and 0.037 in (O.D.), VWR®, Mississauga, ON, Canada) filled with 0.9% saline were implanted into the right jugular vein. Surgery lasted for approximately 15 minutes. After surgery, all rats were singly housed to recover from anesthesia for at least 24 hours with *ad libitum* access to food and water. Rats were not fasted for the radiotracer infusions.

### 4.3.4 Free-living intravenous tracer infusion

Twenty-four hours post-surgery, a second catheter (I.V. Catheter 24 GA/0.75 in, Angiocath™, Becton Dickinson, Franklin Lakes, NJ, USA) was implanted into the tail vein where rats received either vehicle or 10 mg/kg of MEP. Ten minutes post injection of vehicle or MEP, the tail vein catheter was connected to a syringe containing radiolabeled $^{14}$C-palmitate ($n = 8$), $^{14}$C-DHA ($n = 6$) or $^{14}$C-EPA ($n = 7$) attached to a computer-controlled variable speed pump (PHD 22/2000; Harvard Apparatus, Holliston, MA, USA). The radiotracers were infused for 5 minutes via the tail vein catheter at a rate of $0.223(1+e^{-19.2t})$ ml/min ($t$ is infusion time in minute) (Washizaki et al. 1994) which achieves a steady-state plasma radioactivity within 1 minute (Robinson et al. 1992; Chang et al. 1999). Each rat received a dose of 76 $\mu$Ci $^{14}$C-palmitate, 76 $\mu$Ci $^{14}$C-DHA or 78 $\mu$Ci $^{14}$C-EPA per rat. Thus a total of 1.39 $\mu$mol of palmitate, 1.42 $\mu$mol of DHA or 1.44 $\mu$mol of EPA were infused over 5 minutes. During the 5-minute infusion, blood samples were collected from the jugular vein at approximately 0, 0.25, 0.5, 0.75, 1.5, 3, 4, 5 minutes while the un-anaesthetized rat was mobile in the infusion box with food and water. In a pilot study, we found that there were no significant differences in the plasma unesterified fatty
acid concentrations between carotid artery (palmitate: 163 ± 20 nmol/ml; EPA: 0.29 ± 0.017 nmol/ml; DHA: 0.63 ± 0.059 nmol/ml) and jugular vein (palmitate: 163 ± 22 nmol/ml; EPA 0.31 ± 0.025 nmol/ml; DHA: 0.70 ± 0.058 nmol/ml). After 5 minutes, the rats were rapidly euthanized by head-focused, high-energy microwave irradiation (13.5 kW for 1.6 seconds; Cober Electronics Inc., Norwalk, CT, USA). The radiotracer infusions continued until rats were euthanized. The brain was excised and cut sagittally. Both hemispheres were stored at -80°C for radioactive and biochemical analyses. Plasma was isolated from whole blood via microcentrifugation at 6200 rpm (2000 g) for 5 minutes and stored at -80°C.

4.3.5 Lipid analysis

Total lipids from one brain hemisphere and from plasma were extracted by the method of Folch, Lee and Sloane Stanley (Folch et al. 1957). Isolation of various neutral lipid and phospholipid classes from the total lipid extract was previously described by Chen et al (Chen et al. 2011).

4.3.6 Unesterified fatty acid analysis

At 15 weeks of age, sixteen rats were randomized to receive vehicle or 10 mg/kg methyl paloxirate (MEP) via tail vein catheter as described above. Fifteen minutes post injection, rats were euthanized by head-focused, high energy microwave irradiation (13.5 kW for 1.6 seconds) and brains were excised and sagittally cut for unesterified fatty acids/lipid mediators and acyl-CoA analyses as described below. Brain hemispheres were homogenized in ethanol to yield a concentration of 100 mg tissue/ml. The unesterified fatty acids from 100 mg of brain tissue were isolated. The unesterified fatty acid bands were collected and extracted twice from silica by hexane:isopropanol (3:2 by vol.) with 5.5% water. Extracted unesterified fatty acids were dried down with nitrogen gas and added with 100 μl of freshly made pentafluorobenzylbromide (PFB) cocktail consisted of acetonitrile:N,N Diisopropylethylamine:PFB (1 ml:100 μl:10 μl by vol.). The mixtures were shaken for 15 minutes and dried down with nitrogen gas. Fatty acid-PFB esters (FAPE) were reconstituted in 100 μl hexane for GC-MS analysis as described below.
For measurements of unesterified EPA, brain samples were homogenized in ethanol on ice. An aliquot of 100 mg tissue was spiked with ARA-d8 (20 ng; Cayman Chemical, Ann Arbor, MI, USA) and dried under nitrogen gas in reduced light conditions. Residues were dissolved in ethanol, acidified to pH 4 with 1 N HCl and extracted three times with ethyl acetate. After washing to neutrality with water, the ethyl acetate fraction was dried under nitrogen and transferred to siliconized minivials for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as described below.

4.3.7 Acyl-CoA analysis

Long chain acyl-CoA were extracted from the brains of vehicle and MEP-treated rats as well as the brains of radiotracer infused rats using a modified affinity chromatography method (Deutsch et al. 1994). Brains were homogenized by a probe sonicator in isopropanol:25 mM KH₂PO₄:acetonitrile (1:1:2 by vol.) with 10 nmol of added internal standard, heptadecanoyl-CoA (17:0-CoA). Subsequently, protein was precipitated from the homogenate by the addition of saturated (NH₄)₂SO₄. After centrifugation for 5 minutes at 3000 rpm (2000 g), supernatant containing acyl-CoA was extracted and diluted with a 1.25 fold volume of 25 mM KH₂PO₄. The diluted supernatant was then repeatedly passed through an oligonucleotide purification cartridge (ABI Masterpiece™, OPC®, Applied Biosystems; Foster City, CA, USA) for three times at a rate of 5 ml/min. Afterwards, the cartridge was washed with 25 mM KH₂PO₄ and the bound acyl-CoA were eluted with isopropanol/1 mM glacial acetic acid (75:25 by vol.). Acyl-CoA samples were reconstituted in elution buffer (100 μl) and stored at -80°C until HPLC analysis as described below.

4.3.8 High performance liquid chromatography (HPLC)-UV photodiode array detection and liquid scintillation counting (LSC)

Fatty acid methyl esters (FAME) and radioactive acyl-CoA analysis were previously described by Chen et al.
4.3.9 Liquid chromatography (LC)-tandem mass spectrometry (MS/MS)

Acyl-CoA concentrations were detected using an Agilent 1200 Binary LC Pump (Agilent Technologies, Wilmington, DE, USA) equipped with a Zorbax SB-Phenyl column (3 x 50 mm, 3.5 μm spherical size; Chromatographic Specialties, Brockville, ON, Canada). The initial conditions of elution were set at 600 μl/min gradient system consisting of (A) 70% 10 mM ammonium acetate in water and (B) 30% 10 mM ammonium acetate:acetonitrile (10:90 by vol.). The gradient started with 70% (A) and 30% (B) and maintained for 1 minute, decreased to 30% (A) and 70% (B) over 5-minute period where it was maintained for 6 minutes before returning to 70% (A) and 30% (B) over 6.2-minute period and maintained for 10 minutes to complete the total run of 28.2 minutes. Mass spectrometry analyses were carried out on API4000 QTRAP (AB SCIEX, Concord, ON, Canada) quadruple-linear ion trap (QqLIT) mass spectrometers. The QTRAP analyses were conducted in positive ion mode under multiple reaction monitoring (MRM) conditions. The turbospray temperature was set to 600°C, the curtain gas flow to 30 psi, and the ion spray voltage to 5500 V. The collision energy (CE), de-clustering potential (DP) and collision cell exit potential (CXP) were optimized and were set to 45, 42, 10 for palmitoyl-CoA; 45, 40, 7 for palmitoleoyl-CoA; 40, 40, 15 for heptadecanoyl-CoA; 47, 45, 10 for stearoyl-CoA; 47, 50, 10 for oleoyl-CoA and linoleoyl-CoA; 48, 40, 14 and 75, 40, 7 for α-linolenoyl-CoA; 43, 40, 15 for arachidonoyl-CoA; 50, 40, 12 and 50, 40, 15 and 85, 40, 8 for EPA-CoA; and 40, 40, 15 for DHA-CoA. Peaks were identified and quantified by mass transitions, compound specific parameters and standard curves of authentic acyl-CoA standards (Avanti, Alabaster, AL, USA) (Figure 4.3-1). Concentrations were corrected for percent recovery of heptadecanoyl-CoA and expressed as nmol/g brain.
Figure 4.3-1. EPA-CoA mass spectra.

LC-MS/MS profile of palmitoyl-CoA (top), DHA-CoA (top) and EPA-CoA (bottom). (A) For palmitate, the MRM transition is 1006 to 499 m/z. (B) For DHA, the MRM transition is 1078 to 571 m/z. (C) For EPA, there are three MRM transitions: 1052 to 545, 1052 to 428 and 1052 to 136 m/z.
Brain unesterified EPA was detected using an Agilent HPLC 1200 equipped with a Zorbax SB-Phenyl column. HPLC solvent contained 4 μl/l propionic acid. The initial conditions of elution were set at 400 μl/min gradient system consisting of (A) water and (B) acetonitrile. The gradient started with 80% (A) and 20% (B) and maintained for 2 minute, decreased to 75% (A) and 25% (B) for 0.5 minutes, then further decreased to 50% (A) and 50% (B) for 5 minutes, then to 45% (A) and 55% (B) for 6.2 minutes and 100% (B) for 11 minutes. Mass spectrometry analyses were carried out on API5500 triple quadruple mass spectrometer (AB SCIEX, Concord, ON, Canada). The QTRAP analyses were conducted in electrospray ionization negative ion mode. The turbospray temperature was set to 500°C and the ion spray voltage to 4500 V. The collision energy (CE), de-clustering potential (DP) and collision cell exit potential (CXP) were optimized and were set to 15, 50 and 11, respectively. Concentration was quantified by comparing the deuterium-to-protium ratio of brain unesterified EPA with standard lines generated from authentic standards. Authentic standards in appropriate dilutions (0.002 to 2 ng) were prepared and analyzed simultaneously with brain samples. The lower limit of quantification (LLQ) was 0.002 ng (6.3 fmol of EPA).

4.3.10 Gas chromatography (GC)-flame ionization detection (FID)

Brain fatty acid concentrations from total and phospholipid classes were quantified as described by Chen et al (Chen et al. 2011).

4.3.11 Gas chromatography (GC)-mass spectrometry (MS)

FAPE were identified with an Agilent 7890A gas chromatograph (Agilent Technologies) equipped with a SP-2380 (Supelco) fused silica column (Agilent Technologies; 30 m x 0.25 mm i.d. x 0.2 μm film thickness) and an Agilent 5975C quadruple mass spectrometry detector (Agilent Technologies). The sample was injected in split mode (10:1). The injection port temperature was set to 240°C and the ionization mode was set to negative chemical ionization using methane. FAPE were eluted using a temperature program initially set at 150°C for 1
minute, increase at 12°C/min to 270°C and then at 40°C/min to 275°C for 3 minutes. The carrier helium gas was set to a constant flow of 1 ml/min. The LLQ for n-3 docosapentaenoate was 1 ng/20 mg brain. The LLQ for palmitate, palmitoleate, α-linolenate and EPA was 5 ng/20 mg brain. The LLQ for ARA and linoleate was 10 ng/20 mg brain. The LLQ for oleate, stearate and DHA was 20 ng/20 mg brain.

4.3.12 Kinetics

Total and phospholipid class radioactivity were adjusted by the percentage of radiolabeled palmitate, DHA and EPA as measured by HPLC and LSC for kinetic modeling. The model for in vivo kinetics of brain fatty acids in rats has been previously described (Robinson et al. 1992; Washizaki et al. 1994; Grange et al. 1995; Rapoport 1999; Contreras et al. 2000; Rapoport et al. 2001; DeMar et al. 2005).

The unidirectional incorporation coefficient, \( k_i^{\text{palmitate}, \text{DHA or EPA}} \) (ml plasma/day/g brain), which represents the incorporation of plasma radiotracers into stable brain lipid pools \( i \), was calculated as:

**Equation 4-1.** Incorporation coefficient of radiotracers.

\[
k_i^{\text{palmitate}, \text{DHA or EPA}} = \frac{c_i^{\text{brain}}(\text{palmitate}, \text{DHA or EPA}) (T)}{\int_0^T c_i^{\text{plasma}}(\text{palmitate}, \text{DHA or EPA}) \, dt}
\]

where \( c_i^{\text{brain}}(\text{palmitate}, \text{DHA or EPA}) \) is the radioactivity in \( i \) (nCi/g brain) from palmitate, DHA or EPA at time, \( T \), and \( c_i^{\text{plasma}}(\text{palmitate}, \text{DHA or EPA}) \) is the plasma radioactivity (nCi/ml plasma) of \(^{14}\text{C-}\)palmitate, \(^{14}\text{C-DHA} \) or \(^{14}\text{C-EPA}\)-infused rats.

Because elongation/desaturation products of \(^{14}\text{C-EPA}\), n-3 docosapentaenoic acid (n-3 DPA) and DHA, were detected, we can determine their incorporation into stable lipid pools \( i \) with following adjustment to the equation:
Equation 4-2. Incorporation of coefficient of elongated/desaturated radiotracers.

\[ k^*_i (\text{EPA} \to \text{n-3 DPA or DHA}) = \frac{c^*_\text{brain} (\text{n-3 DPA or DHA}) (T) }{\int_0^T c^*_\text{plasma} (\text{EPA}) dt} \]

where \( k^*_i (\text{EPA} \to \text{n-3 DPA or DHA}) \) is the conversion-incorporation coefficient and \( c^*_\text{brain} (\text{n-3 DPA or DHA}) \) is the brain radioactivity of n-3 DPA or DHA as determined by HPLC and LSC.

Since the incorporation coefficient applies to both radiolabeled and non-radiolabeled fatty acids, we can determine the rate of incorporation of non-radiolabeled plasma fatty acids into stable brain lipid pools as un-metabolized fatty acids, \( J_{in,i} (\text{palmitate, DHA, EPA}) \), or as elongation/desaturation products \( J_{in,i} (\text{EPA} \to \text{n-3 DPA or DHA}) \) (nmol/g brain/day).

Equation 4-3. Rate of incorporation of non-radiolabeled plasma fatty acids.

\[ J_{in,i} (\text{palmitate, DHA or EPA}) = k^*_i (\text{palmitate, DHA or EPA}) c^*_\text{plasma} (\text{palmitate, DHA or EPA}) \]

Equation 4-4. Rate of incorporation of elongated/desaturated products.

\[ J_{in,i} (\text{EPA} \to \text{n-3 DPA or DHA}) = k^*_i (\text{EPA} \to \text{n-3 DPA or DHA}) c^*_\text{plasma} (\text{EPA}) \]

where \( c^*_\text{plasma} (\text{palmitate, DHA or EPA}) \) is the plasma unesterified fatty acid concentration.

In addition to the rate of incorporation from plasma to stable brain lipid pools, we can also calculate the net rate of incorporation, \( J_{FA,i} (\text{palmitate, DHA or EPA}) \) (nmol/g brain/day) (Robinson et al. 1992; Rapoport et al. 2001), from the brain acyl-CoA pool to stable brain lipid pools via correction for the steady-state ratio of specific activity of acyl-CoA pool over the specific activity of radiotracer in plasma which is defined as the dilution factor, \( \lambda_{\text{palmitate, DHA or EPA}} \).

Equation 4-5. Dilution factor.

\[ \lambda_{\text{palmitate, DHA or EPA}} = \frac{c^*_\text{brain} (\text{palmitate, DHA or EPA})}{c^*_\text{brain} (\text{palmitate, DHA or EPA})} \cdot \frac{c^*_\text{plasma} (\text{palmitate, DHA or EPA})}{c^*_\text{plasma} (\text{palmitate, DHA or EPA})} \]
where the numerator and denominator are the steady-state specific activities of brain acyl-CoA and plasma unesterified fatty acids, respectively. Since the infusion is 5 minutes, contributions of fatty acid from \textit{de novo} synthesis and esterified plasma fatty acid are negligible (Robinson et al. 1992; Purdon et al. 1997; Rapoport et al. 2001; DeMar et al. 2005); thus only contributions from plasma unesterified fatty acids and acyl-CoA pools were considered in the calculation of $\lambda_{\text{palmitate}, \text{DHA}, \text{EPA}}$.

