The Effect of a Low n-6 Polyunsaturated Fatty Acid Diet on the Rate of Loss of Arachidonic Acid and Docosahexaenoic Acid from Rat Brain Phospholipids

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Nutritional Sciences
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Abstract

Arachidonic acid (ARA) and docosahexaenoic acid (DHA) are the predominant n-6 and n-3 polyunsaturated fatty acids (PUFA) in the brain, and alterations in their metabolism are thought to play a role in several neurological disorders. This thesis investigates how lowering dietary n-6 PUFA affects the rate of loss of ARA and DHA from rat brain phospholipids. Rats were fed a low (2% linoleic acid) or high (24% linoleic acid) n-6 PUFA diet for 15 weeks post-weaning. Baseline brain phospholipid fatty acid, eicosanoid and docosanoid concentrations were then measured. The remaining rats underwent intracerebroventricular infusion of $^3$H-ARA /$^3$H-DHA and the loss of radioactivity was measured over time to determine the rate of ARA/DHA loss. Low (vs. high) n-6 PUFA rats exhibited slower loss of ARA and more rapid loss of DHA. These changes in PUFA metabolism may provide insight into mechanisms of dietary-lipid-driven treatments, particularly low n-6 PUFA, for neurological disorders.
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List of Abbreviations

ALA  α-linolenic acid
ARA  arachidonic acid
CD36  cluster of differentiation 36, fatty acid translocase
CHD  coronary heart disease
ChoGpl  choline glycerophospholipids
CoA  coenzyme A
COX  cyclooxygenase
cPLA₂  cytosolic phospholipase A₂
Cyt P450  cytochrome p450
DHA  docosahexaenoic acid
EET  epoxytrienoic acid/epoxides
EPA  eicosapentaenoic acid
EtnGpl  ethanolamine glycerophospholipids
FAME  fatty acid methyl esters
GC-FID  gas chromatography-flame ionization detection
HETE  hydroxyeicosatetraenoic acid
HPETE  hydroperoxyeicosatetraenoic acid
HPLC  high performance liquid chromatography
ICV  intracerebroventricular
iPLA₂  calcium-independent phospholipase A₂
LA  linoleic acid
LC-MS-MS  liquid chromatography-tandem mass spectrometry
LSC  liquid scintillation counting
LO  lipoygenase
LT  leukotrienes
LX  lipoxins
MaR  maresin
MUFA  monounsaturated fatty acids
DPAn-3  n-3 docosapentaenoic acid
DPAn-6  n-6 docosapentaenoic acid
PD  protectin
PG  prostaglandin
PGI₂  prostacyclin
PL  phospholipid
PPAR-γ  peroxisome proliferator-activated receptor gamma
PtdIns  phosphatidylinositol
PtdSer  phosphatidylserine
PUFA  polyunsaturated fatty acids
RvD  resolvin
SFA  saturated fatty acids
sPLA₂  secretory phospholipase A₂
SPM  specialized pro-resolving mediators
TAG  triacylglycerol
TLC  thin layer chromatography
TPL  total phospholipid
TX  thromboxane
CHAPTER 1:
Introduction and Literature Review
1.1 General Introduction and Organization of Thesis

Fatty acid metabolism plays an important role in the maintenance of proper brain function. In order to understand how one can manipulate fatty acid metabolism for the benefit of health, it is critical to fully understand how fatty acid metabolism is regulated, as well as how it can be influenced by external factors including diet, drugs and genetics. This thesis focuses on the external factor of diet, and in particular the effect of differing levels of n-6 polyunsaturated fatty acids (PUFA).

Chapter 1 of this thesis begins with a general overview of fatty acids, and then narrows in on the main PUFA in the brain: arachidonic acid (ARA) and docosahexaenoic acid (DHA). It then provides background on how a low n-6:n-3 PUFA ratio may be beneficial for health, and how the metabolism of ARA and DHA is linked to neurological disorders. This will set the stage for the main question targeted by this thesis: Can dietary alterations in n-6 PUFA affect the metabolism of ARA and DHA in the brain? Chapter 1 will end by discussing the studies that have addressed this question so far, and it will transition into Chapter 2, which states the literature gaps, and establishes the main objectives and hypotheses of this thesis. Chapter 3 is the main experiment chapter, presenting the study design, results, discussion and conclusion. Chapter 4 aims to situate the study’s findings within the larger context of fatty acid metabolism, covering limitations, future directions, significance and overall conclusions.

1.2 Fatty Acid Structure and Transport

1.2.1 What is a Fatty Acid?

A fatty acid is a lipid that consists of a carbon chain with a methyl group at one end and a carboxylic acid group at the other. Short-chain fatty acids are 4 to 7 carbons long, medium-chain fatty acids are 8 to 12 carbons long and long-chain fatty acids are longer than 12 carbons. In nature, the chain length is usually an even number, containing up to 6 double-bonds. These double-bonds are mainly in the cis configuration. Trans fatty acids are predominantly formed by food processing/chemical manipulation, but they can occasionally be found in natural lipids (eg. conjugated linoleic acid in dairy products). Fatty acids are grouped by the number of double-bonds they have into saturated fatty acids (SFA, 0 double-bonds), monounsaturated fatty acids...
MUFA, 1 double-bond), and polyunsaturated fatty acids (PUFA, > 2 double-bonds). Palmitic acid (16:0) is the most abundant SFA found in nature, while oleic acid (18:1n-9) is the most abundant MUFA (AOCS 2014). Mammals, including humans and rats, can enzymatically synthesize all required fatty acids except for linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) (Smedley and Lubrzynska 1913; Rittenberg and Bloch 1945; Ponticorvo, Rittenberg et al. 1949; Williamson and Wakil 1966; Brophy and Vance 1975). These PUFA must be consumed in the diet, and thus, LA and ALA have been classified as essential fatty acids (Cunnane 2003). LA and ALA can then undergo desaturations and elongations to produce other longer-chain n-6 and n-3 PUFA, including arachidonic acid (ARA) and docosahexaenoic acid (DHA) (Figure 1.2-1) (Sprecher 2000; Milligan and Bazinet 2008; Brenna, Salem et al. 2009). Essential fatty acids are important for proper growth, development and physiology (Aaes-Jorgensen, Leppik et al. 1958).

Fatty acids have many functions in the body. They can be esterified to glycerol backbones, forming triacylglycerol (TAG) molecules that are the primary fat storage molecules making up adipose tissue. When esterified to a glycerol molecule that is also attached to a phosphate group with a choline/ethanolamine/serine/inositol head-group, they form the respective phospholipids: choline glycerophospholipid (ChoGpl), ethanolamine glycerophospholipid (EtnGpl), phosphatidylinositol (PtdIns) and phosphatidylserine (PtdSer). Cell membranes are comprised of a phospholipid (PL) bilayer, and membrane properties such as membrane fluidity are largely influenced by the types of fatty acids found in those phospholipids. Due to a selective aminophospholipid translocase, choline glycerophospholipids are more concentrated in the outer leaflet of the bilayer, while EtnGpl and PtdSer are more concentrated in the inner leaflet (Farooqui, Horrocks et al. 2000). In the brain, most PUFA are found esterified to phospholipids. There are also free (unesterified) fatty acids that act as signaling molecules, and are thought to help regulate many different processes. Finally, fatty acid metabolism serves many purposes. For example, fatty acids can undergo β-oxidation to create ATP, and they can also be metabolized into compounds that act as bioactive mediators (AOCS 2014).
**Figure 1.2-1.** Conversion of LA and ALA into longer-chain n-6 and n-3 PUFA.

The conversion of LA and ALA into longer chain n-3 and n-6 PUFA requires the enzymatic activity of Δ6-desaturase, elongase, and Δ5-desaturase. The n-3 and n-6 PUFA compete with each other for these enzymes as both metabolic pathways involve the same steps. The synthesis of the final products, DPAn-6 and DHA, also requires one cycle of β-oxidation.
1.2.2 Absorption and Transport in the Body

Most fat that is eaten gets digested in the small intestine by pancreatic lipases. Fatty acids, along with cholesterol, fat-soluble vitamins, monoglycerides and glycerol form micelles upon mixing with bile from the gallbladder. This facilitates their absorption into the intestinal mucosal cells. Short and medium-chain fatty acids are water-soluble and thus, can be transported directly in the bloodstream as phospholipids or as free fatty acids bound to carrier proteins like albumin. In the mucosa, the rest of the fatty acids are incorporated into lipoproteins called chylomicrons that are transported via the lymph to the thoracic duct, where they are released into the bloodstream (Carlier, Bernard et al. 1991; Wang, Liu et al. 2013).

1.2.3 Fatty Acids in Brain Phospholipids

Although there is little variation in the PL compositions of different lobes (frontal, parietal, temporal and occipital), the distribution across the PL classes in the human brain varies slightly with age (Svennerholm and Stallberg-Stenhagen 1968). The fetal brain is about 50% ChoGpl and 30% EtnGpl. As age increases, there is a shift toward more EtnGpl and less ChoGpl in the grey matter. In the white matter, the proportion of ChoGpl drops to ~25%, replaced by a larger proportion of PtdSer.