$J_{FA, i(\text{palmitate, DHA or EPA})}$ of non-radiolabeled fatty acids and EPA elongation/desaturation products from acyl-CoA pools into stable brain lipid pools $i$ are calculated as followed:

**Equation 4-6.** Rate of incorporation of non-radiolabeled brain acyl-CoA.

$$I_{FA, i(\text{palmitate, DHA or EPA})} = \frac{J_{in,k(\text{palmitate, DHA or EPA})}}{\lambda_{\text{palmitate, DHA or EPA}}}$$

**Equation 4-7.** Rate of incorporation of elongated/desaturated CoA products.

$$I_{FA, i(\text{EPA-n-3DPA or DHA})} = \frac{J_{in,k(\text{EPA-n-3DPA or DHA})}}{\lambda_{\text{EPA}}}$$

The rate of turnover, $F_{FA, i(\text{palmitate, DHA or EPA})}$ (%/day), and half-life, $t_{1/2}$ (day) (Robinson et al. 1992; Rapoport et al. 2001), within stable brain lipid pools $i$ as un-metabolized fatty acids or EPA elongation/desaturation products are quantified as,

**Equation 4-8.** Rate of turnover of non-radiolabeled phospholipid fatty acids.

$$F_{FA, i(\text{palmitate, DHA or EPA})} = \frac{I_{FA, i(\text{palmitate, DHA or EPA})}}{c_{\text{brain, i(\text{palmitate, DHA or EPA})}}}$$

**Equation 4-9.** Rate of turnover of elongated/desaturated phospholipid fatty acids.

$$F_{FA, i(\text{EPA-n-3DPA or DHA})} = \frac{I_{FA, i(\text{EPA-n-3DPA or DHA})}}{c_{\text{brain, i(n-3DPA or DHA)}}}$$
where \( c_{\text{brain, i(palmitate, DHA or EPA)}} \) is the brain fatty acid concentrations of stable brain lipid pools \( i \).

**Equation 4-10.** Half-life of non-radiolabeled phospholipid fatty acids.

\[
\frac{0.693}{t_{1/2}} = \frac{1}{F_{\text{FA}(\text{palmitate, DHA or EPA})}}
\]

**Equation 4-11.** Half-life of elongated/desaturated phospholipid fatty acids.

\[
\frac{0.693}{t_{1/2}} = \frac{1}{F_{\text{FA}(\text{EPA or DHA})}}
\]

### 4.3.13 Statistics

Concentrations and rates are expressed as mean ± SD. HPLC profiles were analyzed as pooled samples and do not have SD. Statistical comparisons of kinetic parameters between \(^{14}\text{C-DHA}\) and \(^{14}\text{C-EPA}\) infusions were performed, \textit{a priori}, using two-tailed \( t \)-test. Differences between \(^{14}\text{C-DHA}\) and \(^{14}\text{C-EPA}\) infusions upon vehicle administration were statistically significant at \( ^{\#}p < 0.05, ^{##}p < 0.01 \) and \( ^{###}p < 0.001 \). Comparisons were not performed with \(^{14}\text{C-palmitate}\) which served as positive control to confirm the activity of MEP (Freed et al. 1994). Statistical comparisons of fatty acid and acyl-CoA concentrations, radioactivity and kinetic parameters between vehicle and MEP-treated rats were performed using two-tailed \( t \)-test. All data had passed the normality and equal variance test (SigmaStats 3.5). Differences between vehicle and MEP-treated rats were statistically significant at \( ^{*}p < 0.05, ^{**}p < 0.01 \) and \( ^{***}p < 0.001 \).
4.4 Results

4.4.1 Brain fatty acid and acyl-CoA concentrations

Upon MEP-treatment, there were selective increases in brain unesterified fatty acids (Table 4.4-1). There were significant 2.5, 1.4, 1.5 and 1.3 fold increases in unesterified palmitate, linoleate (18:2n-6), α-linolenate (18:3n-3) and EPA, respectively. In contrast, ARA (20:4n-6) and DHA were unaffected by MEP.

MEP had no effect on the concentration of palmitoyl-CoA but reduced the concentration of stearoyl-CoA (Table 4.4-1). MEP increased linoleoyl-CoA, α-linolenoyl-CoA and decreased EPA-CoA. Interestingly, while MEP had no effect on unesterified DHA, it significantly reduced DHA-CoA in the brain.

Acute administration of MEP was insufficient to significantly alter total phospholipids fatty acid concentrations (Table 4.4-1). There were no significant difference between the brain fatty acid concentrations of all radiotracer infused rats in each treatment; therefore, data were pooled for comparison between vehicle and MEP-treatments. In accordance to previous reports, palmitate, stearate and oleate were the major constituents of brain phospholipids consisting of 25%, 22% and 21% of the total fatty acids, respectively. Similarly, the major PUFA species in brain phospholipids were ARA and DHA which accounted for 8% and 9% of total fatty acids, respectively. Lastly, the level of EPA in brain total phospholipids was relatively low, as compared to DHA, at 0.2% of total fatty acids which corresponded to 225 ± 12 nmol/g brain in vehicle-treated rats and 202 ± 8.6 nmol/g brain in MEP-treated rats.

After fractionation into the four major phospholipid classes, there was no significant effect of MEP on the fatty acid compositions of individual phospholipid classes with the exceptions of a 9% reduction in linoleate from EtnGpl and a 8% reduction in ARA from PtdSer upon MEP-treatment (Table 4.4-2). After adjustments for pool size, palmitate was primarily esterified to ChoGpl and as the major component in ChoGpl, it accounted for 48% of total ChoGpl fatty acids (Table 4.4-2). As for DHA, it was the primarily esterified to EtnGpl and PtdSer which accounted for 17% of total fatty acids in both EtnGpl and PtdSer (Table 4.4-2). As for EPA, albeit, the esterification of EPA to PtdIns was the lowest among four major phospholipid classes, when
pool size is considered, 0.5% of total PtdIns fatty acids were EPA as compared to 0.1%, 0.2% and 0.4% of total ChoGpl, EtnGpl and PtdSer fatty acids, respectively.
Table 4.4-1. Brain fatty acid concentrations of unesterified fatty acid (n = 8 per treatment), acyl-CoA (n = 8 per treatment) and total phospholipids (total PL; n = 10-11 per treatment) pools.

<table>
<thead>
<tr>
<th></th>
<th>Brain Unesterified FA</th>
<th></th>
<th>Brain Acyl-CoA</th>
<th></th>
<th>Brain Total PL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>MEP</td>
<td>Vehicle</td>
<td>MEP</td>
<td>Vehicle</td>
</tr>
<tr>
<td>16:0</td>
<td>22 ± 16</td>
<td>54 ± 24**</td>
<td>6.2 ± 0.33</td>
<td>6.4 ± 0.59</td>
<td>24559 ± 2030</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>0.36 ± 0.13</td>
<td>0.49 ± 0.1*</td>
<td>1.2 ± 0.11</td>
<td>1.3 ± 0.2</td>
<td>376 ± 65</td>
</tr>
<tr>
<td>18:0</td>
<td>38 ± 10</td>
<td>79 ± 31**</td>
<td>6.1 ± 0.39</td>
<td>5.4 ± 0.68*</td>
<td>22241 ± 2610</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>6.6 ± 2.7</td>
<td>8.2 ± 1.2</td>
<td>8.3 ± 0.54</td>
<td>7.8 ± 0.74</td>
<td>21384 ± 3400</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>0.91 ± 0.36</td>
<td>1.3 ± 0.31*</td>
<td>0.47 ± 0.048</td>
<td>0.63 ± 0.11**</td>
<td>770 ± 49</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.074 ± 0.036</td>
<td>0.11 ± 0.061*</td>
<td>0.022 ± 0.0039</td>
<td>0.043 ± 0.016**</td>
<td>27 ± 6.5</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>3.8 ± 0.95</td>
<td>4.3 ± 1.8</td>
<td>0.90 ± 0.065</td>
<td>0.83 ± 0.01</td>
<td>7921 ± 791</td>
</tr>
<tr>
<td>20:5n-3*</td>
<td>0.019 ± 0.0017</td>
<td>0.025 ± 0.0051**</td>
<td>0.019 ± 0.0012</td>
<td>0.016 ± 0.0041*</td>
<td>225 ± 37</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.13 ± 0.02</td>
<td>0.13 ± 0.061</td>
<td>ND</td>
<td>ND</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>6.0 ± 2</td>
<td>7.0 ± 1.4</td>
<td>0.68 ± 0.028</td>
<td>0.50 ± 0.093***</td>
<td>8789 ± 830</td>
</tr>
</tbody>
</table>

Data are mean ± SD and are expressed in nmol/g brain. Unesterified fatty acid concentrations were quantified by GC-MS with exception of EPA+ which was determined by LC-MS/MS. Total phospholipid fatty acid concentrations were quantified by GC-FID. Acyl-CoA concentrations were quantified by LC-MS/MS. P-values indicated significantly different from vehicle-treated rats; *p < 0.05, **p < 0.01, ***p < 0.001. Brain 22:5n-3 (n-3 DPA)-CoA was not determined (ND).
Table 4.4-2. Brain fatty acid concentrations of four major phospholipid classes (n = 10-11 per treatment).

<table>
<thead>
<tr>
<th></th>
<th>ChoGpl</th>
<th>EtnGpl</th>
<th>PtdIns</th>
<th>PtdSer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>MEP</td>
<td>Vehicle</td>
<td>MEP</td>
</tr>
<tr>
<td>16:0</td>
<td>23818 ± 1531</td>
<td>23762 ± 492</td>
<td>2903 ± 242</td>
<td>2786 ± 107</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>146 ± 14</td>
<td>145 ± 11</td>
<td>148 ± 62</td>
<td>143 ± 45</td>
</tr>
<tr>
<td>18:0</td>
<td>6597 ± 646</td>
<td>6403 ± 150</td>
<td>6590 ± 257</td>
<td>6492 ± 213</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>10893 ± 1098</td>
<td>10705 ± 373</td>
<td>8833 ± 973</td>
<td>8235 ± 507</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>464 ± 32</td>
<td>452 ± 36</td>
<td>182 ± 10</td>
<td>166 ± 11</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>11 ± 1.3</td>
<td>10 ± 0.83</td>
<td>14 ± 3.1</td>
<td>13 ± 1.5</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>2242 ± 169</td>
<td>2255 ± 70</td>
<td>4302 ± 322</td>
<td>4089 ± 154</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>61 ± 9</td>
<td>60 ± 8.7</td>
<td>74 ± 16</td>
<td>65 ± 11</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>3.7 ± 0.65</td>
<td>3.5 ± 0.37</td>
<td>8.6 ± 0.73</td>
<td>8.7 ± 1</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>1351 ± 100</td>
<td>1387 ± 85</td>
<td>5812 ± 485</td>
<td>5448 ± 356</td>
</tr>
</tbody>
</table>

Data are mean ± SD and are expressed in nmol/g brain. Fatty acid concentrations were quantified by GC-FID. P-values indicated significantly different from vehicle-treated rats; *p < 0.05, **p < 0.01, ***p < 0.001. MEP, methyl paloxirate; ChoGpl, choline glycerophospholipid; EtnGpl, ethanolamine glycerophospholipid; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine.
4.4.2 Identification of radioactivity in total and phospholipid fractions

After a 5-minute infusion of radiolabeled $^{14}$C-palmitate, the only radioactive peak in plasma total lipids and brain total phospholipids of the vehicle and MEP-treated rats was palmitate (Figure 4.4-1B,C). Similarly, upon $^{14}$C-DHA infusion, the only radioactive peak in plasma total lipids and brain total phospholipids of vehicle and MEP-treated rats was DHA (Figure 4.4-1B,C). Upon $^{14}$C-EPA infusion, while plasma total lipids of the vehicle and MEP-treated rats only contained radioactive EPA peak (Figure 4.4-1B), brain total phospholipids from the vehicle and MEP-treated rats all contained radiolabeled EPA, DHA, n-3 DPA and palmitate (Figure 4.4-1C). However, we observed higher n-3 DPA and lower DHA in the MEP-treated rats. The major radioactive peak in brain total phospholipids of the vehicle and MEP-treated rats was EPA accounting for 62% and 56% of total radiolabeled fatty acids, respectively; whereas the minor peak was palmitate accounting for 4% and 2% of brain total radiolabeled fatty acids in the vehicle and MEP-treated rats, respectively. In the vehicle-treated rats, 24% and 10% of brain total radiolabeled fatty acids corresponded to n-3 DPA and DHA, respectively, elongation and desaturation products of EPA; while in the MEP-treated rats, the composition of radiolabeled n-3 DPA and DHA was 38% and 3% of brain total radiolabeled fatty acids, respectively.

Because we detected elongation and desaturation products in the $^{14}$C-EPA-infused rats, the percent composition of radiolabeled fatty acids in each major phospholipid class was also measured (Figure 4.4-1D). The radiolabeled fatty acid composition of ChoGpl from the vehicle-treated rats was 65% EPA, 11% DHA, 18% n-3 DPA and 6% palmitate; whereas the composition from the MEP-treated rats was 66% EPA, 4% DHA, 26% n-3 DPA and 3% palmitate. In contrast to ChoGpl, only three of the four radiolabeled fatty acids were detected in EtnGpl. From EtnGpl of the vehicle-treated rats, the radiolabeled fatty acid composition was 24% EPA, 29% DHA and 47% n-3 DPA; while in the MEP-treated rats, the composition was 25% EPA, 14% DHA and 61% n-3 DPA. In PtdIns and PtdSer fractions, there were only two detectable radiolabeled peaks, EPA and n-3 DPA. In PtdIns of the vehicle and MEP-treated rats, EPA accounted for the majority of brain radiolabeled fatty acids at 66% and 65%, respectively. However, in PtdSer, there was more radiolabeled EPA (54% of total radiolabeled fatty acids) in the MEP-treated rats as opposed to the vehicle-treated rats (44% of radiolabeled fatty acids).
Lastly, there was no esterification of $^{14}$C-EPA into CerPCho after five minutes of infusion (data not shown).
Figure 4.4-1. HPLC separation and identification (intravenous infusion).
HPLC separation of brain radioactivity in perfusates and total phospholipids (total PL) of vehicle and MEP-treated rats (top panel) and phospholipid classes of $^{14}$C-EPA-infused rats (bottom panel) at 5-minute post infusion. Peak identified was confirmed with authentic standards, GC-MS and GC-FID. No co-elution with 16:0 (palmitate) and 22:5n-3 (n-3 DPA) was found. Percent composition of each identified radioactive fatty acid was calculated and used to adjust the radioactivity of the total phospholipids. See Table 2 for abbreviations.
4.4.3 Radioactivity in brain aqueous and lipid fractions

MEP significantly decreased radioactivity in the brain aqueous fraction (marker of β-oxidation) for all radiotracers (Figure 4.4-2). Upon vehicle-injections, the radioactivity of aqueous fractions between radiotracer-infused rats were similar (14C-palmitate: 17 nCi/g brain; 14C-DHA: 17 nCi/g brain; 14C-EPA: 21 nCi/g brain) (Figure 4.4-3). However, post MEP-treatment, radioactivity in the brain aqueous fraction of the 14C-palmitate-infused rats was significantly reduced by 74% whereas in the 14C-EPA-infused rats and the 14C-DHA-infused rats, the radioactivity of brain aqueous fractions were reduced by 54% and 23%, respectively.

In 14C-palmitate-infused rats, MEP had no significant effect on esterification into brain total phospholipids (Figure 4.4-2). However, there was a significant 65% increase in radioactivity of PtdIns in MEP-treated rats; while no significant differences were observed in ChoGpl, EtnGpl and PtdSer between the brains of the vehicle and MEP-treated rats (Figure 4.4-2). In 14C-DHA-infused rats, MEP had no significant effect on brain total phospholipids or any individual phospholipid classes (Figure 4.4-2). Finally, upon 14C-EPA infusion, MEP significantly increased total radioactivity in brain total phospholipids (vehicle: 17 ± 0.9; MEP: 22 ± 0.6 nCi/g brain; p < 0.05) (data not shown). However, upon adjusting the radioactivity for percent composition of 14C-EPA, we observed no significant effect of MEP-treatment on esterification of 14C-EPA into brain total phospholipids (p = 0.06), but esterification into PtdSer was increased upon MEP-treatment (Figure 4.4-2). It is possible with a larger sample size that we would have observed a significant effect of MEP on 14C-EPA incorporation into total phospholipids and this result should be interpreted with caution. The increase in total radioactivity upon MEP-treatment was, largely, due to significant increase in the esterification of radiolabeled n-3 DPA into brain total phospholipids (vehicle: 4.1 ± 0.2; MEP: 8.3 ± 0.2 nCi/g brain; p < 0.001) (data not shown). There was also a significant 49% increase in radioactivity of PtdSer with MEP-treatment; while other phospholipid classes were unaffected by MEP (Figure 4.4-2).

Furthermore, to account for the amount of infused radiotracer in the plasma available to the brain, incorporation coefficients ($k_i^*$ - Equation 4-1, 4-2) were determined. There was a significant increase in $k_i^*$ of 14C-palmitate into ChoGpl and PtdIns upon MEP-treatment (Table 4.4-3). Upon 14C-DHA infusion, there was no effect of MEP on $k_i^*$ (Table 4.4-3). In 14C-EPA-
infused rats, there was no effect of MEP on $k_i^*$ of brain total phospholipids. However, there were significant increases in $k_i^*$ for $^{14}$C-EPA into EtnGpl in addition to PtdSer (Table 4.4-3).
Figure 4.4-2. Radioactivity of the aqueous (AQ) and organic fractions including total phospholipids (total PL) and four major phospholipid fractions (n = 3-4) upon HPLC adjustment.

Data are mean ± SD and are expressed in nCi/g brain. Fractions were isolated by TLC and radioactivity counted by LSC. P-values indicated significantly different from vehicle-treated rats; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See Table 2 for abbreviations.
Figure 4.4-3. Kinetic summary of palmitate, DHA and EPA in brain total phospholipids for vehicle (black) and MEP-treated (grey) rats.

Kinetic rates ($J_{\text{in}}$ and $J_{\text{FA}}$) are nmol/g brain/day and radioactivity is nCi/g brain. $P$-values indicated significantly different from vehicle-treated rats; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $'p = 0.06$. $P$-values indicating significant differences between $^{14}$C-DHA-infused and $^{14}$C-EPA-infused rats are †$p < 0.05$, ‡$p < 0.01$, §§$p < 0.001$. See Table 2 for abbreviations.
4.4.4 Net rates of incorporation of palmitate, DHA and EPA from plasma unesterified fatty acid and brain acyl-CoA pools into brain phospholipids

The $J_{in}$ (Equation 4-3, 4-4) of palmitate was significantly higher in ChoGpl and PtdIns upon MEP-treatment (Table 4.4-3). There were no significant effects of MEP on the $J_{in}$ of DHA into brain phospholipids (Table 4.4-3). Upon MEP-treatment, the $J_{in}$ for EPA was significantly increased in EtnGpl and PtdSer (Table 4.4-3). Additionally, there were significant increases in the $J_{in}$ of EPA-synthesized n-3 DPA into brain total phospholipids, ChoGpl and EtnGpl of the MEP-treated rats. (Table 4.4-3) However, this was accompanied by significant decreases in the $J_{in}$ of EPA-synthesized DHA into brain total phospholipids and ChoGpl of the MEP-treated rats (Table 4.4-3).

MEP did not significantly affect the $J_{FA}$ (Equation 4-6, 4-7) of palmitoyl-CoA into brain phospholipids (Table 4.4-3). In $^{14}$C-DHA-infused rats, MEP significantly reduced the $J_{FA}$ of DHA-CoA in brain total phospholipids, ChoGpl and EtnGpl (Table 4.4-3). Similarly, in $^{14}$C-EPA-infused rats, there were significant reductions in the $J_{FA}$ of EPA-CoA in brain total phospholipids and ChoGpl with MEP-treatment (Table 4.4-3). Additionally, MEP did not affect the $J_{FA}$ of EPA-synthesized n-3 DPA-CoA into brain phospholipids (Table 4.4-3). However, in accordance with MEP’s effect on the $J_{FA}$ of DHA-CoA, MEP also significantly reduced the $J_{FA}$ of EPA-synthesized DHA-CoA into brain total phospholipids, ChoGpl and EtnGpl (Table 4.4-3).

In comparing $k_i^*$ between DHA and EPA, there was no significant difference between the $k_i^*$ (Table 4.4-3). However, the $J_{in}$ of DHA into brain total phospholipids was 3 fold higher than EPA upon vehicle-injection (Table 4.4-3). In addition, the $J_{FA}$ of DHA-CoA into total phospholipids was 156 fold higher as compared to EPA-CoA upon vehicle-injection (Table 4.4-3).
4.4.5 Rate of turnover of palmitate, DHA and EPA in brain phospholipids

While MEP did not affect the rate of turnover, $F_{FA}$ (Equation 4-8, 4-9), of palmitate and EPA in brain total phospholipids, MEP significantly reduced the $F_{FA}$ of DHA in brain total phospholipids by 24% per day (Table 4.4-3). When individual phospholipid classes were analyzed, MEP did not significantly alter the $F_{FA}$ of palmitate in any phospholipid classes (Table 4.4-3). However, MEP significantly reduced the $F_{FA}$ of EPA in ChoGpl and DHA in ChoGpl (Table 4.4-3). Similar to MEP’s effect on the $F_{FA}$ for DHA, MEP also significantly reduced the $F_{FA}$ of EPA-synthesized DHA to brain total phospholipids, ChoGpl and EtnGpl; whereas there was no effect on EPA-synthesized n-3 DPA (Table 4.4-3). The $F_{FA}$ of DHA into brain total phospholipids was 4 fold higher as compared to EPA upon vehicle-injection (Table 4.4-3).

In regards to half-life, $t_{1/2}$ (Equation 4-10, 4-11), MEP did not significantly affect the half-life of palmitate, DHA or EPA in brain total phospholipids (Table 4.4-3). Similarly, there was no effect of MEP on the $t_{1/2}$ of palmitate, DHA and EPA in phospholipid fractions except for EPA in ChoGpl where MEP-treatment significantly increased the $t_{1/2}$ of EPA by 1.7 fold (Table 4.4-3). In addition, MEP did not significantly affect the $t_{1/2}$ of EPA-synthesized n-3 DPA except in PtdSer where there was a significant increase in the $t_{1/2}$ by 1.6 fold (Table 4.4-3). Lastly, MEP significantly increased the $t_{1/2}$ of EPA-synthesized DHA in brain total phospholipids, ChoGpl and EtnGpl by 4.2, 4.3 and 2.5 fold, respectively (Table 4.4-3). The $t_{1/2}$ of DHA was 4 fold lower than EPA upon vehicle-injection (Table 4.4-3).
Table 4.4-3. Kinetic parameters in rat brain total phospholipids (total PL) and four major phospholipid classes.

<table>
<thead>
<tr>
<th></th>
<th>$k_i^*$ (ml/day/g)</th>
<th>$J_{in}$ (nmol/g/day)</th>
<th>$J_{FA}$ (nmol/g/day)</th>
<th>$F_{FA}$ (%/day)</th>
<th>$t_{1/2}$ (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>MEP</td>
<td>Vehicle</td>
<td>MEP</td>
<td>Vehicle</td>
</tr>
<tr>
<td><strong>Palmitate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total PL</td>
<td>12 ± 2.9</td>
<td>15 ± 2.8</td>
<td>2004 ± 468</td>
<td>2478 ± 458</td>
<td>32728 ± 17692</td>
</tr>
<tr>
<td>ChoGpl</td>
<td>8.0 ± 1.3</td>
<td>10 ± 0.82*</td>
<td>1297 ± 214</td>
<td>1649 ± 134*</td>
<td>20713 ± 9692</td>
</tr>
<tr>
<td>EtnGpl</td>
<td>1.9 ± 0.35</td>
<td>2.4 ± 0.4</td>
<td>303 ± 56</td>
<td>393 ± 65</td>
<td>4926 ± 2623</td>
</tr>
<tr>
<td>PtdIns</td>
<td>1.0 ± 0.23</td>
<td>1.9 ± 0.61*</td>
<td>161 ± 38</td>
<td>310 ± 100*</td>
<td>2679 ± 1655</td>
</tr>
<tr>
<td>PtdSer</td>
<td>0.49 ± 0.28</td>
<td>0.65 ± 0.33</td>
<td>80 ± 46</td>
<td>105 ± 54</td>
<td>1253 ± 792</td>
</tr>
<tr>
<td><strong>DHA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total PL</td>
<td>28 ± 2.9</td>
<td>25 ± 1.9</td>
<td>17 ± 1.8</td>
<td>16 ± 1.2</td>
<td>7259 ± 958</td>
</tr>
<tr>
<td>ChoGpl</td>
<td>8.2 ± 0.99</td>
<td>6.9 ± 0.61</td>
<td>5.2 ± 0.62</td>
<td>4.3 ± 0.38</td>
<td>2146 ± 225</td>
</tr>
<tr>
<td>EtnGpl</td>
<td>13 ± 2.2</td>
<td>12 ± 1.9</td>
<td>7.9 ± 1.4</td>
<td>7.2 ± 1.2</td>
<td>3233 ± 184</td>
</tr>
<tr>
<td>PtdIns</td>
<td>2.8 ± 0.12</td>
<td>2.7 ± 0.48</td>
<td>1.8 ± 0.078</td>
<td>1.7 ± 0.3</td>
<td>688 ± 144</td>
</tr>
<tr>
<td>PtdSer</td>
<td>0.90 ± 0.084</td>
<td>1.1 ± 0.18</td>
<td>0.56 ± 0.053</td>
<td>0.68 ± 0.11</td>
<td>237 ± 50</td>
</tr>
<tr>
<td><strong>EPA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total PL</td>
<td>20 ± 4</td>
<td>26 ± 2.4</td>
<td>5.7 ± 1.1**</td>
<td>7.3 ± 0.7*</td>
<td>47 ± 5.7**</td>
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<tr>
<td>ChoGpl</td>
<td>9.9 ± 1.9</td>
<td>13 ± 1.*</td>
<td>2.8 ± 0.55**</td>
<td>3.7 ± 0.31*</td>
<td>23 ± 3.3**</td>
</tr>
<tr>
<td>EtnGpl</td>
<td>2.7 ± 0.66*</td>
<td>4.3 ± 0.61*</td>
<td>0.78 ± 0.19**</td>
<td>1.2 ± 0.17*</td>
<td>6.4 ± 1.3***</td>
</tr>
<tr>
<td>PtdIns</td>
<td>1.8 ± 0.3*</td>
<td>2.5 ± 1.4</td>
<td>0.52 ± 0.087**</td>
<td>0.73 ± 0.41</td>
<td>4.5 ± 0.2*</td>
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<tr>
<td>PtdSer</td>
<td>0.60 ± 0.19</td>
<td>1.0 ± 0.2*</td>
<td>0.17 ± 0.055**</td>
<td>0.29 ± 0.057*</td>
<td>1.4 ± 0.36*</td>
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<tr>
<td><strong>EPA-&gt;n-3DPA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total PL</td>
<td>7.6 ± 1.5</td>
<td>17 ± 1.7***</td>
<td>2.2 ± 0.44</td>
<td>5.0 ± 0.49**</td>
<td>18 ± 2.2</td>
</tr>
<tr>
<td>ChoGpl</td>
<td>2.7 ± 0.53</td>
<td>5.0 ± 0.43**</td>
<td>0.79 ± 0.15</td>
<td>1.4 ± 0.12**</td>
<td>6.5 ± 0.92</td>
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<td>EtnGpl</td>
<td>5.2 ± 1.3</td>
<td>11 ± 1.5**</td>
<td>1.5 ± 0.36</td>
<td>3.0 ± 0.43**</td>
<td>12 ± 2.5</td>
</tr>
<tr>
<td>PtdIns</td>
<td>0.93 ± 0.16</td>
<td>1.4 ± 0.78</td>
<td>0.27 ± 0.045</td>
<td>0.39 ± 0.22</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>PtdSer</td>
<td>0.75 ± 0.24</td>
<td>0.88 ± 0.17</td>
<td>0.21 ± 0.068</td>
<td>0.25 ± 0.05</td>
<td>1.8 ± 0.45</td>
</tr>
<tr>
<td><strong>EPA-&gt;DHA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total PL</td>
<td>3.4 ± 0.67</td>
<td>1.6 ± 0.15*</td>
<td>1.0 ± 0.19</td>
<td>0.45 ± 0.044*</td>
<td>7.9 ± 0.97</td>
</tr>
</tbody>
</table>

*Significant differences compared to Vehicle MEP. **Significant differences compared to Vehicle. ***Significant differences compared to EPA->n-3DPA.
<table>
<thead>
<tr>
<th></th>
<th>ChoGpl</th>
<th>1.6 ± 0.31</th>
<th>0.80 ± 0.069**</th>
<th>0.46 ± 0.089</th>
<th>0.23 ± 0.02**</th>
<th>3.8 ± 0.54</th>
<th>0.98 ± 0.14**</th>
<th>0.30 ± 0.035</th>
<th>0.070 ± 0.01**</th>
<th>233 ± 28</th>
<th>1010 ± 146**</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtnGpl</td>
<td>3.2 ± 0.78</td>
<td>2.5 ± 0.35</td>
<td>0.92 ± 0.22</td>
<td>0.71 ± 0.1</td>
<td>7.6 ± 1.5</td>
<td>3.1 ± 0.81*</td>
<td>0.13 ± 0.023</td>
<td>0.054 ± 0.017*</td>
<td>556 ± 93</td>
<td>1370 ± 415*</td>
<td></td>
</tr>
<tr>
<td>PtdIns</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<td>ND</td>
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</tbody>
</table>

Data are mean ± SD. *P*-values indicated significantly different from vehicle-treated rats; *p < 0.05, **p < 0.01, ***p < 0.001, ′p = 0.06. *P*-values indicating significant differences between 14C-DHA-infused and 14C-EPA-infused rats are ′′p < 0.05, ′′′p < 0.01, ′′′′p < 0.001. See Table 2 for abbreviations.
4.5 Discussion

When β-oxidation was inhibited by MEP, we observed significant reductions in radioactivity of the aqueous fractions for 14C-palmitate-infused rats by 74%, 14C-EPA-infused rats by 54% and 14C-DHA-infused rats by 23% (Figure 4.4-2 and 4.4-3). The relatively small reduction in the brain aqueous fraction of 14C-DHA-infused rats suggests that β-oxidation products are a small percentage of the radioactivity in the aqueous fraction and the majority of radioactivity in the aqueous fraction may be attributed to other water soluble DHA metabolites or glycolipids. We also observed significant increases in the esterification of 14C-palmitate into PtdIns and of 14C-EPA into PtdSer; whereas there was no significant change in esterification of 14C-DHA into brain phospholipids (Figure 4.4-3). The lack of increase in esterification of DHA into brain phospholipids, upon MEP-treatment, is similar to a previous report with ARA which is the major n-6 PUFA in rat brain phospholipids (Freed et al. 1994). While there was no effect of MEP on the esterification of 14C-palmitate into cholesteryl esters, there was significant a 2 fold increase in the esterification of 14C-palmitate into diacylglycerol ($J_{in}$, vehicle: 106 ± 19 nmol/g/day; MEP: 238 ± 34 nmol/g/day; $p < 0.01$) and 2 fold increase into triacylglycerol ($J_{in}$, vehicle: 140 ± 23 nmol/g/day; MEP: 276 ± 19 nmol/g/day; $p < 0.01$) (data not shown) which was previously observed by Freed et al (Freed et al. 1994). Furthermore, there were significant increases in unesterified fatty acids known to be extensively β-oxidized upon entry to the brain including palmitate (Dhopeshwarkar and Mead 1969; Freed et al. 1994), linoleate (DeMar et al. 2006), α-linolenate (DeMar et al. 2005) and EPA (Chen et al. 2009; Chen et al. 2011); whereas the concentration of relatively metabolically stable unesterified PUFA, including ARA (Green et al. 2010) and DHA (DeMar et al. 2004), were unaffected. Previously, using GC-MS, we did not detect unesterified EPA in the brain, but our detection limit was 60 pmol (Chen et al. 2011). In the current study, again we did not detect EPA by GC-MS (data not shown), but upon more sensitive LC-MS/MS we estimated, for the first time, the brain unesterified EPA pool to be 19 pmol/g brain.