In the ChoGpl fraction, the predominant fatty acids are 16:0 (greater in grey matter) and 18:1n-9 (greater in white matter). 18:0 and 18:1n-9 are the major fatty acids found in the EtnGpl pool of grey matter and white matter respectively. ARA (20:4n-6) makes up ~16-18% of the EtnGpl pool, but this decreases with age. In grey matter, DHA (22:6n-3) increases with age to ~30% by adulthood. In white matter, however, it decreases over time. The composition of PtdSer is similar to that of EtnGpl, except for its lower concentration of n-6 PUFA. Conversely, the PtdIns pool has a high concentration of 18:0 and ARA, but a low concentration of n-3 PUFA (Svennerholm and Stallberg-Stenhagen 1968).

The brain, like other tissues, can synthesize SFA and MUFA de novo, but not PUFA. Thus, it obtains its PUFA through uptake from the plasma fatty acid pool. The mammalian brain has a unique composition of fatty acids, maintaining high levels of certain PUFA (eg. ARA and DHA) but low levels of other PUFA (eg. LA, ALA and EPA) (O'Brien, Fillerup et al. 1964; Svennerholm 1964; Svennerholm 1968; Svennerholm and Vanier 1972; Chen, Ma et al. 2008;
Chen, Liu et al. 2009; Chen, Liu et al. 2011). Studies suggest that the conversion of LA and ALA into ARA and DHA within the brain is not as efficient as it is in the liver (DeMar, Lee et al. 2006; Igarashi, DeMar et al. 2007; Igarashi, DeMar et al. 2007). In addition, the brain does not retain as much substrate for ARA and DHA synthesis, as 67% of LA and 59% of ALA is $\beta$-oxidized upon entry into the brain (Demar, Ma et al. 2005; DeMar, Lee et al. 2006). Thus, to allow for the accumulation of ARA and DHA, the brain relies heavily on its uptake of PUFA from the plasma, and on its controlled metabolism of PUFA.

At the blood brain barrier, PUFA uptake occurs via mechanisms that have not been fully elucidated. A few mechanisms have been proposed and investigated so far. The Edmond theory is that low-density lipoproteins interact with low-density lipoprotein receptors on the blood brain barrier to facilitate their endocytosis, and the release of PUFA into the brain (Edmond 2001). Although studies afterward have demonstrated that neither the low-density or very-low-density lipoprotein receptors are necessary for maintaining brain PUFA levels, this mechanism may still contribute to the process (Chen, Ma et al. 2008; Rahman, Taha et al. 2010). According to the Hamilton theory, unesterified fatty acids bound to albumin may enter the brain via a passive diffusion “flip-flop” mechanism (Hamilton, Johnson et al. 2001). The transporter fatty acid translocase CD36 was found to be unnecessary, in support of this passive diffusion method (Ouellet, Emond et al. 2009; Song, Elbert et al. 2010). Lagarde’s theory suggests that the brain preferentially obtains albumin-bound PUFA only when it is esterified in the lysophosphatidylcholine pool (Lagarde, Bernoud et al. 2001). So far, this mechanism has only been investigated in young rats and remains to be characterized in adult rats in case there may be a switch in method of PUFA uptake after development (Thies, Delachambre et al. 1992; Thies, Pillon et al. 1994). Finally, the most recent theory proposed is the Eckel theory, in which lipoprotein lipase releases PUFA from the triacylglyceride molecules of circulating lipoproteins, and the unesterified PUFA are transported across the blood-brain-barrier by CD36 (Goldberg, Eckel et al. 2009). As established previously, CD36 is not required, and PUFA may enter via passive diffusion in this model as well (Song, Elbert et al. 2010). It was also shown that this method may be region-specific, being used predominantly in the hypothalamus (Wang, Astarita et al. 2011). As of yet, no one mechanism is completely agreed upon.

After entering the brain, PUFA are transferred to the endoplasmic reticulum, where they are then activated by attachment to coenzyme A (CoA) through a reaction carried out by acyl-CoA
synthetase (Farooqui, Horrocks et al. 2000; Rapoport, Rao et al. 2007). Next, the fatty acyl-CoA are incorporated into the PL bilayer by acyltransferase, though they can also be transported to the mitochondria/peroxisome for β-oxidation (Rapoport, Rao et al. 2007; Rapoport 2008). There is also an active recycling of the PUFA in the membrane that contributes to fatty acid turnover and allows for constant remodeling of the cell membranes. This deacylation-reacylation process, called the Lands cycle, involves the hydrolysis of PUFA from the PL bilayer by phospholipase A₂, creating lysophospholipids that are readily available for re-esterification with other new/recycled PUFA (Lands 1960; Farooqui, Horrocks et al. 2000). The hydrolyzed PUFA are transported back to the endoplasmic reticulum by fatty acid binding protein. They are either reincorporated into the membrane or β-oxidized as described above. In such a way, the brain is able to continuously regulate and alter the composition of its esterified fatty acids.

1.3 The n-6:n-3 Polyunsaturated Fatty Acid (PUFA) Ratio

Over the past 10,000 years, the food supply has experienced a shift towards containing higher ratios of n-6 to n-3 PUFA. Humans are thought to have evolved on an n-6:n-3 PUFA ratio ~1:1, but current Western diets have ratios that have risen to an average of 15-17:1 (Simopoulos 2002). This is likely the result of the increased consumption of cereal grains, as well as the increased use of vegetable oils, especially those that are high in n-6 PUFA, in order to replace saturated fats (Cordain 1999; Simopoulos 1999; Simopoulos 2002). For example, between the years 1960 and 2000 in France, which is thought to have undergone similar changes as other developed/Western countries, the fatty acid profile of main plant-derived oils and margarines went from 24% to 42% LA, and the n-6:n-3 PUFA ratio rose from 20 to 33 (Ailhaud, Massiera et al. 2006). Plant oil consumption increased from 5 to 11 to 14 kg/inhabitant/year from 1950 to 1985 to 1996 (Ailhaud, Massiera et al. 2006). There has also been an increased use of grains in livestock feed, causing an increase in the n-6:n-3 PUFA ratio in meats and fish (Crawford, Gale et al. 1969; Simopoulos 2002; Weill, Schmitt et al. 2002). Compared to wild fish that rely on eating algae and smaller marine organisms, farmed fish have higher amounts of n-6 PUFA, as their fish meal often contains vegetable oils (Strobel, Jahreis et al. 2012). In addition, popular methods of food processing, such as frying or breading in vegetable oils, also contributes significantly towards raising the n-6:n-3 PUFA ratio in the food supply as n-3 PUFA are more susceptible to degradation (Strobel, Jahreis et al. 2012).
There is a growing concern that diets with a high n-6:n-3 PUFA ratio may have adverse health consequences. Since n-6 and n-3 PUFA compete for the same enzymes, the balance of their often opposing physiological functions depends heavily on the amount of each type of PUFA present in a tissue at any given time. A high n-6:n-3 PUFA ratio has been associated with the pathogenesis of many conditions/diseases, including cardiovascular disease, inflammatory disease, autoimmune disease, liver disease, cancer and more recently, major depression (Broughton, Johnson et al. 1997; James and Cleland 1997; Simopoulos 2002; El-Badry, Graf et al. 2007; Lucas, Mirzaei et al. 2011). In patients who had a primary myocardial infarction, randomization onto an ALA-rich diet with an n-6:n-3 PUFA ratio of 4:1 versus a control post-infarction diet with a 20:1 ratio caused an 80% reduction in cardiac mortality (de Lorgeril, Renaud et al. 1994). It is not clear, however, if the beneficial effects of lowering the n-6:n-3 PUFA ratio are mainly attributed to the increase of n-3 PUFA alone, as opposed to the combination of the high n-3 PUFA with the low n-6 PUFA intake. A meta-analysis of 25 studies by Harris, Poston and Haddock only showed an association between lower n-3 PUFA status and risk of coronary heart disease (CHD) (Harris, Poston et al. 2007). Interestingly, only ARA in TAG-rich adipose tissue, as opposed to other PL-rich tissues, was only associated with CHD risk, and LA even had a significant protective effect against non-fatal CHD endpoints. Similarly, a recent pilot clinical study did not show a significant advantage of a low versus high n-6:n-3 PUFA ratio in CHD risk (Lee, Dart et al. 2012). Thus, the effect of the n-6:n-3 PUFA ratio on CHD is not entirely conclusive. Looking at other diseases, patients with non-alcoholic fatty liver disease had a 6.6 fold higher n-6:n-3 PUFA ratio in their liver phospholipids than healthy controls, and high ratios have generally been associated with increased macrosteatosis (Araya, Rodrigo et al. 2004; Sanyal 2005; El-Badry, Graf et al. 2007). Studies have also shown that high n-6:n-3 PUFA ratios promote tumor angiogenesis and increase the risk of high grade prostate cancer as well as premenopausal breast cancer (Maillard, Bougnoux et al. 2002; Goodstine, Zheng et al. 2003; Williams, Whitley et al. 2011; Kang and Liu 2013).