In accordance with previous studies (Chen et al. 2009; Chen et al. 2011), β-oxidation of palmitate, EPA and DHA were confirmed by the synthesis of radiolabeled cholesterol at 1.4, 1.1 and 0.8 nCi/g brain, respectively (data not shown). Moreover, we observed that within five minutes, EPA was metabolized into longer chain PUFA such as n-3 docosapentaenoate (n-3...
DPA) and DHA via elongation and desaturation as well as β-oxidized and resynthesized into palmitate via fatty acid synthase. When β-oxidation was inhibited by MEP, there was an increase in EPA elongation to n-3 DPA, but not DHA. This suggests that without β-oxidation to remove the influx of EPA, the brain compensates by elongating some EPA to n-3 DPA which emerging evidence suggests may be bioactive in the brain (Figure 4.4-3) (Phang et al. 2009; Kaur et al. 2011). Subsequently, the significant increase in total radioactivity of brain total phospholipids with MEP-treatment was partly the result of increased esterification of radiolabeled n-3 DPA. However, the brain synthesis of DHA from EPA appears inadequate to maintain the turnover of DHA in brain phospholipids because it would require 768 days to replace phospholipid DHA with DHA-CoA synthesized from EPA; whereas it only required about 1 day to replace phospholipid DHA with intact DHA-CoA.

In addition to the metabolism of EPA via elongation and desaturation, we also observed a 12% reduction in λ (vehicle: 0.12 ± 0.014; MEP: 0.24 ± 0.022; p < 0.01) and a 16% reduction in EPA-CoA upon MEP-treatment suggesting that inhibition of β-oxidation further reduced the recycling of EPA into brain total phospholipids (Figure 4.4-3). Interestingly, upon MEP-treatment, DHA-CoA was also reduced by 26%; whereas linoleoyl-CoA and α-linolenoyl-CoA concentrations increased by 25% and 49%, respectively. Although unclear, these changes in acyl-CoA concentrations may be explained by the selectivity of long chain fatty acyl-CoA synthetases where the inhibition of β-oxidation leads to an influx of un-metabolized linoleate and α-linolenate which competes for long chain fatty acyl-CoA synthetase thereby reducing synthesis of EPA-CoA and DHA-CoA (Bazinet et al. 2006b; Shimshoni et al. 2011).

Previously, we had demonstrated that there were differences between DHA and EPA metabolism in the brain including β-oxidation (Chen et al. 2009) and loss kinetics (Chen et al. 2011) that may partially explain the 250-300 fold difference in their brain phospholipid levels. In this study, we further explored additional differences in the metabolism of DHA and EPA to explain large differences in their levels. Firstly, there were no significant differences in the $k_i^*$ between DHA and EPA which recapitulated our previous in situ finding (Chen et al. 2009). Although there was no difference in the $k_i^*$ of DHA and EPA, the net rate of incorporation ($J_{in}$) of plasma unesterified DHA into brain phospholipids was 3 fold higher than EPA. Specifically, we found that the most striking difference was in the net rate of incorporation ($J_{FA}$) of brain DHA-CoA and EPA-CoA into brain phospholipids. The $J_{FA}$ of DHA-CoA into brain phospholipids was 154 fold
higher than EPA-CoA (Figure 4.4-3). This implies that the major difference in brain DHA and EPA concentration is not due to uptake from the plasma, but rather from recycling within the brain acyl-CoA pool. This large difference in esterification from acyl-CoA pool may be attributed to a 36 fold difference in brain acyl-CoA concentrations and a 50 fold difference in $\lambda$ (DHA: $0.0024 \pm 0.0003$ versus EPA: $0.12 \pm 0.01$; $p < 0.01$). This translated to 75% recycling of DHA per day (t1/2 of 22 hours) and 21% recycling of EPA per day (t1/2 of 3.3 days) in brain phospholipids. The lack of EPA recycling in brain phospholipids may explain the rapid loss of EPA (loss t1/2: 5 days or 14% per day) (Chen et al. 2011) from brain phospholipids as compared to DHA (loss t1/2: 33 days or 2% per day) (DeMar et al. 2004). Although not measured in this study, it would be interesting to investigate if EPA lost from brain phospholipids is converted to bioactive lipid mediators including E-series resolvins (Oh et al. 2011a; Oh et al. 2011b).

In comparison to previous reports calculating the $J_{in}$ of palmitate (724-822 nmol/g/day) (Grange et al. 1995; Chang et al. 1996; Contreras et al. 1999), our calculated $J_{in}$ of palmitate (2004 nmol/g/day) for adult rats was 2.4-2.8 fold higher. However, this difference appears to be driven by the plasma unesterified palmitate concentration as the $k_{i*}$ of palmitate in our study was comparable to previous reports (Freed et al. 1994; Grange et al. 1995; Purdon et al. 1997; Golovko et al. 2005). As compared to our previously reported $J_{out}$ for palmitate (469 nmol/g/day) (Chen et al. 2011), the $J_{in}$ exceeded the $J_{out}$, but the $J_{in}$ may be an overestimate. Albeit we only detected radiolabeled palmitate in the brains of $^{14}$C-palmitate-infused rats, we did not identify the position of radiolabeled carbon. Therefore, it is not possible to differentiate between intact infused $^{14}$C-palmitate and re-synthesized $^{14}$C-palmitate from recycling of $^{14}$C in de novo fatty acid synthesis (Carey 1975; Miller et al. 1987; Marbois et al. 1992; Lee et al. 1994a; Lee et al. 1994b; Edmond et al. 1998). Similarly, the $J_{out}$ may be underestimated because of extensive palmitate $\beta$-oxidation and active re-synthesis. Future studies that identify the position of radiolabeled carbons could improve the quantification of $J_{in}$ and $J_{out}$ for palmitate. The $J_{in}$ of DHA (17 nmol/g/day) was 89-91% lower than the reported $J_{in}$ of 150-190 nmol/g/day (Chang et al. 1999; Contreras et al. 2000). The difference was again driven by a discrepancy in plasma unesterified DHA concentration. There are several possible explanations for this discrepancy including: 1) different strains of rats, 2) our rat chow did not contain EPA and DHA which may decrease circulating unesterified EPA and DHA and/or 3) the use of heparin. In this study as opposed to previous kinetic reports, we did not administer heparin which activates endothelial
and hepatic lipoprotein lipase which promotes lipolysis of triacylglycerol into unesterified fatty acids (Grossman et al. 1954; Grossman et al. 1955). This highlights the importance of comparing kinetics within the same model strains with same dietary regimen and under the same experimental conditions. In comparison to the previous reported $J_{\text{out}}$ for DHA (58-257 nmol/g/day) (DeMar et al. 2004), the $J_{\text{out}}$ exceeded the $J_{\text{in}}$ suggesting that multiple plasma pools may be required to maintain DHA levels in brain phospholipids. However, as mentioned previously, differences in experimental conditions may account for the discrepancy; hence comparison of the $J_{\text{in}}$ and the $J_{\text{out}}$ under similar experimental conditions is warranted. In the case of EPA, the calculated $J_{\text{in}}$ of EPA (5.7 nmol/g/day) accounted for 36% of the $J_{\text{out}}$ (16 nmol/g/day) (Chen et al. 2011). This implies that the maintenance of EPA levels in brain phospholipids may require other plasma pools in addition to the unesterified fatty acid pool.

In our previous investigations, we consistently found higher esterification of $^{14}$C-EPA into PtdIns as compared to other phospholipid classes and DHA (Chen et al. 2009; Chen et al. 2011). This is of interest because PtdIns is a key modulator in several signaling cascades (Berridge et al. 1989) and is a candidate therapeutic target for drugs used to treat bipolar disorder a disorder where EPA may be efficacious (Jope et al. 1996; Silverstone et al. 2002; Ding and Greenberg 2003; Frangou et al. 2006; Frangou et al. 2007). Therefore, an interesting aspect of this study was to examine if EPA in PtdIns would increase upon MEP-treatment. Upon inhibition of β-oxidation by MEP, we observed no significant changes in the $J_{\text{in}}$ for EPA into PtdIns. Furthermore, in contrast to our in situ investigation (Chen et al. 2009), we found that the $k_{i*}$ of $^{14}$C-EPA into PtdIns was significantly lower when compared to $^{14}$C-DHA. The majority of $^{14}$C-EPA esterification was into ChoGpl and EtnGpl as opposed to PtdIns. There are two possible explanations for these discrepancies: 1) the brain concentration of EPA in PtdIns may be tightly regulated acutely in vivo or 2) increased esterification of $^{14}$C-EPA into PtdIns may require phospholipid remodeling that does not occur upon acute intravenous infusion in vivo (Chen et al. 2011). Therefore, a study that traces the time course of the metabolism and remodeling of EPA containing phospholipids is warranted.

In conclusion, the 250-300 fold difference in DHA and EPA brain phospholipid levels may be due to multiple redundant mechanisms including β-oxidation, decreased incorporation from the plasma unesterified fatty acid pool, elongation/desaturation to n-3 DPA and lower recycling within brain phospholipids (Figure 4.4-3). While β-oxidation may play a role in removing EPA
from the brain, this process is not necessary to maintain low levels of EPA because inhibition of 
\(\beta\)-oxidation can be compensated by increasing EPA elongation/desaturation and reducing EPA 
recycling.
Chapter 5
Rapid de-esterification and loss of eicosapentaenoic acid from rat brain phospholipids: an intracerebroventricular study


Contribution: As the first author, I performed all the experimental procedures, data analysis and drafting of the published manuscript. My overall contribution to the publication is 95%.
5 Objective 4: *in vivo* intracerebroventricular infusion

5.1 Abstract

Eicosapentaenoic acid (EPA, 20:5n-3) is being explored as a therapy in neurological diseases and disorders. Although it is known that palmitate is the most abundant fatty acid in the brain while EPA is one of the lowest, the mechanism by which the brain maintains this balance is unclear. Therefore, to trace the metabolism of these fatty acids in the brain, $^{14}$C-palmitate or $^{14}$C-EPA was administered via intracerebroventricular infusion to rats. From 4 to 128 days post-infusion, brains were collected after head-focused, high-energy microwave irradiation for biochemical analysis. At day 4 post-infusion, 57% (82 ± 26 nCi) of the total phospholipid radioactivity in $^{14}$C-palmitate-infused brains was intact palmitate; while in $^{14}$C-EPA-infused brains, 9% (2 ± 0.9 nCi) of the radioactivity was intact EPA. The half-life of esterified $^{14}$C-palmitate and $^{14}$C-EPA was 32 ± 4 (2% loss per day) and 5 ± 0.2 days (14% loss per day), respectively. Radioactivity was also detected in other saturates, monounsaturates and cholesterol, suggesting that the infused radiolabeled fatty acids were $\beta$-oxidized. In conclusion, the low concentration of EPA in brain phospholipids may be the result of extensive metabolism of EPA, in part by $\beta$-oxidation, upon entry into the brain and upon de-esterification from phospholipids.

5.2 Introduction

The brain possesses a unique fatty acid composition with high levels of palmitate and the polyunsaturated fatty acids (PUFA), arachidonic acid (ARA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3), but low levels of other n-3 PUFA, especially eicosapentaenoic acid (EPA, 20:5n-3) (Svennerholm 1964; Crawford and Sinclair 1971; Bazinet et al. 2005; Brenna and Diau 2007). In recent years, EPA has been investigated as a therapy for several neurological diseases and disorders (Burgess et al. 2000; Peet et al. 2001; Peet and Horrobin 2002; Puri et al. 2005; Keck et al. 2006; Ross et al. 2007; Sorgi et al. 2007; Amminger et al. 2010). In the periphery, EPA and its oxygenated derivatives (eicosanoids and E-series resolvins) regulate immune function (Peterson et al. 1998; Thies et al. 2001; Hasturk et al. 2007; Ishida et al. 2010), decrease
platelet aggregation (Ahmed and Holub 1984; Nieuwenhuys and Hornstra 1998; Adan et al. 1999) and increase cerebral blood flow (Black et al. 1984a; Black et al. 1984b; Katayama et al. 1997; Katsumata et al. 1999). However, the function of EPA in the brain is not clear and no study has reported the kinetics of EPA in the brain.

Since endogenous synthesis of PUFA is low within the brain compared to uptake from the plasma unesterified fatty acid pool (DeMar et al. 2005; DeMar et al. 2006), this suggests that the brain must maintain its PUFA concentrations via the uptake from plasma which may be supplied via diet and/or the liver. Using an in situ cerebral perfusion competition assay, Ouellet et al reported that 14C-EPA and 14C-DHA uptake were not saturable at supraphysiological concentrations and that 14C-EPA and 14C-DHA entered the brain at similar rates (Ouellet et al. 2009). While these findings are consistent with the model proposed by Hamilton and colleagues (Hamilton et al. 2001), it is unclear why brain phospholipids are enriched in DHA and low in EPA (Philbrick et al. 1987; Chen et al. 2008b; DeMar et al. 2008). As a follow up, we found that EPA was 2.5-fold more readily β-oxidized than DHA, upon 40 seconds in situ perfusion (Chen et al. 2009). Although EPA β-oxidation was higher than DHA, this difference does not fully explain an up to 300 fold difference in their brain concentrations (Philbrick et al. 1987; Chen et al. 2008b; DeMar et al. 2008). This discrepancy may be due to down-regulated β-oxidation in situ or the brain may be rapidly catabolizing de-esterified EPA from phospholipids.

The kinetics of fatty acid uptake and metabolism in the brain can be quantified in vivo (Stinson et al. 1991; Robinson et al. 1992; Lee et al. 1994a; Rapoport et al. 1997; Rapoport 1999; Contreras et al. 2000; DeMar et al. 2004; DeMar et al. 2005; DeMar et al. 2006; Green et al. 2010). $J_{in}$ describes the net rate of esterification from the plasma unesterified fatty acid pool into brain phospholipids (Robinson et al. 1992; Rapoport et al. 1997; Rapoport 1999; Contreras et al. 2000); whereas $J_{out}$ describes the net rate of fatty acid loss from brain phospholipids to cellular metabolism (Stinson et al. 1991; DeMar et al. 2004; Green et al. 2010). In addition to these kinetic parameters, the rate of synthesis ($J_{syn}$) represents the esterification of either de novo synthesized saturates and monounsaturates or the elongation/desaturation of PUFA precursors (Lee et al. 1994a; DeMar et al. 2005; DeMar et al. 2006). Upon calculation of these kinetic parameters, the model can predict the relative contribution of pools from which fatty acids enter the brain, with the assumption that the sum of $J_{in}$ and $J_{syn}$ approximate $J_{out}$ (Duncan and Bazinet 2010). In this regard, the $J_{in}$ of ARA and DHA are reported to be between 316-563 (Washizaki et
al. 1994; Contreras et al. 2001) and 150-190 nmol/g/day (Chang et al. 1999; Contreras et al. 2000), respectively; while the $J_{\text{out}}$ of ARA and DHA are 248-281 (Green et al. 2010) and 257 nmol/g/day, respectively (DeMar et al. 2004). Because the $J_{\text{in}}$ of ARA and DHA approximate their respective $J_{\text{out}}$, this suggests that $J_{\text{syn}}$’s contribution is relatively minor. When determined experimentally, brain synthesis ($J_{\text{syn}}$) of ARA and DHA from their precursors was $\leq 1\%$ and $\leq 0.2\%$ of their respective $J_{\text{in}}$ (DeMar et al. 2005; DeMar et al. 2006). Therefore, the kinetic model predicted that uptake from the plasma unesterified fatty acid pool ($J_{\text{in}}$) is likely a major contributor to brain ARA and DHA concentrations (Rapoport et al. 2007; Duncan and Bazinet 2010; Green et al. 2010).

With regards to saturated fatty acids, upon entry into the brain, palmitate is more readily β-oxidized compared to ARA and DHA (Dhopeshwarkar and Mead 1969; Dhopeshwarkar et al. 1973; Sun and Horrocks 1973; Kawamura and Kishimoto 1981; Miller et al. 1987; Gnaedinger et al. 1988). Furthermore, de novo synthesis of palmitate is active in the brain (Carey 1975; Miller et al. 1987; Marbois et al. 1992; Lee et al. 1994a; Lee et al. 1994b; Edmond et al. 1998); thus $J_{\text{syn}}$ may be a significant contributor of palmitate in brain phospholipids. In developing rats, $J_{\text{syn}}$ is 1992 nmol/g/day (Lee et al. 1994a); whereas in adult rats, $J_{\text{in}}$ is 724-822 nmol/g/day (Grange et al. 1995; Chang et al. 1996; Contreras et al. 1999). Similar to ARA and DHA, if the plasma unesterified palmitate pool is a major plasma contributor to brain phospholipids, then the sum of $J_{\text{syn}}$ and $J_{\text{in}}$ should approximate $J_{\text{out}}$. If $J_{\text{out}}$ exceeds the sum of $J_{\text{syn}}$ and $J_{\text{in}}$, then it would suggest that other plasma fatty acid pools contribute to palmitate uptake into brain phospholipids. However, if the sum of $J_{\text{syn}}$ and $J_{\text{in}}$ exceeds $J_{\text{out}}$, then it would imply that the known kinetic parameters may be overestimated or that the current model requires modification. In this study we administered $^{14}$C-palmitate or $^{14}$C-EPA intracerebroventricularly to rats in order to calculate $J_{\text{out}}$ for these fatty acids.
5.3 Materials and Methods

5.3.1 Animals

All procedures were performed in accordance with the policies set out by the Canadian Council on Animal Care and were approved by the Animal Ethics Committee at the University of Toronto. Male Sprague Dawley rats were purchased from Charles Rivers (Saint-Constant, QC, Canada) at 12 weeks of age and kept at an animal facility with a 12 hour light-dark cycle and a constant temperature of 22°C for three weeks. The rats received *ad libitum* access to standard chow (Teklad 2018, Harlan, Madison, WI, USA) and water. The linoleate (18:2n-6) and α-linolenate (18:3n-3) composition of the diet (measured by gas chromatography-flame ionization detection (GC-FID)) was 53% and 6%, respectively, and longer-chain PUFA (20:3n-3, 20:4n-6, EPA, 22:4n-6, 22:5n-6, 22:5n-3 and DHA) were <0.5%. At 15 weeks of age, seven rats were euthanized by head-focused, high-energy microwave irradiation (13.5 kW for 1.75 seconds; Cober Electronics Inc., Norwalk, CT, USA) to calculate baseline fatty acid concentrations and 45 rats were randomized to receive either 14C-palmitate ([1-14C]-palmitate, specific activity: 53 mCi/mmol, Moravek Biochemical Inc, Brea, CA, USA) or 14C-EPA ([1-14C]-EPA, specific activity: 54 mCi/mmol, Moravek Biochemical Inc, Brea, CA, USA) intracerebroventricularly. The purity of the radiotracers was confirmed to be > 99% by high performance liquid chromatography (HPLC) and liquid scintillation counting (LSC).

5.3.2 Intracerebroventricular tracer infusion

Rats were anesthetised with isoflurane inhalation (3% induction, 1-2% maintenance) and then placed in a stereotaxic frame (Stoelting, IL, USA). Before the incision was made, 50 μl of 0.5% sensorcaine was injected subcutaneously at the incision site. The skull was exposed and a small hole was drilled (+1.5 mm lateral and -1 mm anterior/posterior from bregma). A 5 μl injection containing 10 μCi of radiolabeled 14C-palmitate or 14C-EPA dissolved in 5 mM HEPES buffer (pH 7.4) containing 50 mg/ml fatty acid-free bovine serum albumin was infused at a constant rate of 0.17 μl/minute into the right lateral ventricle (-4 mm dorsal/ventral) of the brain using a 33-gauge beveled injection needle (World Precision Instruments, Sarasota, FL, USA). Thus 185
nmol of palmitate and 189 nmol of EPA were injected over 29.4 minutes. Equivalent doses of radiolabeled palmitate and EPA were administered because the uptake rate of these fatty acids into the brain appears to be similar (Chen et al. 2008a; Ouellet et al. 2009). During the surgery, rats were placed on a heating pad and given a subcutaneous injection of 1 ml 0.9% NaCl. Five minutes after the infusion, the needle was removed at a rate of 1 mm/minute. The skull was mended with cranioplastic cement and the incision was stitched with self-dissolving sutures. After surgery, rats recovered under a heating lamp for 20-30 minutes before returning to their cages. At 4, 16, 32, 64 and 128 days post-infusion, rats were euthanized by head-focused, high-energy microwave irradiation (13.5 kW for 1.75 seconds). The brain was excised and stored at -80°C until further biochemical analyses.

5.3.3 Lipid extraction

Total lipids were extracted according to the method of Folch, Lees and Sloane Stanley using chloroform: methanol: 0.88% KCl (2:1:0.75 by vol.) (Folch et al. 1957). Isolation of various lipid classes from the total lipid extract was achieved by thin-layer chromatography (TLC). All TLC plates were washed in chloroform: methanol (2:1 by vol.) and activated by heating at 100°C for one hour prior to use. Neutral lipids were separated with TLC G-plates (EMD Chemical, Gibbstown, NJ, USA) along with authentic standards (Avanti, Alabaster, AL, USA) in heptane: diethyl ether: glacial acetic acid (60:40:2 by vol.). TLC H-plates (Analtech, Newark, DE, USA) were used to separate phospholipid fractions along with authentic standards in chloroform: methanol: 2-propanol: 0.25% KCl: triethylamine (30:9:25:6:18 by vol.). Bands corresponding to authentic standards for total phospholipids, cholesterol, unesterified fatty acids, triglycerides, cholesteryl esters, ceramide phosphocholine (CerPCho), choline glycerolphospholipids (ChoGpl), phosphatidylserine (PtdSer), phosphatidylinositol (PtdIns) and ethanolamine glycerolphospholipids (EtnGpl) were visualized under UV light after spraying with 0.1% 8-anilino-1-naphthalene sulfonic acid.

Bands corresponding to total phospholipids and phospholipid fractions were collected into test tubes with a known amount of heptadecanoic acid (17:0), then converted to fatty acid methyl esters (FAME; ester-linked fatty acids) and fatty aldehyde dimethyl acetal (FADMA; vinyl
ether-linked fatty acids) with 14% boron trifluoride-methanol at 100°C for one hour. FAME from rats at 15 weeks of age (baseline) were quantified by GC-FID as described below. FAME and FADMA from 14C-palmitate- and 14C-EPA-infused brains were separated by HPLC, counted by LSC and identified by gas chromatography-mass spectrometry (GC-MS) and GC-FID as described below.

5.3.4 Cholesterol Analysis

Cholesterol bands from neutral lipid TLC were collected and saponified in 1 M methanolic NaOH at 90°C for one hour. Subsequently, the addition of saline and hexane separated the non-saponifiable cholesterol from saponifiable materials. After centrifugation at 1600 rpm (275 g units) for 4 minutes, the upper hexane phase containing cholesterol was transferred to a separate test tube. The hexane wash and centrifugation were repeated to enhance yield. Cholesterol extracts were reconstituted and transferred into scintillation vials with 5 ml of scintillation cocktail and counted by LSC.

5.3.5 High-performance liquid chromatography

FAME and FADMA from total and individual phospholipid pools were separated by HPLC (Waters 2690, Boston, USA) equipped with an in-line UV photodiode array detector (Waters 996, Boston, USA) set at a wavelength of 242 nm. Initial conditions were set at 1 ml/minute gradient system consisting of (A) 100% H2O and (B) 100% acetonitrile. The gradient commenced with 85% (B) for 30 minutes, then increased to 100% (B) over a 10 minute period where it was maintained for 20 minutes before returning to 85% (B) over a 5 minute period (Aveldano et al. 1983). Two columns were selected for total phospholipid separation (Luna C18, 4.6 x 250 x 5 μm; Phenomenex, Torrance, CA, USA and Symmetry C18, 4.6 x 250 x 5 μm; Waters, Mississauga, ON, Canada). Fractions from the Luna column were collected every minute for 55 minutes. The Luna column provided general separation of various saturates, monounsaturates and polyunsaturates. Fractions from the Symmetry column were collected every 10 seconds from 26 to 46 minutes of the 55 minute run. The Symmetry column separated
peaks that co-elute with the Luna column (palmitate/oleate and palmitoleate/n-3 docosapentaenoate). Radioactivity was quantified by LSC and peak identity was confirmed by GC-FID and GC-MS.

The Symmetry column resolved the co-elusion of palmitoleate and n-3 docosapentaenoate but was not capable of separating palmitate and oleate baseline to baseline. Thus after palmitate/oleate fractions from the Symmetry column were collected, one third of each fraction was removed for fatty acid quantification by GC-FID; while the other two thirds was counted by LSC. Multiple linear regression analysis was performed to determine the relative radioactivity of palmitate and oleate.

5.3.6 Liquid scintillation counting

Total phospholipid and phospholipid fraction bands as well as HPLC fractions from radiotracer infused brains were added to scintillation vials with 5 ml of scintillation cocktail (GE Healthcare Life Sciences, Baie d’Urfe, QC, Canada). Radioactivity was quantified by a Packard TRI-CARB2900TR liquid scintillation analyzer (Packard, Meriden, CT, USA) with a detector efficiency of 48.8%. Radioactivity was expressed in units of decays per minute (dpm); then converted to nCi/brain.

5.3.7 Gas chromatography-flame ionization detection

FAME were analyzed using a Varian-430 gas chromatograph (Varian, Lake Forest, CA, USA) equipped with a Varian FactorFour capillary column (VF-23ms; 30 m x 0.25 mm i.d. x 0.25 μm film thickness) and a FID. Samples were injected in splitless mode. The injector and detector ports were set at 250°C. FAME were eluted using a temperature program set initially at 50°C for 2 minutes, increasing at 20°C/minute and held at 170°C for 1 minute, then at 3°C/minute and held at 212°C for 5 minutes to complete the run at 28 minutes. The carrier gas was helium, set to a constant flow rate of 0.7 ml/minute. Peaks were identified by retention times of authentic FAME standards (Nu-Chek-Prep., Elysian, MN, USA). The concentration of each fatty acid
from phospholipids and unesterified fatty acids at baseline was calculated by comparison to the internal standard (17:0) and expressed as nmol/g brain (Chen et al. 2009).

5.3.8 Gas chromatography-mass spectrometry

Radioactive fractions and unesterified fatty acids were analyzed with an Agilent 6890 series gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a HP-5 ms capillary column (Agilent Technologies; 30 m x 0.25 mm i.d. x 0.25 μm film thickness) and an Agilent 5973 Network Mass Selective Detector (Agilent Technologies). The sample was injected in splitless mode. The injector port, ion source, and interface were 250, 230 and 280°C, respectively. FAME and FADMA were eluted using a temperature program initially set at 50°C for 1 minute, increased at 15°C/minute to 240°C for 5 minutes and then at 15°C/minute to 280°C for 8 minutes to complete the run in 29.33 minutes. The carrier gas was helium, set to a 1 ml/minute constant flow. A mass range from 50 to 700 amu was scanned using electron ionization energy of 70 eV. Peaks were identified on basis of selected fragmented ions. Peak identification was also confirmed by their retention time via GC-FID.

5.3.9 Kinetics

Radioactivity of total phospholipids and phospholipid fractions were adjusted by the percentage of radiolabeled palmitate and EPA determined via HPLC and LSC. The ^14^C-palmitate and ^14^C-EPA radioactivity was log-transformed and plotted against day post intracerebroventricular infusion, then the data were fitted by linear regression to provide slope (day^-1^) (GraphPad Prism version 4.0, La Jolla, CA, USA).

Loss half-lives of ^14^C-palmitate and ^14^C-EPA in brain phospholipids were calculated from the slopes of the regression lines from total phospholipids and phospholipid fractions by the following equation (Stinson et al. 1991):
**Equation 5-1.** Loss half-life of non-radiolabeled phospholipid fatty acids.

\[
    t_{1/2} = \frac{\log_{10} 2}{(\text{slope of regression line})}
\]

Then the half-lives (t\(_{1/2}\)) of palmitate and EPA were used to calculate their rate of loss (\(J_{\text{out}}\), nmol/g brain/day) from brain phospholipids, by the following equation (Rapoport et al. 2001):

**Equation 5-2.** Rate of loss of non-radiolabeled phospholipid fatty acids.

\[
    J_{\text{out}} = \frac{0.693 C_{\text{FA}}}{t_{1/2}}
\]

where \(C_{\text{FA}}\) is the baseline fatty acid concentration of palmitate or EPA in a phospholipid pool (nmol/g brain).

The fractional loss was then calculated by the following equation (Green et al. 2010):

**Equation 5-3.** Fractional loss of non-radiolabeled phospholipid fatty acids.

\[
    \text{Fractional loss} = \frac{J_{\text{out}}}{C_{\text{FA}}} \times 100
\]

5.4 Results

5.4.1 Baseline fatty acid concentrations

At 15 weeks of age, the concentration of palmitate in rat brain total phospholipids was 21358 ± 609 nmol/g brain (Table 5.4-1). After phospholipid fractionation, palmitate concentrations were 14393 ± 183, 2118 ± 75, 252 ± 9 and 232 ± 10 nmol/g brain in ChoGpl, EtnGpl, PtdIns and PtdSer, respectively. The concentration of EPA in brain total phospholipids was 116 ± 12 nmol/g brain. Upon phospholipid fractionation, EPA concentrations were 20 ± 1, 4 ± 0.3, 8 ± 0.3 and 17
± 0.9 nmol/g brain in ChoGpl, EtnGpl, PtdIns and PtdSer, respectively. Whereas DeMar et al found that PUFA concentrations remained consistent post-^{3}H-DHA infusion, we found that the concentration of measured fatty acids did not differ significantly between baseline and radiotracer-infused brains at day 4 implying that intracerebroventricular infusion did not affect brain fatty acid concentrations (data not shown, \( P > 0.1 \)).

We were unable to detect unesterified EPA in the rat brain (Figure 5.4-1). To estimate a threshold concentration of unesterified EPA in the brain, the plasma unesterified EPA concentration (2.18 nmol/ml) and cerebral blood volume (2.3%) were used to calculate a contamination of 50 pmol plasma unesterified EPA in the whole brain. Since the GC-MS detection limit was 60 pmol, the concentration of unesterified EPA in the brain was estimated to be less than 10 pmol/brain (Figure 5.4-1).
Table 5.4-1. Baseline concentration of brain phospholipid fatty acids (n = 7).