The effect of altering the n-6:n-3 PUFA ratio has also been investigated in the brain. A low n-6:n-3 PUFA ratio in rodents is associated with increased expression of PPAR-γ (peroxisome proliferator-activated receptor gamma), which is thought to be protective against neurodegenerative diseases and brain ischemic/oxidative injury (Tian, Fan et al. 2011). In rats, lowering the dietary n-6:n-3 PUFA ratio from 26.45 to 4.47 caused a 25% increase (p < 0.05) in
the size of neurons in the CA1 region of the hippocampus (Hajjar, Goh et al. 2013). This was accompanied by an increase in the expression of synaptophysin, a pre-synaptic protein that is important for synaptic plasticity. A case-control study found that patients with major depression had significantly higher ARA:EPA ratios in their serum phospholipids compared to healthy controls (23.4 ± 14.5 versus 16.9 ± 10.2, mean ± SD, p = 0.001) (Maes, Smith et al. 1996; Simopoulos 2002). This suggests that a lower n-6:n-3 PUFA diet, with specific emphasis on lowering ARA while increasing EPA, may contribute to the treatment of major depression (Lucas, Mirzaei et al. 2011). The regulation of nociception is also influenced by n-6:n-3 PUFA intake. A recent clinical study found that feeding patients with chronic daily headache a diet that is high in n-3 PUFA and low in n-6 PUFA causes a reduction in the number of headache days per month, as well as a reduction in the severity and length of the headaches (Ramsden, Faurot et al. 2013). This was in comparison to a group of patients that was fed a diet low in n-6 PUFA alone, highlighting the importance of the high level of n-3 PUFA over the low level of n-6 PUFA.

More studies are needed to confirm whether associations with brain health are more related to high n-3 PUFA and low n-6 PUFA levels individually, or to the ratio (i.e. the combination of the two components). It can be difficult to differentiate between the two, as an alteration in the level of n-3 PUFA can affect the level of n-6 PUFA and vice versa. As one increases, the other one often decreases due to their competition for the same enzymes. When considering this “seesaw effect”, more attention should also be given to the specific n-3 PUFA or n-6 PUFA that is being manipulated. For example, Garg and Li have shown that an EPA-rich diet lowers serum total lipid ARA, while a DHA-rich diet does not (Garg, Reddi et al. 1994). The same study found however, that the DHA-rich diet specifically lowered PL ARA more than the EPA-rich diet.

1.4 Major PUFA in the Brain

Arachidonic acid (ARA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3) are the predominant PUFA in the brain, each making up around 10% of total brain fatty acids (Rapoport 2008). Like other PUFA, they are found mainly esterified in the stereospecifically numbered (sn)-2 position of membrane phospholipids (Farooqui, Hirashima et al. 1992). The effects of bioactive mediators produced by the metabolism of ARA and DHA have been of great interest in relation to health and disease, especially over the past couple decades. In the body, ARA and DHA supplementation are capable of preventing essential fatty acid deficiency, which occurs
when less than 1-2% of total calories is obtained from essential fatty acids (Aaes-Jørgensen, Leppik et al. 1958; Le, Meisel et al. 2012). This highlights the importance of ARA and DHA, over their precursors LA and ALA, in maintaining normal physiology, and it is particularly interesting that the brain is enriched in ARA and DHA, but not LA and ALA.

1.4.1 Arachidonic Acid (ARA)

ARA is the predominant n-6 PUFA in the brain. It was first isolated and identified in the early 1900s, but was not purified from the brain until 1924 (Hartley 1907; Hartley 1909; Wesson 1924). Its complete structure and connection to linoleic acid was determined in the mid-1900s (Dolby, Nunn et al. 1940; Arcus and Smedley-Maclean 1943). The main food sources of linoleic acid in Western countries like the United States are vegetable oils like sunflower/corn oil, meats and grain products (Hadders-Algra 2008). ARA can be found in meats, eggs and lean fish (Kris-Etherton, Taylor et al. 2000). In lower income countries, n-6 PUFA are mainly obtained from cereals and vegetable oils (Michaelsen, Dewey et al. 2011). Despite the large focus on DHA in brain neurodevelopment, adequate ARA consumption is particularly important for early brain development. The pre-natal brain accretes more ARA than DHA, especially during the first two trimesters (Svennerholm 1968; Martinez 1992). Supplementation of DHA can actually be detrimental to pre-natal and early post-natal development if the level of DHA is high enough to suppress the level of ARA. Despite having improved retina development, increased intakes of DHA at such a young age have been associated with poorer auditory and motor development in animals (Amusquivar, Ruperez et al. 2000; Haubner, Stockard et al. 2002; Auestad, Stockard-Sullivan et al. 2003; Heinemann, Waldron et al. 2005). Observational studies show that a higher prenatal level of ARA leads to better neurodevelopment in early infancy, but not in later life (Hadders-Algra 2008). Thus, for early brain development, an intake of at least around 2:1 ARA:DHA is recommended to ensure that ARA levels remain high enough (Brenna, Varamini et al. 2007; Hadders-Algra 2008).

1.4.2 Docosahexaenoic Acid (DHA)

DHA is the main n-3 PUFA in the brain. It was isolated in the early-mid 1900s from various fish oils (Whitcutt 1957). In the later 1900s, techniques for an easier and more efficient isolation of DHA from cod oil were discovered (Wright, Kuo et al. 1987). Fatty fish are the main dietary sources of DHA along with EPA, and levels are particularly high in mackerel, herring and
salmon (Kris-Etherton, Taylor et al. 2000). Sources of the DHA precursor ALA include nuts, seeds, flaxseed oil and soybean oil (Hadders-Algra 2008). After gestation, DHA accretion overtake ARA accretion in the brain, and as a result, DHA levels are often higher than ARA levels in adult brains (Martinez and Mougan 1998). So far, DHA is recognized as being particularly important during the pre/peri-natal period of brain development (McCann and Ames 2005; Innis 2008). Studies comparing babies that were fed formulas with/without DHA show that DHA is needed for proper development of visual skills as well as cognitive and motor skills (Heird and Lapillonne 2005; Fewtrell 2006). DHA has also been associated with improved neurogenesis in rodents (Katakura, Hashimoto et al. 2009). Fewer studies have looked at the effects of DHA supplementation on neurogenesis in adulthood/post-maturation. Although animal studies seem to show that DHA improves age-related decreases in neurogenesis, human studies have not been as conclusive (Dyall, Michael et al. 2010; Karr, Grindstaff et al. 2012).

1.5 ARA and DHA Metabolism

There are both ARA-selective and DHA-selective enzymes that control the metabolism and recycling of each of these PUFA independently from one another. As a result, the ARA and DHA cascades can be separately influenced/targeted by drug administration, genetic manipulations, diet alterations and disease pathology (Rapoport 2008).

1.5.1 ARA Metabolism into Eicosanoids

ARA is selectively hydrolyzed from the phospholipid membrane by calcium-dependent cytosolic phospholipase A$_2$ (cPLA$_2$, type IVA) (Clark, Lin et al. 1991; Shikano, Masuzawa et al. 1994; Strokin, Sergeeva et al. 2003; Farooqui, Ong et al. 2006; Burke and Dennis 2009). In its now unesterified, deacylated form, it can be metabolized into bioactive lipid-derived mediators called eicosanoids (Yue, Jansen et al. 2007; Harizi, Corcuff et al. 2008). Unesterified ARA can be metabolized by cyclooxygenases (COX-1, COX-2), lipoxygenases (5-LO, 12-LO, 15-LO) and cytochrome p450, creating three pathways of eicosanoid synthesis (Figure 1.5-1) (Harizi, Corcuff et al. 2008). Metabolism by the COX enzymes produces prostaglandin H$_2$ (PGH$_2$), which is quickly converted into other prostaglandins (PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, and PGI$_2$) and thromboxanes (TXA$_2$ and THB$_2$). These eicosanoids are thought to be mostly pro-inflammatory, and they regulate blood flow, pain, thrombosis and hormone secretion (Weissmann 1991; Serhan, Clish et al. 2000; Harizi, Corcuff et al. 2008). Through the LO-mediated pathway, ARA
produces hydroperoxyeicosatetraenoic acids (HpETE), which are converted into hydroxyeicosatetraenoic acids (HETE), and then leukotrienes (LT) and lipoxins (LX) (Masoodi, Eiden et al. 2010). Although leukotrienes, including LTC₄ and LTB₄ are associated with causing inflammation, it has now become clear that lipoxins (predominantly LXA₄ and LXB₄) are involved into the resolution of inflammation, a process that may help ameliorate the neuroinflammation that is associated with many neurological diseases (Serhan and Petasis 2011). Finally, the cytochrome p450 pathway produces 20-HETE and epoxides (EET). These eicosanoids help with the regulation of cerebral vascular function including blood flow and neurovascular coupling (Attwell, Buchan et al. 2010; Imig, Simpkins et al. 2011).