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Total PL</th>
<th>ChoGpl</th>
<th>EtnGpl</th>
<th>PtdIns</th>
<th>PtdSer</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>215 ± 17</td>
<td>105 ± 4.6</td>
<td>63 ± 3.3</td>
<td>3.1 ± 0.2</td>
<td>10 ± 1.2</td>
</tr>
<tr>
<td>16:0</td>
<td>21358 ± 609</td>
<td>14393 ± 183</td>
<td>2118 ± 75</td>
<td>252 ± 9.0</td>
<td>232 ± 10</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>344 ± 12</td>
<td>119 ± 13</td>
<td>79 ± 2.1</td>
<td>6.1 ± 0.2</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>18:0</td>
<td>19518 ± 393</td>
<td>4139 ± 82</td>
<td>5245 ± 131</td>
<td>485 ± 9.2</td>
<td>4168 ± 62</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>15799 ± 423</td>
<td>6899 ± 100</td>
<td>4850 ± 160</td>
<td>491 ± 15</td>
<td>2262 ± 65</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>3226 ± 79</td>
<td>1804 ± 21</td>
<td>786 ± 24</td>
<td>79 ± 3.1</td>
<td>159 ± 5.0</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>561 ± 16</td>
<td>288 ± 9.3</td>
<td>87 ± 2.7</td>
<td>27 ± 1.2</td>
<td>20 ± 1.5</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20:0</td>
<td>525 ± 35</td>
<td>65 ± 3.2</td>
<td>76 ± 5.8</td>
<td>15 ± 0.8</td>
<td>47 ± 2.8</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>1646 ± 110</td>
<td>350 ± 10</td>
<td>966 ± 50</td>
<td>70 ± 3.2</td>
<td>211 ± 37</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>112 ± 9.5</td>
<td>46 ± 1.0</td>
<td>57 ± 3.1</td>
<td>2.5 ± 0.2</td>
<td>10 ± 0.8</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>5862 ± 129</td>
<td>1590 ± 32</td>
<td>3546 ± 48</td>
<td>156 ± 5.6</td>
<td>367 ± 9.4</td>
</tr>
<tr>
<td>22:0</td>
<td>402 ± 36</td>
<td>54 ± 3.5</td>
<td>ND</td>
<td>17 ± 0.9</td>
<td>51 ± 3.6</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>126 ± 13</td>
<td>37 ± 2.1</td>
<td>41 ± 2.6</td>
<td>12 ± 0.6</td>
<td>38 ± 2.3</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>116 ± 12</td>
<td>20 ± 1.0</td>
<td>4.3 ± 0.3</td>
<td>8.1 ± 0.3</td>
<td>17 ± 0.9</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>1505 ± 67</td>
<td>163 ± 4.0</td>
<td>1169 ± 17</td>
<td>38 ± 1.0</td>
<td>248 ± 6.2</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>91 ± 8.3</td>
<td>43 ± 3.6</td>
<td>41 ± 14</td>
<td>6.4 ± 0.3</td>
<td>76 ± 3.9</td>
</tr>
<tr>
<td>24:1n-9</td>
<td>1294 ± 104</td>
<td>23 ± 2.5</td>
<td>ND</td>
<td>9.2 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>77 ± 2.0</td>
<td>12 ± 1.2</td>
<td>47 ± 1.8</td>
<td>1.6 ± 0.1</td>
<td>12 ± 0.8</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>6015 ± 274</td>
<td>957 ± 27</td>
<td>3929 ± 58</td>
<td>93 ± 4.7</td>
<td>1650 ± 37</td>
</tr>
</tbody>
</table>

Data are mean ± SEM and are expressed in nmol/g brain. Fatty acid concentrations were quantified by GC-FID. Total PL, total phospholipids; ChoGpl, choline glycerophospholipid; EtnGpl, ethanolamine glycerophospholipid; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine.
Figure 5.4-1. Identification and estimation of baseline unesterified EPA concentration in the brain.

A) GC-MS separation of unesterified fatty acids in the brain. B) GC-MS separation of brain unesterified fatty acids spiked with an authentic EPA standard. C) GC-MS separation of unesterified fatty acids in the plasma. EPA was 2.18 nmol/ml and plasma accounts for 2.3% of the brain volume. D) Mass spectrum of the peak at retention time of 15.95.
5.4.2 Distribution of radiolabeled fatty acid in brain regions

Four days after infusion of $^{14}$C-palmitate or $^{14}$C-EPA into the right lateral cerebral ventricle, brains were removed and dissected for radiotracer analysis. Prefrontal cortex (PFC), cortex and rest of brains (ROB) were separated into left and right hemispheres; while hippocampus, cerebellum, brainstem and olfactory bulbs were analyzed as whole tissues. The distribution of radioactivity (%/g tissue) in $^{14}$C-palmitate infused brains was 28.5% (right ROB), 11.9% (left ROB), 12.3% (right cortex), 1.6% (left cortex), 1.8% (right PFC), 0.9% (left PFC), 25.4% (brainstem), 9.2% (hippocampus), 8.3% (cerebellum) and 0.05% (olfactory bulbs). The distribution of radioactivity in $^{14}$C-EPA infused brains was 28% (right ROB), 5.5% (left ROB), 6.7% (right cortex), 3.4% (left cortex), 0.9% (right PFC), 0.4% (left PFC), 5.7% (brainstem), 43% (hippocampus), 6.7% (cerebellum) and 0.2% (olfactory bulbs). In accordance with a previous report (DeMar et al. 2004), the radioactivity in the right hemisphere (site of intracerebroventricular infusion) was greater than the left.

5.4.3 Radioactivity identification in total phospholipids and phospholipid fractions

At day 4 post-infusion, 72% of radioactivity detected in brain total phospholipids from $^{14}$C-palmitate-infused brains corresponded to a co-eluting palmitate and oleate peak at 45 minutes (Figure 5.4-2). Further separation revealed that 56% of radioactivity in brain total phospholipids was palmitate and 16% was oleate. In addition to palmitate and oleate, radiolabeled palmitoleate (1%), stearate/20:1n-9 (22%) and fatty aldehydes (5%) were detected. A gradual loss of radiolabeled fatty acids from total phospholipids was observed over the 128-day period (data not shown).

The majority of radioactivity in brain total phospholipids of $^{14}$C-EPA-infused brains was distributed among three peaks, eluting at 28, 37 and 45 minutes corresponding to DHA (27%), n-3 docosapentaenoate (21%) and palmitate/oleate (26%), respectively (Figure 5.4-2). Radiolabeled EPA only accounted for 9% of radioactivity in brain total phospholipids at day 4 post-infusion. The remaining radioactivity was palmitoleate (4%), stearate/20:1n-9 (9%) and
fatty aldehydes (13%). Similar to $^{14}$C-palmitate-infused brains, a gradual loss of radiolabeled fatty acids was observed over the 128-day period. Moreover, by 64 days post-infusion, the radioactivity of EPA in total phospholipids was below our detection threshold ($\leq$ 14 pCi/brain).

The percent composition of radiolabeled fatty acids in each phospholipid fraction was also determined. For $^{14}$C-palmitate-infused brains at day 4 post-infusion, the majority of radioactivity in ChoGpl and EtnGpl was palmitate (58% and 43%, respectively). The remainder of radioactivity in ChoGpl and EtnGpl included radiolabeled palmitoleate (0.9%; non-detectable), oleate (29%; 7%), stearate/20:1n-9 (12%; 31%) and fatty aldehyde (0.3%; 19%). The majority of radioactivity in PtdIns and PtdSer was stearate/20:1n-9 (39% and 75%, respectively); while the remainder of radioactivity was distributed among palmitate (36%; 9%) and oleate (25%; 15%) (Figure 5.4-3).

For $^{14}$C-EPA-infused brains at day 4 post-infusion, the identity of radioactivity varied between phospholipid fractions (Figure 5.4-3). In ChoGpl, 55% of radioactivity was palmitate/oleate while the rest was distributed amongst EPA (4%), DHA (15%), n-3 docosapentaenoate/palmitoleate (18%), stearate/20:1n-9 (7%) and fatty aldehyde (1%). In EtnGpl, DHA and n-3 docosapentaenoate/palmitoleate represented 38% and 37% of total EtnGpl radioactivity, respectively; whereas the remainder was associated with EPA (7%), palmitate/oleate (10%), stearate/20:1n-9 (5%) and fatty aldehyde (4%). In PtdIns, 44% of radioactivity was EPA; while DHA, n-3 docosapentaenoate/palmitoleate, palmitate/oleate and stearate/20:1n-9 corresponded to 2%, 33%, 11% and 10% of total PtdIns radioactivity, respectively. In PtdSer, the majority of radioactivity was found to be in DHA (36%) and n-3 docosapentaenoate/palmitoleate (35%); whereas the remainder consisted of EPA (3%), palmitate/oleate (4%) and stearate/20:1n-9 (22%). For both $^{14}$C-palmitate and $^{14}$C-EPA-infused brains, the pattern of radiolabeled fatty acid was broadly consistent from 4 to 128 days.
**Figure 5.4-2.** HPLC separation and identification in perfusate and brain total phospholipids (intracerebroventricular infusion).

HPLC separation of radioactivity in perfusates and of brain total phospholipids from $^{14}$C-palmitate- (left panel) and $^{14}$C-EPA-infused (right panel) brains at day 4 post-infusion. Each peak was identified by GC-MS and GC-FID. The ratio of n-3 docosapentaenoate and palmitoleate in $^{14}$C-EPA infused brains is 6:1; while the same peak from $^{14}$C-palmitate brains was exclusively palmitoleate. The ratio of palmitate to oleate in $^{14}$C-palmitate-infused brains was 3.4:1. Percent composition of each identified radioactive fatty acid peak was calculated and used to adjust the radioactivity of the total phospholipid fraction.
Figure 5.4-3. HPLC separation and identification in four major phospholipid classes (intracerebroventricular infusion).

HPLC separation of radioactivity in four major phospholipid fractions from $^{14}$C-palmitate- (upper panel) and $^{14}$C-EPA-infused (lower panel) brains at day 4 post-infusion. Each peak was identified by GC-MS and GC-FID. The ratio of palmitate to oleate in $^{14}$C-palmitate-infused brains was 1.97:1 (ChoGpl), 6.2:1 (EtnGpl), 1:1.66 (PtdSer) and 1.46:1 (PtdIns). Percent composition of each identified radioactive fatty acid was calculated and used to adjust the radioactivity of individual phospholipid fractions.
5.4.4 Rate of loss of phospholipid-esterified palmitate and EPA

At day 4 post-infusion, $82 \pm 26$ nCi of radiolabeled palmitate and $2 \pm 0.9$ nCi of radiolabeled EPA were esterified into brain phospholipids as compared to the initial infusion of 10 μCi of each radiotracer. When individual phospholipid fractions were analyzed, radiolabeled palmitate was predominantly esterified to ChoGpl (72% of total palmitate radioactivity); whereas radiolabeled EPA was esterified into PtdIns (47% of total EPA radioactivity).

For calculations of loss half-lives ($t_{1/2}$, Equation 5-1) and rate of loss ($J_{out}$, Equation 5-2), log transformed fatty acid radioactivity was plotted against days post-infusion (Figure 5.4-4). All slopes for $^{14}$C-palmitate were significantly different from zero ($P<0.05$); while three of the five slopes (total phospholipid, ChoGpl and EtnGpl) for $^{14}$C-EPA were significantly different from zero ($P<0.05$; Table 5.4-2). In total phospholipids, $^{14}$C-palmitate and $^{14}$C-EPA were lost at 2% ($t_{1/2} = 32$ days, $J_{out} = 469$ nmol/g/day) and 14% per day ($t_{1/2} = 5$ days, $J_{out} = 16$ nmol/g/day), respectively, according to Equation 5-3 (Table 5.4-2). Since there may be phospholipid class specific differences in the kinetics of fatty acid metabolism, individual fractions were examined. When phospholipid fractions were analyzed, $^{14}$C-palmitate loss $t_{1/2}$ ranged from $21 \pm 3$ (PtdIns) to $90 \pm 34$ days (PtdSer) and $^{14}$C-EPA loss $t_{1/2}$ ranged from $6 \pm 0.4$ (EtnGpl) to $25 \pm 16$ days (PtdSer). The net rate of loss from brain phospholipids ($J_{out}$) of $^{14}$C-palmitate and $^{14}$C-EPA ranged from $2 \pm 0.7$ (PtdSer) to $416 \pm 51$ nmol/g/day (ChoGpl) and $0.5 \pm 0.3$ (PtdSer) to $1.5 \pm 0.5$ nmol/g/day (ChoGpl), respectively. Since two of the five slopes (PtdIns and PtdSer) for $^{14}$C-EPA-infusion did not reach statistical significance, the calculated kinetic parameters of these fractions represents threshold values.
Figure 5.4-4. Linear regression analysis of log_{10}Radioactivity (nCi/brain) and days post-infusion.

$S = 0.036548 \pm 0.001307 \left( ^{14}C\text{-Palmitate} \right)$
$S = 0.06105 \pm 0.001858 \left( ^{14}C\text{-EPA} \right)$

14C-palmitate and 14C-EPA slopes, indicated by the line of best fit, were significantly different from zero ($P<0.05$) with the exception of 14C-EPA slopes in PtdIns ($P=0.1$) and PtdSer ($P=0.2$) fractions. 14C-palmitate and 14C-EPA slopes are significantly different from each other for total PL, ChoGpl and EtnGpl ($P<0.05$). Data are mean ± SE; $n = 3$ independent samples per fatty acid per time point. See Table 1 for abbreviations.
Table 5.4-2. Palmitate and EPA kinetic parameters in rat brain total phospholipids and phospholipid fractions.

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>Lipid Class</th>
<th>Slope (day(^{-1}))</th>
<th>SE (day(^{-1}))</th>
<th>P-Value</th>
<th>Loss t(_{1/2}) (day)</th>
<th>J(_{out}) (nmol/g/day)</th>
<th>Fractional Loss (%/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitate</td>
<td>Total PL</td>
<td>-0.009548</td>
<td>0.001307</td>
<td>&lt;0.0001</td>
<td>32</td>
<td>469</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>ChoGpl</td>
<td>-0.012550</td>
<td>0.001527</td>
<td>&lt;0.0001</td>
<td>24</td>
<td>416</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>EtnGpl</td>
<td>-0.012820</td>
<td>0.000870</td>
<td>&lt;0.0001</td>
<td>23</td>
<td>63</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>PtdIns</td>
<td>-0.014390</td>
<td>0.002090</td>
<td>&lt;0.0001</td>
<td>21</td>
<td>8</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>PtdSer</td>
<td>-0.003357</td>
<td>0.001258</td>
<td>0.0175</td>
<td>90</td>
<td>2</td>
<td>0.008</td>
</tr>
<tr>
<td>EPA</td>
<td>Total PL</td>
<td>-0.06105</td>
<td>0.001858</td>
<td>&lt;0.0001</td>
<td>5</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>ChoGpl</td>
<td>-0.03205</td>
<td>0.011100</td>
<td>0.0446</td>
<td>9</td>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>EtnGpl</td>
<td>-0.04773</td>
<td>0.003319</td>
<td>0.0001</td>
<td>6</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>PtdIns</td>
<td>-0.03981</td>
<td>0.019040</td>
<td>0.1047</td>
<td>8</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>PtdSer</td>
<td>-0.01197</td>
<td>0.007749</td>
<td>0.1971</td>
<td>25</td>
<td>0.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Half-lives of \(^{14}\)C-palmitate and \(^{14}\)C-EPA were derived from the slopes using t\(_{1/2} = \log_{10}2/(\text{slope of regression line})\). Baseline fatty acid concentration were used to derived rates of loss (J\(_{out}\)) from brain lipid classes using J\(_{out} = 0.693C_{FA}/t_{1/2}\), where C\(_{FA}\) is the baseline fatty acid concentration. P-values indicate significant difference of slope from zero. \(^{14}\)C-palmitate and \(^{14}\)C-EPA slopes are significantly different from each other for total PL, ChoGpl and EtnGpl (P\(<0.05\)). See Table 1 for abbreviations.
5.5 Discussion

Similar to previous reports (Philbrick et al. 1987; Chen et al. 2008b; DeMar et al. 2008), palmitate was the most abundant fatty acid in brain total phospholipids at $21358 \pm 609$ nmol/g brain or 27% of total fatty acids. Upon adjustment for pool size, palmitate was predominately esterified into ChoGpl (46% of total ChoGpl fatty acid) as compared to EtnGpl (9.1%), PtdIns (14%) and PtdSer (2.4%). As expected, the concentration of EPA was relatively low in brain phospholipids at $116 \pm 12$ nmol/g brain or 0.15% of total fatty acids, which is comparable to the reported human brain EPA concentration of $122 \pm 102$ nmol/g brain or 0.13% of total fatty acids (Igarashi et al. 2010). Although EPA appeared to be selectively esterified into ChoGpl ($20 \pm 1.0$ nmol/g brain) and PtdSer ($17 \pm 0.9$ nmol/g brain), when the concentration of EPA was adjusted for the total fatty acid content of each phospholipid fraction, 0.45% of total PtdIns fatty acid was EPA; whereas 0.06%, 0.02% and 0.17% of ChoGpl, EtnGpl and PtdSer fatty acids were EPA, respectively. In addition, when the esterification pattern of radiotracers was analyzed, similar to the fatty acid percent composition, $^{14}$C-palmitate and $^{14}$C-EPA were predominantly esterified to ChoGpl (72%) and PtdIns (47%), respectively. Thus, consistent with our previous in situ investigation, EPA appears to be preferably esterified to PtdIns (Chen et al. 2009). This is of interest as PtdIns modulates several signaling cascades (Berridge et al. 1989) and is a candidate target of therapies for bipolar disorder (Jope et al. 1996; Silverstone et al. 2002; Ding and Greenberg 2003) where EPA may be efficacious (Frangou et al. 2006; Frangou et al. 2007).