### 1.5.2 DHA Metabolism into Docosanoids

DHA is cleaved from the membrane by calcium-independent phospholipase A₂ (iPLA₂, type VIA) (Shikano, Masuzawa et al. 1994; Yang, Mosior et al. 1999; Strokin, Sergeeva et al. 2003; Farooqui, Ong et al. 2006; Green, Orr et al. 2008; Burke and Dennis 2009). Once unesterified, it can be converted into docosanoids via 15 and 12-lipoxygenase (Figure 1.5-1). Some of the docosanoids produced include the D-series resolvins, protectins and maresins which are involved in anti-inflammation as well as resolution of inflammation (Serhan and Petasis 2011). This is why they are also called specialized pro-resolving mediators (SPM). Some docosanoids have also been found to indirectly alter the enzymes involved in eicosanoid production. For example, neuroprotectin D1 down-regulates pro-inflammatory interleukin-1β which normally stimulates the expression of COX-2 (Bazan 2009). It also helps protect the brain against oxidative stress by up-regulating the expression of antiapoptotic proteins Bcl-2 and BclxL, as well as down-regulating the expression of pro-apoptotic proteins Bax and Bad (Bazan 2005; Mukherjee, Chawla et al. 2007).
**Figure 1.5-1.** Simplified schematic of the metabolism of ARA and DHA into eicosanoids and docosanoids.

ARA is hydrolyzed from the PL membrane by cPLA₂. It is converted to prostaglandins (PGD₂, PGE₂, PGF₂α), thromboxanes (TXA₂, TXB₂) and prostacyclins (PGI₂) by the COX-enzymes. It can also be converted to hydroxyeicosatetraenoic acids (HETE), leukotrienes (LT) and lipoxins (LX) by lipoxygenase enzymes (LO). Lastly metabolism by cytochrome p450 leads to production of other HETE and eicosatetraenoic acids (EET). DHA is hydrolyzed from the PL membrane by iPLA₂ and is then metabolized by lipoxygenases into resolvins (RvD), protectins (PD) and maresins (MaR). *Figure adapted from: (Harizi, Corcuff et al. 2008; Serhan and Petasis 2011)*
1.6 ARA and DHA Metabolism in Neurological Disorders

Many neurological diseases and disorders are associated with alterations in ARA and DHA metabolism. This includes Alzheimer’s disease, Parkinson’s disease, bipolar disorder.

1.6.1 Alzheimer’s Disease

Alzheimer’s disease is a progressive, neurodegenerative condition characterized by dementia as well as the accumulation of amyloid-β plaques and neurofibrillary tangles in the brain (Bazan, Molina et al. 2011). Neuroinflammation is thought to be important in the pathogenesis of Alzheimer’s disease (Hensley 2010; Hommet, Mondon et al. 2014). For example, a recent in vivo positron emission tomography study has shown that levels of translocator protein, a marker of neuroinflammation, are higher in Alzheimer’s disease patients than in healthy controls and even patients with mild cognitive disorder (Kreisl, Lyoo et al. 2013). Thus, an increased metabolism of DHA into resolvins, protectins and maresins, as well as a selective increase in the metabolism of ARA into lipoxins could potentially help to resolve the neuroinflammation in an Alzheimer’s disease setting (Serhan and Petasis 2011). The disease even appears to be characterized by an increased metabolism of ARA. For instance, post-mortem brains of Alzheimer’s patients display an up-regulated expression of ARA-selective enzymes (cPLA2, sPLA2, and COX-2) (Sun, Horrocks et al. 2007; Rapoport 2008). PET imaging has also revealed a more rapid incorporation of ARA into the brains of patients with Alzheimer’s disease compared to the brains of healthy controls (Esposito, Giovacchini et al. 2008). Not surprisingly, this increased incorporation was especially found in regions typically having higher levels of inflammatory cytokines and activated microglia (Rapoport 2008). Furthermore, neuroprotectin D1, which is depleted in the brains of Alzheimer’s patients, inhibits cell death, as well as the synthesis of amyloid-β plaques that are characteristic of the disease. In a cell culture of human neuronal-glial cells, neuroprotectin D1 had both an anti-apoptotic and anti-amyloidogenic effect, signifying its potential therapeutic effect in Alzheimer’s disease (Zhao, Calon et al. 2011).

1.6.2 Parkinson’s Disease

Parkinson’s disease is another neurodegenerative disorder in which the death of dopamine neurons in the substantia nigra region of the brain causes impaired balance, movement, and fine motor control (Bazan, Molina et al. 2011). It is also characterized by a progressive over-
accumulation of abnormal cytoplasmic aggregates of α-synuclein in the brain, along with neuroinflammation and increased oxidative stress (Vijitruth, Liu et al. 2006; Yakunin, Loeb et al. 2012). Thus, inflammation-resolving docosanoids or eicosanoids may contribute towards the therapy for Parkinson’s disease. In addition, studies have shown that COX-2 may play a critical role in microglia activation as well as dopaminergic cell death (Vijitruth, Liu et al. 2006). This may be in part due to the reactive oxygen species that are created as by-products of prostaglandin production (Nikolic and van Breemen 2001). As mentioned above, neuroprotectin D1 can downregulate the activation of COX-2, and thus, may be beneficial in this disease setting (Bazan 2009).

### 1.6.3 Bipolar Disorder

Bipolar disorder is characterized by alternating manic and depressive periods, and as a result, it is often accompanied by relatively high rates of suicide (Rapoport 2008). Studies suggest that bipolar disorder is, at least in part, caused by an up-regulated metabolism of ARA. First, ARA-metabolism enzymes, including cPLA₂, sPLA₂ and COX-2 are overexpressed in the frontal cortex of post-mortem brains from patients with bipolar disorder (Rao, Kim et al. 2007). Second, the main mood stabilizers (lithium, carbamazepine and valproic acid) used to treat bipolar disorder all seem to reduce ARA metabolism, without affecting the metabolism of DHA. Lithium and carbamazepine down-regulate the transcription of cPLA₂ and decrease the expression of its transcription factor, activator protein-2 (Chang, Grange et al. 1996; Bazinet, Rao et al. 2006; Rao, Bazinet et al. 2007). Reduced levels of cPLA₂ results in a reduced hydrolysis of ARA from the membrane and a lower ARA turnover. Valproate was found to selectively inhibit the long-chain fatty acyl-CoA synthetase that is responsible for the conversion of ARA to arachidonyl-CoA, a process required for ARA esterification into the PL membrane (Bazinet, Weis et al. 2006). Again, this effect was selective for ARA, and no effect of valproate was seen on the metabolism of DHA (Bazinet, Rao et al. 2005). In addition, chronic valproate in rats was found to decrease COX-2 transcription, also leading to decreased metabolism of ARA into prostaglandins (Rao, Bazinet et al. 2007). This further supports the involvement of neuroinflammation in bipolar disorder, and as discussed above, neuroinflammation is a process that is directly affected by the metabolism of both ARA and DHA (Stertz, Magalhaes et al. 2013). Lastly, when testing the therapeutic effect of a new potential drug called topiramate, studies were able to predict its inefficacy in preliminary clinical trials by its inability to alter
ARA metabolism (Lee, Ghelardoni et al. 2005; Kushner, Khan et al. 2006). Thus, ARA metabolism appears to play a significant role in both the pathology and the treatment of bipolar disorder.

1.7 Tracking Fatty Acid Metabolic Consumption with Infusion of Radiolabelled Fatty Acid in Rats

Aside from the brain, there is also a high level of DHA in the retina, and this level does not change greatly in the face of a dietary n-3 PUFA deprivation. Thus, researchers suspected that the retina was somehow conserving DHA in response to the dietary challenge, and they became interested in how they could measure the rate of DHA loss from the retina (i.e. the rate of metabolic consumption of DHA). In 1991, a study addressed this task by infusing a radiolabelled (tritiated) DHA into the vitreous humor of rat eyes, in vivo, and measuring the rate of loss of the tritiated DHA post-infusion (Stinson, Wiegand et al. 1991). The study also examined the effects of different levels of PUFA on the rates of loss. More specifically, rats were fed one of the following diets: 1) a 10% hydrogenated coconut oil diet (low n-3, low n-6 PUFA), 2) a 10% flaxseed oil diet (high n-3, low n-6 PUFA), or 3) a 10% safflower oil diet (high n-6, low n-3 PUFA). After 15 weeks of feeding, 20 μCi of tritiated DHA was intravitreally infused and it became evenly incorporated into the rat retina. A subset of rats was killed 1, 2, 3, 4 and 6 days after the infusion. The specific activities of the phosphatidyl choline and phosphatidyl ethanolamine fractions from the rod outer segments in the retinas were determined, and these measures of radioactivity were plotted against time. As expected, there was a loss of radioactivity from the retina over time. After logarithmically transforming the plotted curve, a linear regression analysis was done to determine the slope of this rate of loss. Next, the loss half-life ($t_{1/2}$) of DHA in the retina was calculated by this slope using Equation (1).