As fatty acids enter the brain, they can be esterified or metabolized. At day 4 post-infusion, $82 \pm 26$ nCi of $^{14}$C-palmitate and $2 \pm 0.9$ nCi of $^{14}$C-EPA were esterified to total phospholipids, the major brain fatty acid pool. Therefore, 99.2 and 99.9% of $^{14}$C-palmitate and $^{14}$C-EPA, respectively, disappeared upon entry into the brain. Albeit the concentration of infused EPA was higher than the unesterified fatty acid pool initially, after four days the amount of esterified radiolabeled palmitate and EPA was 1.52 and 0.039 nmol/brain or 0.007 and 0.03% of the phospholipid pool, respectively. To test if fatty acids were transferred from brain to peripheral tissues, the liver was analyzed for radioactivity. Similar to ARA and 20:1n-9 (Golovko and Murphy 2006), for both $^{14}$C-palmitate- and $^{14}$C-EPA-infused brains, 0.02% of radioactivity was detected in the liver at day 4 post-infusion suggesting that a negligible amount of the radiotracers
left the brain intact. This implies that the disappearance of fatty acids from the brain occurred via metabolism rather than diffusion from brain to periphery.

Unlike the intracerebroventricular administration of radiolabeled DHA (DeMar et al. 2004) or ARA (Green et al. 2010) where the majority of radioactivity detected in brain phospholipids was intact DHA and ARA, respectively, infused $^{14}$C-EPA was metabolized into radiolabeled n-3 docosapentaenoate and DHA in brain phospholipids suggesting that the brain can synthesize longer chain PUFA using EPA as a precursor (Kaduce et al. 2008). However, it is important to note that the radioactivity in n-3DPA and DHA was 0.048% and 0.064% of the initial dose, respectively. Furthermore, the appearance of radiolabeled saturates and monounsaturates in $^{14}$C-EPA-infused brain suggests that EPA was also β-oxidized into acetyl-CoA which can enter various synthetic pathways including fatty acid synthesis (Cunnane et al. 2003). In regards to $^{14}$C-palmitate-infused brains, radiolabeled stearate, palmitoleate, oleate and 20:1n-9 could originate from elongation and desaturation of infused $^{14}$C-palmitate or the β-oxidation product of $^{14}$C-palmitate, radiolabeled acetyl-CoA. At day 4 post-infusion, cholesterol from both $^{14}$C-palmitate- (4 ± 1 nCi/ brain) and $^{14}$C-EPA-infused (3 ± 1 nCi/brain) brains was radiolabeled; thus β-oxidation of palmitate and EPA were confirmed (Cunnane et al. 1994). In addition to metabolism via mitochondrial β-oxidation and elongation/desaturation, EPA may have been converted to oxygenated derivatives like eicosanoids and E-series resolvins and future studies examining this are warranted.

Along with various radiolabeled saturates, monounsaturates and PUFA, radiolabeled fatty aldehydes such as palmitaldehyde, olealdehyde and octadecanal were identified. The appearance of radiolabeled fatty aldehyde suggested that fatty acids were incorporated into plasmalogens, which are specialized pools of ChoGpl and EtnGpl with vinyl ether-linked fatty acids rather than ester-linkages. Therefore, as expected, ChoGpl and EtnGpl fractions, but not PtdSer and PtdIns, had radiolabeled fatty aldehydes when individual phospholipid fractions were examined (Sun and Horrocks 1968; Diagne et al. 1984). In accordance to previous reports (Miller et al. 1987; Cunnane et al. 2006), the appearance of de novo synthesized radiolabeled fatty acids, fatty aldehydes and cholesterol suggested that the brain conserved carbons from fatty acid β-oxidation.
In addition to the initial rapid disappearance of EPA in the brain, esterified $^{14}$C-EPA in brain total phospholipids was also rapidly lost to de-esterification. The EPA half-life of 5 days was 6-9 times faster than palmitate (half-life = 32 days), ARA (half-life = 44 days) (Green et al. 2010) and DHA (half-life = 33 days) (DeMar et al. 2004). Since de novo synthesis ($J_{\text{syn}}$) of PUFA in the brain is negligible, the $J_{\text{out}}$ of EPA (16 nmol/g/day) should approximate the rate of incorporation ($J_{\text{in}}$) of EPA from the plasma unesterified pool into brain phospholipids. This prediction could be tested with intravenous infusion of radiolabeled EPA.

The reported $J_{\text{in}}$ of palmitate (724-822 nmol/g/day) is 1.5-1.8 fold higher than our calculated $J_{\text{out}}$ for palmitate (469 nmol/g/day). However, the $J_{\text{in}}$ for palmitate may be overestimated as it was assumed that total radioactivity detected in brain phospholipids was intact $^{14}$C-palmitate (Grange et al. 1995; Chang et al. 1996; Contreras et al. 1999). Since some fatty acids are rapidly metabolized by the brain as evident by linoleate (DeMar et al. 2006) and $\alpha$-linolenate (DeMar et al. 2005), where 5 minutes after intravenous infusion, 71% and 63% of radioactivity in brain phospholipids were fatty acid metabolites, respectively, it is possible that palmitate is also rapidly metabolized. Future studies correcting $J_{\text{in}}$ for intact palmitate are warranted. Furthermore, due to recycling of $^{14}$C into de novo fatty acids, the half-life of infused $^{14}$C-palmitate quantified in this study may be underestimated. Future studies identifying the position of radiolabeled carbon could improve the estimation of $J_{\text{out}}$ for palmitate (Corso and Brenna 1997; Huang et al. 2000). Once these kinetic parameters are established, the difference between $J_{\text{in}}$ and $J_{\text{out}}$ could be used to estimate the $J_{\text{syn}}$ of palmitate. Although $J_{\text{syn}}$ of palmitate has been calculated to be 1992 nmol/g/day, this value was obtained from developing rats which have an increased requirement for palmitate due to myelination during brain growth (Dhopeshwarkar et al. 1969; Marbois et al. 1992); whereas mature rats have a lower palmitate requirement and down-regulated fatty acid synthetase activity in the brain (Cantrill and Carey 1975).

In conclusion, the low concentration of EPA in brain phospholipids appears to be the results of rapid metabolism, in part via $\beta$-oxidation, upon entry into the brain and the rapid de-esterification ($t_{1/2} = 5$ days) and loss (14%/day) of phospholipid esterified EPA. In contrast to EPA, palmitate is stably incorporated into brain phospholipids with half-life (32 days) and fractional loss (2%/day) comparable to ARA and DHA.
Inhibiting mitochondrial β-oxidation selectively reduces levels of non-enzymatic oxidative polyunsaturated fatty acid metabolites in the brain


Contribution: As the first author, I performed all the experimental procedures with exceptions to the lipidomics quantification via liquid chromatography-tandem mass spectrometry which was outsourced to Dr. Denis Reynaud. All data analysis and drafting of the published manuscript was done by me. My overall contribution to the publication is 85%.
6 Objective 5: *in vivo* intravenous injection

6.1 Abstract

Schoenfeld and Reiser recently hypothesized that fatty acid β-oxidation is a source of oxidative stress in the brain. To test this hypothesis, we inhibited brain mitochondrial β-oxidation with methyl palmitoxirate (MEP) and measured oxidative PUFA metabolites in the rat brain. Upon MEP-treatment, levels of several non-enzymatic auto-oxidative PUFA metabolites were reduced with few effects on enzymatically-derived metabolites. Our finding confirms the hypothesis that reduced fatty acid β-oxidation decreases oxidative stress in the brain and β-oxidation inhibitors may be a novel therapeutic approach for brain disorders associated with oxidative stress.

6.2 Introduction

While the brain constitutes only 2% of total body mass, it consumes 20% of total body energy. In contrast to the heart and liver, the brain is largely fueled by the oxidation of glucose rather than fatty acids, leading to a respiratory quotient of 0.97 to 1 (Dickens 1936; Sokoloff 1960). Even though, glucose yields 20% less ATP per carbon than palmitate, glucose oxidation consumes 15% less oxygen per ATP, suggesting that the brain’s dependence on glucose for energy over fatty acids reduces the risk of hypoxia in neurons where oxygen is limited (Erecinska and Silver 2001). According to Schoenfeld and Reiser, the selection of glucose over fatty acids may be evolutionarily beneficial to the brain as mitochondrial fatty acid β-oxidation generates superoxides via multiple pathways including; 1) increased binding to complexes I and III of electron transport chain, and 2) increased flavoprotein-ubiquinone oxidoreductase activity due to the high FADH2/NADH ratio generated by fatty acid β-oxidation (Seifert et al. 2010; Dumont and Beal 2011; Rodrigues and Gomes 2012). Fatty acid β-oxidation might increase peroxidation of polyunsaturated fatty acids (PUFA). Peroxidized PUFA, which are present in several brain disorders, may be especially problematic in the brain given the high levels of arachidonic acid (ARA; 20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3) in the phospholipid membrane (Igarashi et al. 2010; Chen et al. 2011). Lastly, given the relatively low expression of superoxide
dismutase and glutathione peroxidase, the brain may not have the capacity to handle oxidative stress induced by fatty acid \(\beta\)-oxidation (Cand and Verdetti 1989).

Therefore, to test the hypothesis of Schönfeld and Reiser that fatty acid \(\beta\)-oxidation increases oxidative stress in the brain (Schonfeld and Reiser 2013), we administered a carnitine palmitoyltransferase I inhibitor, methyl palmoxirate (MEP), to rats and analyzed the brain levels of non-enzymatic auto-oxidative PUFA metabolites and enzymatically-derived metabolites. Upon MEP-treatment, there was a selective decrease in brain basal levels of non-enzymatic auto-oxidative PUFA metabolites.

6.3 Materials and Methods

6.3.1 Animals

All procedures were performed in accordance with the policies of the Canadian Council on Animal Care and were approved by the Animal Ethics Committee at the University of Toronto. Male Sprague Dawley rats were purchased from Charles Rivers (Saint-Constant, QC, Canada) at 12 weeks of age and kept at the animal facility within an automated 12 hour light-dark cycle and a constant temperature of 22°C. The rats received ad libitum access to standard chow (Teklad 2018; Harlan, Madison, WI) and water. At 15 weeks of age, six rats were subjected to either high-energy, head-focused microwave irradiation or CO\(_2\) asphyxiation.

6.3.2 Tail vein intravenous injection

A separate group of eleven rats were implanted with a tail vein catheter (iv catheter 24 gauge/0.75 inch, Angiocath™; Becton Dickinson) and received either vehicle or 10 mg/kg of methyl palmoxirate (MEP; donated by S.I. Rapoport). Fifteen minutes post-injection, rats were rapidly euthanized by high-energy, head-focused microwave irradiation (13.5 kW for 1.6 s; Cober Electronics Inc., Stratford, CT) to avert ischemia for accurate quantification of \textit{in vivo} basal levels of non-enzymatic auto-oxidative PUFA metabolites and enzymatically-derived metabolites. Previously, we reported that this method reduced \(\beta\)-oxidation of fatty acid by 23%
to 74% (Chen et al. 2013). MEP readily crosses the blood-brain barrier with a plasma half-life of 0.6 min in the rat. The brain was excised and stored at -80°C for lipidomics profiling.

### 6.3.3 Eicosanoid/docosanoid preparation and extraction

Composite standards of lipid metabolites (natural or deuterated; Cayman Chemicals Company, Ann Arbor, MI) were diluted from stock solutions in ethanol for performing an eight-point calibration curve (0.05 to 5 ng). The internal standard mixtures were prepared in ethanol and added to all composite standards and samples prior to extraction. Siliconized glassware was used for extraction and sample preparations. Auto-oxidation of PUFA were minimized by extracting on ice, in reduced light conditions and with solvents containing 0.1% BHT. Frozen brain hemispheres were homogenized in methanol. Aliquots of 250 mg of homogenized brain were combined with 1 ng of internal standard mixture. In addition, external ARA, EPA and DHA standards were prepared along with homogenized brain samples. Samples were mixed for one minute, incubated on ice for 30 minutes, and then centrifuged at 1000 g for 10 minutes. Supernatants were collected; while the pellet was resuspended and mixed in ethanol for one minute, then centrifuged again. The ethanolic supernatants were collected and combined with previously extracted methanolic supernatant. The supernatants were evaporated under nitrogen gas, suspended in 10% ethanol, acidified to pH 3 with 1 N HCl and extracted three times with ethyl acetate. The ethyl acetate layer was washed to neutrality with water and dried under nitrogen gas. Residues from brain and external standard samples were reconstituted in acetonitrile:water (1:1 by vol.), transferred to inserts in amber vials, caped and immediately analyzed by LC-MS-MS.

### 6.3.4 LC-MS-MS based lipidomics

LC-MS-MS was performed on a 1290 UHPLC System (Agilent Technologies, Santa Clara, CA) and a QTRAP5500 Mass Spectrometer (ABSciex, Framingham, MA). Chromatography ran at a flow rate of 600 μl/min on a Zorbax SB-Phenyl column (Agilent Technologies; 3.0 x 50 mm, 3.5 μm) with a gradient initiating at 80% water and ramping up to 100% acetonitrile over 9 minutes.
The Mass Spectrometer was operated in negative ESI mode with a source voltage setting of 4500 V and a source temperature setting of 600°C. Precursor to product ion mass transitions were acquired by scheduled MRM. Quantitative analysis was performed by Analyst 1.5.2 Software (ABSciex). Area ratios of integrated peaks (natural to deuterated standard) were plotted against standard curves for quantification. The limit of quantification was 0.025 ng per sample and values below this point down to approximately 0.005 ng per sample were semi-quantitative. Measured lipid mediators were classified as non-enzymatic or enzymatic metabolites based on the appearance of auto-oxidative metabolites in external PUFA standards (ARA, EPA and DHA) upon extraction with ethanol. Metabolites exceeding the threshold of 0.005 ng were classified as non-enzymatic auto-oxidative metabolites while those below are considered as enzymatically-derived metabolites. Because 8-iso-PGF\textsubscript{3\alpha} is non-enzymatically produced from free-radical-induced peroxidation, it is categorized as a non-auto-oxidative metabolite.

6.3.5 Statistics

Data are presented as percent change in concentration relative to vehicle-injected rats and are expressed as mean ± SD. Statistical comparisons of eicosanoids/docosanoids between microwave fixed and CO\textsubscript{2} asphyxiated rats as well as vehicle-injected and MEP-treated rats were performed, a priori, using two-tailed Student’s t-test. Statistically significant differences are set at *\(p < 0.05\), **\(p < 0.01\) and ***\(p < 0.001\).

6.4 Results

6.4.1 Brain lipidomics of microwave fixed and CO2 asphyxiated rats

When rats were asphyxiated with CO\textsubscript{2}, there were significant increases in the levels of both non-enzymatic auto-oxidative metabolites and enzymatically-derived metabolites as compared to microwave fixed rats (Figure 6.4-1). This elevation ranged from a 5-fold increase in 5-hydroxyeicosatetraenoic acid (5-HETE) to a 633-fold increase in PGD\textsubscript{2}.
Figure 6.4-1. ARA-derived eicosanoid profiles of rat brains fixed with high-energy, head-focused microwave irradiation (white; n = 3) and CO₂ asphyxiation (black; n = 3).

Non-enzymatic auto-oxidative PUFA metabolites and enzymatically-derived metabolites for ARA were expressed as percent of vehicle concentration. P-values denote significant differences compared to vehicle injection at *P < 0.05, **P < 0.01, ***P < 0.001. Refer to methods for classification of non-enzymatic auto-oxidative PUFA metabolites. HETE, hydroxyeicosatetraenoic acids; EET, epoxytrienoic acid; PG, prostaglandin.
6.4.2 Brain lipidomics of vehicle-injected and MEP-treated rats

Inhibition of brain mitochondrial β-oxidation by MEP significantly reduced the levels of all measured HETE and epoxytrieneoic acids (EET), non-enzymatic auto-oxidative metabolites of ARA, by 23-44% and 32-50% compared to vehicle-injected rats, respectively, except for 15-HETE which was unaffected (Figure 6.4-2A). In contrast, there were no significant changes to the enzymatically-derived series 2 prostaglandins between the brains of vehicle-injected and MEP-treated rats. However, there was a significant 34% reduction in the level of 6-keto-PGF$_{1\alpha}$, a byproduct of PGI$_2$ (prostacyclin) in MEP-treated rats. Similarly, the brain level of hydroxyeicosapentaenoic acids (HEPE), non-enzymatic auto-oxidative metabolites of EPA, were reduced by 35% to 76% upon MEP-treatment relative to vehicle. There was also a significant 30% reduction of 17(18)-epoxyeicosatetraenoic acids (EpETE) with MEP-treatment. No significant differences were detected in the level of non-auto-oxidative EPA isoprostane, 8-iso-PGF$_{3\alpha}$, and enzymatically derived D17-6-keto-PGF$_{1\alpha}$, a byproduct of PGI$_3$ (Figure 6.4-2B). Lastly, with inhibition of brain fatty acid β-oxidation via MEP, the levels of 7- and 14-hydroxydocosahexaenoic acid (HDHA) were reduced by 49% and 28%, respectively; whereas 4-HDHA and 17-HDHA levels were not affected upon MEP-treatment relative to vehicle-injection (Figure 6.4-2C).
Figure 6.4-2. Eicosanoid and docosanoid profiles with vehicle-injection (white; n = 5) or MEP-treatment (black; n = 6).

A. ARA

Non-enzymatic auto-oxidative PUFA metabolites and enzymatically-derived metabolites for (A) ARA, (B) EPA and (C) DHA were expressed as percent of vehicle concentration. P-values denote significant differences compared to vehicle-injection at *P < 0.05, **P < 0.01, ***P < 0.001. Refer to methods for classification of non-enzymatic auto-oxidative PUFA metabolites. While 8-iso-PGF$_{3\alpha}$ is a recognized non-enzymatic EPA metabolite, based on our classification criteria, it was categorized as enzymatically-derived metabolite. HETE, hydroxyeicosatetraenoic acids; EET, epoxytrienoic acid; PG, prostaglandin; HEPE, hydroxyeicosapentaenoic acid; EpETE, epoxyeicosatetraenoic acid; HDHA, hydroxydocosahexaenoic acid.

B. EPA

C. DHA
6.5 Discussion

Schönfeld and Reiser proposed that fatty acid β-oxidation leads to increased oxidative stress in the brain (Schönfeld and Reiser 2013). Therefore, we examined the role of mitochondrial β-oxidation in the brain on concentrations of oxidative metabolites of ARA, EPA and DHA which are susceptible to formation of lipid radicals due to their multiple double bonds. The presence of auto-oxidative PUFA metabolites from pure PUFA standards were used to classify non-enzymatic auto-oxidative PUFA metabolites and enzymatically-derived lipid metabolites extracted from microwaved brains. Consistent with literature, we found that microwave fixation is important to avert artifacts from ischemia-induced synthesis of non-enzymatic auto-oxidative PUFA metabolites and enzymatically-derived metabolites (Figure 6.4-1) (Golovko and Murphy 2008). Thus, using microwave fixation, we were able to measure in vivo basal levels of oxidative lipid metabolites in the brain. We then observed that inhibition of brain mitochondrial β-oxidation with MEP, largely reduced the levels of non-enzymatic auto-oxidative PUFA metabolites of ARA, EPA and DHA (Figure 6.4-2), suggesting that brain reduced fatty acid β-oxidation decreased oxidative stress. It is unclear why 15-HETE was unaffected, but the MEP-treated rats exhibited relatively large variation in 15-HETE concentration as compared to other lipidomics measures. In addition, this study is limited to a static picture of HETE metabolism; therefore, it is possible that while no difference was observed with 15-HETE levels, there may be difference in its turnover or longer inhibition of β-oxidation is necessary to significantly reduce 15-HETE levels. We previously reported that MEP inhibition of β-oxidation in the brain does not alter the brain concentrations of unesterified ARA and DHA while it increased unesterified EPA (Chen et al. 2013). Furthermore, MEP does not affect the uptake of DHA and EPA into the brain (Chen et al. 2013). Thus, the reduction in non-enzymatic oxidative PUFA metabolites is unlikely due to decreases in levels of unesterified PUFA.

A limitation of this study was the use of the whole brain for lipidomics profiling. The circumventricular organs including the neurohypophysis and adenohypophysis of pituitary gland and pineal gland β-oxidize fatty acids instead of glucose (Vannucci and Hawkins 1983). Therefore, it would be of interest to see if subregions of the brain that rely on fatty acid β-oxidation have increased oxidative stress or higher antioxidant capacity.
The novel finding that MEP decreased markers of oxidative stress in the brain, may have potential therapeutic implications in traumatic brain injury, epilepsy, stroke, Alzheimer’s disease and Parkinson’s disease where, following the initial insult, the production of lipid peroxides increases (Adibhatla and Hatcher 2010; Hall et al. 2010). Inhibition of mitochondrial fatty acid β-oxidation in the brain, and decreasing oxidized PUFA may prevent the subsequent generation of lipid peroxides which can inflict secondary damage to the brain. However, the link between mitochondrial fatty acid β-oxidation and oxidative stress requires further detailed investigation. Especially, it would be interesting to examine if rotenone or paraquat-induced oxidative stress would increase PUFA auto-oxidative metabolites and if MEP is protective in these models.

In conclusion, consistent with the hypothesis of Schönfeld and Reiser, inhibiting brain fatty acid β-oxidation decreased non-enzymatic auto-oxidative PUFA metabolites in the rat brain. Therefore, mitochondrial fatty acid β-oxidation could be a novel therapeutic target for brain disorders associated with increased oxidative stress.
Chapter 7
General Discussion
7 Discussion

7.1 Overall findings

The research presented in this thesis supports the hypothesis that metabolic differences in DHA and EPA upon entry into the brain can result in large differences in brain phospholipid levels. The selective esterification and turnover of DHA in brain phospholipid over EPA, the selective shuttling of EPA for mitochondrial $\beta$-oxidation and the selective de-esterification of phospholipid-bound EPA, collectively, regulate the low levels of EPA in brain phospholipids.

7.1.1 Biological significance

Similar to DHA, EPA and its enzymatically-derived lipid mediators are regulator of inflammation and its resolution (Tjonahen et al. 2006; Ishida et al. 2010). In addition, the EPA-derived lipid mediators are anti-thrombotic and can increase cerebral blood flow (Bannenberg and Serhan 2010). Even though, DHA and its enzymatically-derived lipid mediators can regulate neuroinflammation and its resolution (Orr et al. 2013), the biological roles of EPA and its enzymatically-derived lipid mediators have yet to be elucidated. Furthermore, when fatty acid $\beta$-oxidation activity was reduced, the brain decreased EPA recycling in brain phospholipids and increased elongation of EPA to n-3 DPA; thereafter, eliminating excess non-$\beta$-oxidized unesterified EPA. This implies that the accretion of EPA in the brain, despite its potent biological effects, may be evolutionarily disadvantageous.

It is well recognized that EPA can compete with ARA for the binding of enzymes involved in lipid mediator synthesis including COX, LOX and cytochrome P450 as well as sterol O-acyltransferase and lysophosphatidylinositol acyltransferase which catalyzes remodeling of cholesteryl esters and PtdIns (Lee et al. 2008). Upon pulse-labeling of brain phospholipid in situ, we observed relatively large incorporation of $^{14}$C-EPA in cholesteryl esters and PtdIns which are enriched with ARA (Chen et al. 2009). This competition with ARA may be disadvantageous because ARA and its oxygenated lipid mediators, ARA-derived eicosanoids, are required secondary messengers involved in complex signaling cascades including inflammation and
synaptic transmission (Bergstrom 1967; Moncada and Vane 1979; Samuelsson 1979; Samuelsson 1981; Samuelsson et al. 1987; Axelrod 1990).

At a cellular level, ARA is involved in synaptic signaling including neuronal firing and neurotransmitter release (Ojeda et al. 1989; Kaufmann et al. 1996). ARA is also involved in the regulation of circadian rhythms and appetite (Baile et al. 1973; Hayaishi and Matsumura 1995). Lastly, ARA is essential for the induction of long term potentiation (LTP) via activation of the NMDA receptor (Axelrod 1990; Miller et al. 1992; Nishizaki et al. 1999). LTP mediates the plasticity of neuronal synapses, thus regulating crucial executive functions such as learning and memory which may confer a survival advantage (Markowitsch 1995; Cohen et al. 1997; Burgess et al. 2001; Colombel et al. 2004). Consequently, competition with ARA during postnatal development and in adulthood may interfere negatively on the proper functioning of many physiological processes (Du et al. 2010; de Souza et al. 2011).

Similar to the unique fatty acid composition in the adult brains, fetal brain PUFA are also composed predominantly of DHA and ARA; while levels of EPA are low (Harauma et al. 2010). The blood supply of EPA to the fetal brain is limited to 0.6 μmol/L of unesterified EPA in umbilical artery; whereas DHA and ARA are found more highly concentrated in all lipid pools of the blood including unesterified fatty acid, triacylglycerides, cholesteryl esters and phosphatidylcholine (Lewis et al. 2011). The supply of ARA and DHA is a mixture of maternal PUFA and in utero fetal liver synthesis of ARA and DHA from LA and ALA. However, the accretion of preformed DHA in the brain and retina is considerably higher than ALA-synthesized DHA (Su et al. 2001); whereas, the accretion of LA-synthesized ARA and ALA-synthesized EPA remains unclear. The low supply of EPA in fetal circulation may be caused by selective binding of DHA and ARA to placental membrane FABP and the high fatty acid oxidation capacity in the placenta (Campbell et al. 1998; Shekhawat et al. 2003; Oey et al. 2005). However, currently, no studies have examined the binding affinity of FABP for EPA nor the extent of EPA β-oxidation in the placenta. Therefore, the placenta may behave like the blood-brain barrier for the fetus and regulate the levels of EPA entering the fetal circulation.

Interestingly, in contrast to fetal tissues, EPA levels of endometrial phospholipids are relatively high in early gestational age (Zhang et al. 1995). The level of EPA steadily decreases in ChoGpl and EtnGpl through gestation; whereas, the level of EPA in PtdIns is stable through gestation but
lower at labor (Zhang et al. 1995). The loss of EPA from PtdIns at labor is consistent with the role of PtdIns in initiating parturition where PGI₃, synthesized from EPA, may aid to relax uterine smooth muscle and protect the fetus during labor (Needleman et al. 1979; Zhang et al. 1995).

### 7.1.2 Clinical implication

Under normal conditions, the competition of ARA and EPA may be problematic in the brain; however, in diseased state, decreasing ARA and increasing EPA may be therapeutic. Based on disease models in genetic knockouts, reduced synthesis of ARA-derived lipid mediators, either via reduction in ARA deacylation (cPLA₂ α-/-) or eicosanoid synthesis (COX-2-/-), decreases the susceptibility to ischemia-reperfusion brain injury and the resistance to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which damages neurons in substantia nigra to reduce striatal dopamine concentration in a model of Parkinson’s disease. Hence, the release of ARA from phospholipids and subsequent conversion to enzymatic-derived lipid mediators appears to exacerbate pathogenesis in disease models.

Hypothetically, by increasing EPA to replace ARA esterification in signaling phospholipids like PtdIns in disease models, may increase the probability of EPA release from PtdIns during disease progression which may reduce uncontrolled neuroinflammation via synthesis of anti-inflammatory EPA-derived lipid mediators (Tjonahen et al. 2006; Ishida et al. 2010). In addition, increasing unesterified EPA levels in disease models may also confer similar benefits. However, in attempts to increase the synthesis of EPA-derived eicosanoids via inhibition of mitochondrial β-oxidation, we found no effects on the levels of enzymatically derived metabolites from ARA, DHA and EPA; while the synthesis of non-enzymatic auto-oxidative metabolites decreased. Recently, Schönfeld and Reiser hypothesized that mitochondrial fatty acid β-oxidation might be correlated to oxidative stress which was supported by our observation of decreases in the levels of non-enzymatic auto-oxidative metabolites, precursors to lipid peroxides, upon inhibition of mitochondrial β-oxidation. In addition to potential therapeutic effects of EPA-derived eicosanoids, inhibition of mitochondrial β-oxidation may be new therapeutic approach to reduce lipid peroxide-induced secondary damages in diseases with oxidative stress.
7.2 Limitations

One of the major limitations of this research is the application in humans. While animal models provide a good platform to examine *in vivo* kinetics, all the findings may not translate to humans. Nevertheless, the differences in brain DHA and EPA levels is observed in rodents and humans alike. Therefore, even though it is unclear what the mechanism regulating EPA in human brains are, one would expect that human brains would have the same capacity and mechanisms to maintain low levels of EPA in brain phospholipids as rodents given similar observations of large differences in brain PUFA concentrations.

Another limitation in this thesis is the quantification of fatty acid β-oxidation. Although the aqueous fraction is a biomarker of fatty acid β-oxidation because it contains water soluble β-oxidation products including acetyl-CoA, glucose and amino acids (Miller et al. 1987), it does not account for the acetyl-CoA shunted to complete oxidation via the Krebs cycle. Hence, without quantification of radiolabeled carbon dioxide, we cannot accurately quantify the complete extent of PUFA β-oxidation. While we hypothesized that fatty acid β-oxidation is the major determinant in the resulting large differences in DHA and EPA concentrations, we were unable to provide an accurate *in vivo* measurement for the difference in PUFA β-oxidation. Instead, we were only able to infer a lowest estimation of the β-oxidation based on tracer recovery (DeMar et al. 2004; Chen et al. 2011).

7.3 Future research

Having characterized all the kinetic parameters associated with fatty acid uptake and metabolism for EPA in healthy rodent models, it would be interesting to examine the regulation of EPA as well as other PUFA in rodent models of disease, particularly, models of mood disorders including depressive disorder and bipolar disorder such as the chronic variable stress/chronic social defeat stress model (depressive-like symptoms) and psychostimulant-induced hyperactivity model (mania-like symptoms). While these are symptomatic models, changes in EPA regulation in the brain would potentially reveal target mechanisms by which EPA are
improving behavioural outcomes. In addition, in diseases associated with mitochondrial dysfunctions and disruption in blood-brain barrier integrity, brain fatty acid regulation may be altered and not able to handle overwhelming influx of non-metabolized PUFA which poses a high risk for oxidative stress. Therefore, by understanding the changes in the regulation of PUFA in the brain, we can identify novel PUFA targets and mechanisms of which development of treatment may be therapeutic.

7.4 Conclusion

Research presented in this thesis supported the hypothesis that relative low EPA levels in brain phospholipids as compared to DHA are maintained via multiple mechanisms including:

1. Increased EPA β-oxidation in the brain
2. Decreased EPA esterification and recycling in brain phospholipids
3. Increased EPA loss from brain phospholipids.

When β-oxidation is inhibited, other mechanisms can, in parts, compensate to maintain low EPA levels in the brain by:

3. Decreased EPA recycling in brain phospholipids and increased elongation to n-3 DPA.

Even though, we hypothesized that increasing unesterified EPA via inhibiting β-oxidation would increase levels of EPA-derived eicosanoids, we found that:

5. Levels of non-enzymatic auto-oxidative PUFA metabolite were decreased; while, levels of enzymatic-derived metabolites were unaffected.
References


composition and prostaglandin E2 production but have different effects on lymphocyte functions and cell-mediated immunity." Lipids 33(2): 171-180.