Equation (1): $t_{1/2} = \frac{\log_{10}(2)}{\text{slope}}$

Stinson and colleagues found that the half-lives of DHA in the coconut oil group and flaxseed oil group were 54 and 19 days respectively. The half-life of the safflower oil group was nondeterminable because the slope of DHA loss was not significantly different from zero. Thus, this provided evidence that n-3 PUFA deprivation caused a conservation of DHA in rat retinas.
1.8 The Effect of Altering Dietary n-3 and n-6 PUFA on Brain ARA and DHA Metabolism

With a method to measure the rate of PUFA metabolic consumption in the retina, researchers began considering how to use similar methods in the brain. Since a clear connection between PUFA metabolism and neurological disorders has been established, being able to directly measure diet-induced changes in PUFA metabolism is highly valuable. This is important for gaining a mechanistic understanding of how alterations in dietary n-3 or n-6 PUFA can lead to metabolic changes that confer benefit to patients in clinical trials.

1.8.1 Low n-3 PUFA Diet and Brain ARA and DHA Metabolism

In 2004, DeMar and colleagues investigated the effects of a low n-3 PUFA diet on the rate of loss of brain ARA and DHA (DeMar, Ma et al. 2004). For 15 weeks post-weaning they fed rats either an n-3 PUFA adequate diet containing ALA at 4.4% of total fatty acids, or an n-3 PUFA deprived diet containing ALA at 0.04% of total fatty acid. By 15 weeks, there was a 95% lower level of ALA and a 90% lower level of DHA in the serum of n-3 PUFA deprived rats compared to the n-3 PUFA adequate rats. There was no significant change in the level of ARA or LA. This corresponded to a 10 fold increase in the n-6:n-3 PUFA ratio. The brain total PL pool of n-3 PUFA deprived rats had a 37% lower DHA concentration and a 95% higher DPAn6 concentration. Following the method developed in the retina by Stinson and colleagues in 1991, 40 μCi of tritiated DHA was infused into the right lateral ventricle of each rat, and the radioactivity of brain phospholipids were measured at 2, 6, 12, 24, 36, 48, and 60 days post-infusion. The loss half-lives from total PL as well as all of the PL fractions were significantly lower in the n-3 PUFA deprived rats versus the n-3 PUFA adequate rats. This showed that the n-3 PUFA deprived rats were conserving DHA. The half-lives were used to calculate the rates of loss ($J_{out}$) for each PL fraction. $J_{out}$ is a measure of how many fatty acid molecules per gram of brain are lost each day, and it is calculated using Equation (2):

Equation (2): $J_{out} = \frac{0.693 * C_{DHA}}{t_{1/2}}$ where $C_{DHA}$ is the baseline concentration of DHA in un-infused rats after 15 weeks of their dietary treatment.
Since the same level of n-3 PUFA deprivation did not change the rate of loss of ARA, it was concluded that the conservation response was likely selective for DHA (Green, Liu et al. 2010). Changes in enzymatic activity are in support of these changes in the metabolism of DHA. In 2007, a study by Rao and colleagues found that below an ALA level of 0.8%, there is a decrease in the activity of enzymes that metabolize DHA (iPLA₂ and COX-1), while there is a complimentary increase in the activity of ARA-metabolizing enzymes (cPLA₂, sPLA₂, and COX-2) (Rao, Ertley et al. 2007). Another study found that n-3 PUFA deprivation reduces the incorporation of DHA into brain PL 40-fold, which accounts for the lower levels of DHA in brain PL (Contreras, Greiner et al. 2000). Likely as another conservation response, there is also a 30-70% reduction the turnover and recycling of DHA in brain PL. Changes in uptake and turnover were not seen in ARA (Contreras, Chang et al. 2001).

1.8.2 Low n-6 PUFA Diet and Brain ARA and DHA Metabolism

Contrary to lowering n-3 PUFA in the diet, a few studies have also been looking at the effects of a low n-6 PUFA diet. In 2009, Igarashi and colleagues compared rats that were fed a high n-6 PUFA diet (28% LA) to rats fed a low n-6 PUFA diet (2% LA) (Igarashi, Gao et al. 2009). They found that there were no differences in overall growth or body weight aside from a 10% decrease in testis size. ARA concentrations decreased by 86%, 27%, 68%, 39%, 25% and 77% in the serum, brain, liver, heart, testis and epididymal adipose tissues respectively. There was an 8 to 9-fold increase in serum, brain and liver eicosatrienoic acid (20:3n-9), which is a marker of LA deficiency. There was also a significant increase in eicosapentaenoic acid (EPA, 20:5n-3). In addition, DHA concentrations in the brain increased by 11%. In 2011, a study found that low n-6 PUFA rats had an increased expression of DHA-metabolizing enzymes (iPLA₂ and 15-LOX), accompanied by a decreased expression of ARA-metabolizing enzymes (cPLA₂ and COX-2) (Kim, Rao et al. 2011). This may have been due to changes in their respective transcription factors. SREBP-1, which is a transcription factor for iPLA₂ was present at higher levels, and transcription factors for cPLA₂ and COX-2 (AP-2α and NF-κB p65) were present at lower levels in low n-6 PUFA rats. Finally, in 2012, an IV- infusion of tritiated DHA was done in low versus high n-6 PUFA rats, and the rates of incorporation/uptake (Jₘ) were calculated (Igarashi, Kim et al. 2012). The low n-6 PUFA rats had a 45% higher rate of DHA incorporation into total PL than the high n-6 PUFA rats. This corresponded to a 49% higher Jₘ in ChoGpl, a 39% higher Jₘ in EtnGpl, a 45% higher Jₘ in PtdSer and a 40% higher Jₘ in PtdIns. The turnover of DHA was
increased by 30% in ChoGpl, by 65% in PtdIns, and by 84% in the PtdSer fraction, but not in the EtnGpl fraction or total PL as a whole.
CHAPTER 2:
Thesis Rationale, Objectives and Hypotheses
2.1 Highlighted Literature Gaps: Metabolic loss of ARA and DHA in Rats on a Low n-6 PUFA Diet

So far, studies have only investigated the effects of a low n-6 PUFA diet on the uptake and turnover of DHA in the rat brain. As of yet, no one has directly examined the effects it may have on the rate of loss ($J_{out}$) of both ARA and DHA from rat brain phospholipids. This is important to determine because the amount of ARA and DHA in the brain phospholipids depends not only on the amount that enters the phospholipids, but also on the amount that leaves.

2.2 Thesis Objective

To address the highlighted literature gaps, the objective of this thesis is to determine the effect of a low versus high n-6 PUFA diet on the rate of loss ($J_{out}$) of ARA and DHA in rat brain phospholipids.

2.3 Hypotheses

As discussed in Chapter 1, previous studies have shown that a low n-6 PUFA diet leads to a down-regulation of ARA-metabolizing enzymes, an up-regulation of DHA-metabolizing enzymes and an up-regulated incorporation and turnover of DHA (Igarashi, Gao et al. 2009; Kim, Rao et al. 2011; Igarashi, Kim et al. 2012). Thus, it was hypothesized that rats fed a low n-6 PUFA diet would:

1. Conserve ARA by having a slower loss (smaller $J_{out}$) of ARA in brain phospholipids

2. Have a more rapid loss (larger $J_{out}$) of DHA in brain phospholipids
CHAPTER 3:
A Low n-6 PUFA Diet Leads to Conservation of ARA and a More Rapid Loss of DHA in Rat Brain Phospholipids

Contributions: I performed all experimental protocol, including animal husbandry, weekly body weight and food intake measurements, surgeries, euthanizations, tissue collection, brain PL and diet fatty acid concentration measurements, radioactivity measurements and HPLC. The exception was the LC-MS-MS measurements of eicosanoids and docosanoids, which were performed by our collaborators Michael Leadley and Dr. Denis Reynaud at the SickKids Analytical Facility for Bioactive Molecules. However, I did compile, analyze and run statistical analyses on all of the data, including the eicosanoid and docosanoid concentrations.
3.1 Abstract

Arachidonic acid (ARA) and docosahexaenoic acid (DHA) are not only the most abundant PUFA (polyunsaturated fatty acids) in the brain, but they are also crucial for many processes including neuroinflammation, blood flow regulation and pain perception. Dietary n-3 PUFA deprivation in rats leads to conservation of DHA in brain phospholipids (PL), but does not alter the rate of loss of ARA. However, it is poorly understood how the level of dietary n-6 PUFA regulates the metabolism of brain ARA and DHA. This study investigates the effect of low dietary n-6 PUFA on the rate of loss of ARA and DHA from rat brain PL. Rats were fed either a low (2% LA) or high (24% LA) n-6 PUFA diet for 15 weeks post-weaning. At 15 weeks, baseline brain eicosanoids, docosanoids and PL fatty acid concentrations were measured in 8 rats per dietary group. The remaining rats underwent an intracerebroventricular infusion of $^3$H-ARA or $^3$H-DHA, and the loss of this radioactivity from their brain PL was tracked for 128 days. The loss half-lives ($t_{1/2}$) as well as the rates of loss of ARA and DHA from brain phospholipids ($J_{out}$) were calculated. Compared to the high n-6 PUFA rats, the low n-6-PUFA rats had a 15% lower concentration of ARA and an 18% higher concentration of DHA in their total brain PL. The low n-6 PUFA diet also lead to decreased levels of the eicosanoids PGF2α, 5-HETE, 8-HETE, 11-HETE, 12-HETE and 15-HETE. Loss half-lives of ARA for the total brain PL, and PL fractions (except phosphatidylserine), were longer in the low versus high n-6 PUFA rats. There were no differences in DHA half-lives between the dietary groups. The rate of loss of ARA ($J_{out}$) was lower in the low n-6 PUFA rats, while the $J_{out}$ of DHA was higher. In conclusion, chronic low dietary n-6 PUFA decreases the metabolic consumption of ARA and increases the consumption of DHA in brain PL. Thus, a low n-6 PUFA diet can be used to target brain ARA and DHA metabolism.

3.2 Introduction

As the most abundant PUFA in the brain, ARA and DHA metabolism is critical to proper brain function. Their bioactive mediators, eicosanoids and docosanoids respectively, regulate many processes including neuroinflammation, pain perception and blood flow (Bazan 2003; Orr and Bazinet 2008; Attwell, Buchan et al. 2010; Bazan, Molina et al. 2011; Serhan and Petasis 2011; Ramsden, Faurot et al. 2013). As a result, the levels of these PUFA have implications in many
neurological disorders, including Alzheimer’s disease, Parkinson’s disease and bipolar disorder (Rapoport 2008; Yakunin, Loeb et al. 2012).

ARA and DHA can either be obtained directly from the diet or synthesized from their nutritionally essential precursor fatty acids LA and ALA, which are the main dietary n-6 and n-3 PUFA. Thus, it is of interest to determine whether changes in the level of dietary n-3 and n-6 PUFA can alter the concentrations of ARA/DHA in the brain, and more importantly, whether it can alter their metabolism. In fact, clinical trials in humans are underway, with the assumption that lowering n-6 PUFA decreases brain ARA and its metabolism (Ramsden, Faurot et al. 2013).

Several studies have investigated the effects of dietary n-3 PUFA deprivation on brain ARA and DHA concentrations/metabolism in rats. Feeding rats an n-3 PUFA deprived diet that contains ALA at a concentration of 0.04% versus an n-3 PUFA adequate diet containing ALA at 4.4% of all fatty acids results in a 37% lower DHA and a 95% higher DPan6 concentration in brain total PL, but does not change the brain ARA concentration (DeMar, Ma et al. 2004). This study also found that n-3 PUFA deprivation conserves DHA in rat brain PL. This conservation response was selective for DHA, as n-3 PUFA deprivation did not alter the rate of ARA metabolic consumption (Green, Liu et al. 2010). Alterations in brain DHA concentration and metabolism appear to occur below a threshold of 0.8% ALA in the diet, where there is a decrease in the activity of DHA-metabolizing enzymes (iPLA2 and COX-1), and an increase in the activity of ARA-selective enzymes (cPLA2, sPLA2 and COX-2) (Rao, Ertley et al. 2007; Kim, Rao et al. 2011). Lower concentrations of DHA in n-3 PUFA deprived rats is also likely due to a reduction in the incorporation of DHA into brain PL, as well as a reduction in DHA recycling (Contreras, Greiner et al. 2000).

In regards to dietary n-6 PUFA, lowering the level of n-6 PUFA in the rat’s diet by reducing the percentage of total fatty acid from 28% LA to 2% LA resulted in a 28% decrease in brain ARA concentration, and an 11% increase in brain DHA concentration (Igarashi, Gao et al. 2009). Low n-6 PUFA rats also had a reduced expression of ARA-selective enzymes (cPLA2 IVA and COX-2), and an increased expression of DHA-selective enzymes (iPLA2 VIA and 15-LOX) (Kim, Rao et al. 2011). There was a corresponding decrease in the protein levels of AP-2α and NF-κB p65 (transcription factors for cPLA2 and COX-2), as well as an increase in the levels of SREBP-1 (an iPLA2 transcription factor) (Kim, Rao et al. 2011). DHA uptake rates into brain PL were
increased by 45% in rats fed a low n-6 PUFA diet, which could account for the increase in brain total PL DHA (Igarashi, Kim et al. 2012). In the same study, the turnover of DHA was increased 30-84%, but only in the ChoGpl, PtdIns and PtdSer fractions.

As of yet, no study has looked directly at the effect of n-6 PUFA deprivation on the rate of loss of ARA and DHA in brain PL. The goal of this study was to determine the rate of loss of both ARA and DHA from brain PL in rats that are fed either the high or low n-6 PUFA diet as used in previous studies (Igarashi, Gao et al. 2009; Kim, Rao et al. 2011; Igarashi, Kim et al. 2012).

Rats were fed either a high or low n-6 PUFA diet for 15 weeks post-weaning, after which they were infused with either $^3$H-ARA or $^3$H-DHA via an intracerebroventricular (ICV) infusion. At set time-points post ICV infusion, rats were euthanized and the radioactivity of their brains was measured to plot a curve depicting the loss of radioactive ARA or DHA over time. After a logarithmic transformation of the curves, a linear regression analysis was done and the regression slopes were used to calculate the ARA and DHA half-lives in brain PL ($t_{1/2}$), as well as the rate of loss of ARA and DHA on a molecular basis ($J_{out}$). ARA was found to be conserved in the n-6 PUFA deprived rats, while DHA appeared to be lost from the brain more rapidly.

### 3.3 Materials and Methods

#### 3.3.1 Animals

All procedures were approved by the Animal Ethics Committee at the University of Toronto (Protocol # 20010100), in accordance with policy statements of the Canadian Council on Animal Care. 112 male Fischer (CDF) rats were purchased from Charles River Laboratories (Saint-Constant, QC, Canada) and arrived at the Division of Comparative Medicine animal facility at 21 days of age. Rats were housed in a 22°C environment with a 12 h light-dark cycle and they received ad libitum access to water and food throughout the study. Upon arrival, they were randomized to receive either the low n-6 PUFA (n = 56) or high n-6 PUFA diet (n= 56) (Figure 3.3-1). Measurements of body weight and food intake were carried out on a weekly basis for 15 weeks. Following 15 weeks of feeding, 8 rats from each dietary group were euthanized by high-energy, head-focused microwave irradiation (13.5 kW for 1.6 s; Cober Electronics Inc., Norwalk, CT, USA). Their brains were removed, dissected sagittally and stored at -80°C for measurements of eicosanoid levels, docosanoid levels and baseline brain phospholipid fatty acid concentrations.
Figure 3.3-1. Study design indicating samples sizes and general procedures.

A)

Time-course of study for (A) $^3$H-ARA-infused rats and (B) $^3$H-DHA-infused rats. Rats arrived at weaning and were randomized onto a high or low n-6 PUFA diet. Food intake and body weight changes were measured on a weekly basis for 15 weeks. At 15 weeks, a portion of animals were euthanized for baseline brain analyses and the rest were infused with radiolabelled PUFA.
3.3.2 High and Low n-6 PUFA Diets

The high n-6 PUFA and low n-6 PUFA rodent diets were based on the AIN-93G formulation with a 10% fat composition (Reeves, Nielsen et al. 1993; Reeves, Rossow et al. 1993) as used by others (Igarashi, Gao et al. 2009; Kim, Rao et al. 2011; Igarashi, Kim et al. 2012). They were purchased from Dyets Inc. (Bethlehem, PA), under the following product names: Revised Modified n-6 PUFA Adequate Diet (Dyet# 180780) and Custom Modified n-6 PUFA Deficient Diet (Dyet #180784). The words “high n-6 PUFA” and “low n-6 PUFA” were selected to differentiate between two levels of LA in the diet: LA at 24% of total fatty acid (high) and LA at 2% of total fatty acid (low). The compositions of both diets are shown in (Table 3.3-1). As in previous studies (Igarashi, Gao et al. 2009; Kim, Rao et al. 2011; Igarashi, Kim et al. 2012), the high n-6 PUFA diet contained safflower oil (32.3 g/kg), hydrogenated soybean oil (5 g/kg) and coconut oil (55 g/kg). The low n-6 PUFA diet did not contain safflower oil, a significant source of linoleic acid (LA). Instead, it contained hydrogenated coconut oil (87.3 g/kg) and olive oil (5 g/kg). Both diets had equal and adequate amounts of flaxseed oil (7.7 g/kg).

Total lipids were extracted from approximately 0.5 g of each diet (n=3) and analyzed by gas chromatography with flame-ionization detection (GC-FID) as described below (section 3.3.6). Resultant fatty acid concentrations are shown in (Table 3.3-2). LA accounted for about 24% of total fatty acids in the high n-6 PUFA diet, and only 2% of total fatty acids in the low n-6 PUFA diet. Approximately 4% of total fatty acids was ALA in both diets. There were negligible amounts of both ARA and DHA (< 0.05 %).
Table 3.3-1. Composition of high and low n-6 PUFA diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>High n-6 PUFA Diet (g/kg of diet)</th>
<th>Low n-6 PUFA Diet (g/kg of diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>99.98</td>
<td>99.98</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Maltose Dextrin</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Dextrose</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Hydrogenated Coconut Oil</td>
<td>55</td>
<td>87.3</td>
</tr>
<tr>
<td>Olive Oil</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Flaxseed Oil</td>
<td>7.7</td>
<td>7.7</td>
</tr>
<tr>
<td>Safflower Oil</td>
<td>32.3</td>
<td>0</td>
</tr>
<tr>
<td>Hydrogenated Soybean Oil</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Cellulose</td>
<td>49.5</td>
<td>49.5</td>
</tr>
<tr>
<td>Mineral Mix #210025</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin Mix #310025</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The only difference between the two diets is the amount of hydrogenated coconut oil, olive oil, safflower oil and hydrogenated soybean oil. The safflower and soybean oil in the high n-6 PUFA diet was replaced by hydrogenated coconut oil and olive oil.
Table 3.3-2. Fatty acid percent composition of high and low n-6 PUFA diets.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>High n-6 PUFA Diet</th>
<th>Low n-6 PUFA Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% total fatty acid</td>
<td>% total fatty acid</td>
</tr>
<tr>
<td>8:0</td>
<td>1.01 ± 0.159</td>
<td>1.08 ± 0.405</td>
</tr>
<tr>
<td>10:0</td>
<td>3.23 ± 0.054</td>
<td>4.79 ± 0.316</td>
</tr>
<tr>
<td>12:0</td>
<td>29.26 ± 0.237</td>
<td>46.14 ± 0.450</td>
</tr>
<tr>
<td>14:0</td>
<td>11.09 ± 0.089</td>
<td>17.62 ± 0.174</td>
</tr>
<tr>
<td>14:1n-7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>16:0</td>
<td>8.65 ± 0.042</td>
<td>9.79 ± 0.249</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>ND</td>
<td>0.12 ± 0.003</td>
</tr>
<tr>
<td>18:0</td>
<td>10.76 ± 0.079</td>
<td>9.40 ± 0.338</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>6.79 ± 0.056</td>
<td>4.56 ± 0.173</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>0.26 ± 0.002</td>
<td>0.21 ± 0.009</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>24.39 ± 0.170</td>
<td>2.08 ± 0.076</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>4.05 ± 0.026</td>
<td>3.98 ± 0.148</td>
</tr>
<tr>
<td>20:0</td>
<td>0.20 ± 0.002</td>
<td>0.13 ± 0.006</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>0.07 ± 0.002</td>
<td>ND</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>22:0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>24:1n-9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>∑ Saturated</td>
<td>64.21 ± 0.25</td>
<td>88.95 ± 0.41</td>
</tr>
<tr>
<td>∑ MUFA</td>
<td>7.25 ± 0.06</td>
<td>4.93 ± 0.19</td>
</tr>
<tr>
<td>∑ n-3 PUFA</td>
<td>4.10 ± 0.02</td>
<td>4.01 ± 0.15</td>
</tr>
<tr>
<td>∑ n-6 PUFA</td>
<td>24.44 ± 0.17</td>
<td>2.10 ± 0.08</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>5.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. Fatty acid percent compositions were quantified by GC-FID. Non-determinable (ND) indicates that the levels were ≤ 0.05%. MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
3.3.3 Radiotracers

5,6,8,9,11,12,14,15-\(^3\)H arachidonic acid (\(^3\)H-ARA) and 4,7,10,13,16,19-\(^3\)H docosahexaenoic acid (\(^3\)H-DHA), each dissolved in toluene, were purchased from Moravek Biochemicals (Brea, CA). The \(^3\)H-ARA had a specific activity of 207 Ci/mmol and a purity of 98.4%. The \(^3\)H-DHA had a specific activity of 1.0 Ci/mmol and a purity of 97.2%. To create each infusate, the tracer was dissolved in a 5 mM HEPES buffer solution (pH 7.4) with a ratio of tracer molecules to fatty acid-free bovine serum albumin (BSA) that was larger than 3:2. The mixture was sonicated and stored at -80°C (Green, Liu et al. 2010). Tracer purity was confirmed by high performance liquid chromatography (HPLC).

3.3.4 Intracerebroventricular Infusion of \(^3\)H-ARA and \(^3\)H-DHA

After 15 weeks of feeding, the non-baseline rats underwent an intracerebroventricular (ICV) infusion of either \(^3\)H-ARA (\(n = 24\) per dietary group) or \(^3\)H-DHA (\(n = 24\) per dietary group) (Figure 3.3-1). As previously described (Green, Liu et al. 2010; Chen, Liu et al. 2011), rats were anesthetized with isoflurane inhalation (3% induction, 1-2% maintenance). Their heads were shaved at the area of incision and they were positioned onto a stereotaxic frame (Stoelting, Wood Dale, IL, USA). To prevent dehydration, they were injected subcutaneously with 1 mL of sterile saline, and for pain control, 5 mg/mL ketoprofen solution (5 mg/kg of rat weight). At the incision site, 100 μL of local analgesic (0.1% Marcaine solution) was injected subcutaneously. 5 minutes after this injection, the skull was exposed with a small incision on the dorsal side of the rat’s head. A small hole was drilled in the skull (+1.5 mm lateral/medial and -1 mm anterior/posterior to bregma) with a micromotor drill (Stoelting, Wood Dale, IL, USA). Using a 33-gauge beveled needle and syringe (World Precision Instruments, Sarasota, FL), 5 μL of \(^3\)H-ARA or \(^3\)H-DHA infusate solution was infused at a rate of 0.17 μL/min into the right lateral ventricle of the brain (+1.5 mm lateral/medial, -1 mm anterior/posterior, and -4 mm dorsal/ventral to bregma). After the infusion, the needle was slowly removed and the skull was mended with cranioplastic cement (Stoelting, Wood Dale, IL, USA). The incision was closed with self-dissolving sutures. For recovery, rats were placed under a heating lamp for 20-30 minutes before being returned to their respective cages. Rats received a second dose of ketoprofen 1 day post-surgery. For the rest of the study, rats continued to consume their respective diets.
3.3.5 Collection of Radioactive Brains

Rats were euthanized by microwave irradiation (13 kW for 1.6s) at the following time-points: 4, 16, 32, 64 and 128 days post-ICV infusion (Figure 3.3.1). 4 low n-6 PUFA rats and 4 high n-6 PUFA rats were euthanized at each time-point from both the $^3$H-ARA-infused and $^3$H-DHA-infused groups. Brains were removed and stored at -80°C.

3.3.6 Extraction and Isolation of Brain Phospholipids

Brains were homogenized and their total lipids were extracted by the 0.88% KCl : methanol : chloroform (2:1:0.75) Folch, Lees and Stanley method (Folch, Lees et al. 1957). Thin-layer chromatography (TLC) was used to isolate the total phospholipids (TPL), as well as the phospholipid classes from the total lipid extract. TLC plates were washed in chloroform:methanol (2:1 by volume), and were activated for 1 hour at 100°C. TLC G-plates (EMD Chemical, Gibbstown, NJ, USA) in a heptane : diethyl ether : glacial acetic acid solution system (60:40:2 mL by volume) was used for neutral lipid separation. TLC H-plates (Analtech, Newark, DE, USA) in a chloroform : methanol : 2-propanol : 0.25% KCl : triethylamine (30:9:25:6:18 by volume) system was used to separate the phospholipid classes. The plates were sprayed with 0.1% 8-anilo-1-naphthalene sulfonic acid for UV visualization of the fatty acid bands. The bands containing total phospholipids, and the phospholipids fractions (ChoGpl, EtnGpl, PtdSer, and PtdIns) were collected into tubes. For GC-FID analysis, a known amount of heptadecanoic acid (17:0) standard was added. In preparation for both GC-FID and HPLC analysis, the fatty acids were converted into fatty acid methyl esters (FAME) by treatment with 14% boron trifluoride-methanol at 100°C for 1 hour.

3.3.7 Quantitation of Baseline Brain Phospholipid Fatty Acids by GC-FID

FAME were analyzed by a Varian-430 gas chromatograph (Varian, Lake Forest, CA, USA) with a flame ionization detector (FID) and a Varian FactorFour capillary column (VF-23ms; 30 m x 0.25 mm i.d. x 0.25 μm film thickness). The FAME were dissolved in hexane and injected in splitless mode. The injector and detector ports were set at 250°C. The FAME were eluted with increasing temperatures. The temperature program started at 50°C for 2 minutes, increased 20°C/minute, held at 170°C for 1 minute, increased 3°C/minute and finally, held at 212°C for 5 minutes. The helium carrier gas had a flow rate of 0.7 mL/min. Output peaks were identified using known retention times of authentic FAME standards (Nu-Chek Prep, Inc., Elysian, MN,
USA). Fatty acid concentrations were calculated by comparison of the GC fatty acid peak areas to the internal 17:0 standard peak area (Chen, Liu et al. 2009; Green, Liu et al. 2010; Chen, Liu et al. 2011).

### 3.3.8 Quantitation of Baseline Brain Eicosanoids and Docosanoids

As described previously (Chen, Trepanier et al. 2014), composite standards of lipid metabolites (natural or deuterated; Cayman Chemicals Company, Ann Arbor, MI, USA) were diluted in ethanol from stock solutions to perform an eight-point calibration curve (0.05 to 5 ng). Internal standard mixtures in ethanol were added to both the composite standards and the samples prior to extraction. Extraction and sample preparations were performed in siliconized glassware. To minimize auto-oxidation, fatty acids were extracted on ice, in a reduced light condition, using solvents that contained 0.1% butylated hydroxytoluene. The frozen brain halves were homogenized in methanol. 1 ng of internal standard mixture was added to a 250 mg aliquot of each homogenized brain. External ARA, EPA and DHA standards were prepared in a similar way. The samples were mixed for 1 minute, incubated on ice for 30 minutes and centrifuged at 1000 g for 10 minutes. The supernatants were collected. The pellet was re-suspended in ethanol for 1 minute and centrifuged again for a second extraction. The resultant ethanolic supernatants were combined with the methanolic supernatants, previously extracted. After evaporation with nitrogen gas, the supernatants were suspended in 10% ethanol, acidified to pH 3 with 1 N HCl, and triply extracted with ethyl acetate. The ethyl acetate layer was washed to neutrality with water and dried under nitrogen gas. The residues from the brain and external standard samples were reconstituted in acetonitrile : water (1:1 by volume) and transferred into the inserts of amber vials for immediate LC-MS-MS analysis.

LC-MS-MS was performed using a 1290 UHPLC System (Agilent Technologies, Santa Clara, CA, USA) and a QTRAP5500 Mass Spectrometer (ABSciex, Framingham, MA, USA). The chromatography was done at a 600 µL/min flow rate on a Zorbax SB-Phenyl column (Agilent Technologies; 3.0 x 50 mm, 3.5 µm). The gradient started at 80% water and over 9 minutes, ramped up to 100% acetonitrile. The mass spectrometer was operated in negative electrospray ionization mode with a source temperature setting of 600 °C and a voltage setting of 4500 V. The precursor to product ion mass transitions was obtained through scheduled multiple reaction monitoring. Quantitative analysis was performed by Analyst 1.5.2 Software (ABSciex). The
area ratios of the integrated peaks (natural to deuterated standard) were plotted against the standard curves for quantification. 0.025 ng per sample was the limit of quantification and values between 0.005 ng and 0.025 ng were considered semi-quantitative.

3.3.9 Confirmation of Radiotracer Identity by HPLC

Total phospholipids were extracted from brain homogenate and methylated as described above (section 3.3.6). Samples were reconstituted in acetonitrile. Based on previous studies (Aveldano, VanRollins et al. 1983; Chen, Liu et al. 2009; Green, Liu et al. 2010; Chen, Liu et al. 2011) FAME were separated by HPLC (high performance liquid chromatography, Waters 2690, Boston, MA, USA) with a Luna C18 reverse column (4.6 x 250 mm, 100 Å; Phenomenex, Torrance, CA) and an in-line UV photodiode array detector (Waters 996) set at a 242 nm wavelength. The system was first stabilized at a 1 mL/min flow rate with a gradient system consisting of (i) 100% H2O and (ii) 100% acetonitrile. The gradient was then set to 85% (ii) for 30 min, and then increased to 100% (ii) over 10 minutes. It was held there for 20 minutes before returning back to 85% (ii) over a 5 minute period. Fractions were collected at 1 minute intervals for 55 minutes, and each of the 55 fractions was measured for radioactivity by liquid scintillation counting (LSC). Similar to what has previously been reported, ARA and DHA had elution times of 35 and 31 minutes respectively (Igarashi, Ma et al. 2006; Green, Liu et al. 2010).

3.3.10 Quantification of Radioactivity by LSC

Liquid scintillation counting (LSC) was used to measure the radioactivity of the total phospholipids, PL fractions and 4-day brain PL HPLC fractions. Samples were put into scintillation vials and 5 mL of scintillation cocktail (GE Healthcare, Life Sciences, Baie d’Urfe, QC, Canada) were added. Radioactivity was quantified using a Packard TRI-CARB2900TR liquid scintillation analyzer (Packer, Meriden, CT, USA) with a detector efficiency of 61.07% for tritium. The measurements were given in disintegrations per minute and were converted to nCi/brain (Green, Liu et al. 2010; Chen, Liu et al. 2011).

3.3.11 Calculations and Statistics

The data were expressed as means ± SEM. Differences in bodyweights and food intake between the two dietary groups were assessed using repeated-measures ANOVA (SigmaPlot 12.5; SigmaPlot Software, San Jose, CA). Curves depicting the loss of radioactivity over time post-
ICV infusion were logarithmically transformed, and fit with linear regression. The slopes of these linear regressions were tested using an ANOVA to determine if they were significantly different from zero as well as if they differed between the high and low n-6 PUFA groups (GraphPad Prism 5; GraphPad Software, La Jolla, CA). Statistical significance was taken at p < 0.05. Loss half-lives of $^3$H-ARA and $^3$H-DHA were calculated from the slopes of the linear regressions using equation 1 and the rate of loss ($J_{out}$) in nmol/g brain/day was calculated using equation 2 (Stinson, Wiegand et al. 1991; DeMar, Ma et al. 2004; Green, Liu et al. 2010):

$$t_{1/2} = \frac{\ln(2)}{\text{slope of regression line}} \quad (1)$$

$$J_{out} = \frac{0.693 \times C_{FA}}{t_{1/2}} \quad (2)$$

where $C_{FA}$ is the baseline brain PL concentration of the fatty acid in question (ARA or DHA).

For the purpose of calculating $J_{out}$, the half-life that was calculated from the slope of the linear regression was treated as a constant and applied to all measurements of baseline brain PL concentrations (n = 8 baseline measures per dietary group). This produced a distribution of $J_{out}$ values, for which we calculated the mean and SEM. Differences in $J_{out}$ between the low and high n-6 PUFA rats were assessed using the student’s t-test.
3.4 Results

3.4.1 Bodyweights and Food Intake

Bodyweights increased overtime as expected (Figure 3.4-1). Although there were some significant differences between bodyweights of high and low n-6 PUFA rats at certain points in time (p < 0.05), the magnitude of these differences were small (Igarashi, Gao et al. 2009). After 15 weeks of feeding, in the $^3$H-ARA infusion group, the high and low n-6 PUFA rats had a mean weight of 363 ± 2.9 g and 356 ± 2.2 g respectively. In the $^3$H-DHA infusion group, the high n-6 PUFA rats had a mean weight of 347 ± 3.7 g and the low n-6 PUFA rats had a mean weight of 344 ± 3.1 g.

Food intake, like bodyweight, was largely the same between both dietary groups (Figure 3.4-2). The few significant differences at specific time points were small, and the pattern of food intake differences did not match the pattern of bodyweight differences.

The mean weights of the high and low n-6 PUFA rat brains after 15 weeks of feeding were 1.7 ± 0.006 g and 1.7 ± 0.02 g respectively (p > 0.05).
Figure 3.4-1. Rat bodyweight measurements for the 15 weeks of feeding prior to ICV infusion.

A)

Weekly bodyweight measurements of (A) $^3$H-ARA infusion group rats (n = 27 per dietary group) and (B) $^3$H-DHA infusion group rats (n= 28 per dietary group). Data are means ± SEM. Data were analyzed by repeated-measures ANOVA. P-values in the order of time effect, diet effect and interaction effect are as follows: (A) p < 0.001, p = 0.021, p < 0.0001; (B) p < 0.0001, p = 0.26, p = 0.76. *indicates significant difference from high n-6 PUFA group (p < 0.05).
Figure 3.4-2. Rat food intake measurements for the 15 weeks prior to ICV infusion.

Weekly food intake measurements per cage of (A) $^3$H-ARA infusion group rats and (B) $^3$H-DHA infusion group rats. $n = 14$ doubly-housed cages per dietary group. Data are means ± SEM. Data were analyzed by repeated-measures ANOVA. P-values in the order of time effect, diet effect and interaction effect are as follows: (A) $p < 0.001$, $p = 0.533$, $p < 0.024$; (B) $p < 0.001$, $p = 0.45$, $p = 0.021$. *indicates significant difference from high n-6 PUFA group ($p < 0.05$).
3.4.2 Radiotracer Identification

Brain samples (4 days post-infusion) were analyzed by HPLC and LSC to confirm that the tritium was labelling the appropriate fatty acid (ARA or DHA), and to indicate that the proper radioactive tracer had been successfully infused into the brains. ARA elutes at 35 minutes (Green, Liu et al. 2010). All of the radioactivity in the $^3$H-ARA-infused rat brain phospholipids eluted at 35 minutes and was identified as ARA in both the high and low n-6 PUFA rats (Figure 3.4-3). DHA elutes at 31 minutes. All of the radioactivity in the $^3$H-DHA-infused rat brain phospholipids eluted at 31 minutes and was identified as DHA in both dietary groups (Chen, Domenichiello et al. 2013) (Figure 3.4-4).
Figure 3.4-3. HPLC separation of radioactivity in brain total phospholipids of $^3$H-ARA-infused rats.

HPLC separation of radioactivity in brain total phospholipids (TPL) from $^3$H-ARA-infused low and high n-6 PUFA rat brains, 4 days post-infusion (n = 4 in each dietary group). Peaks indicated that radioactivity elutes at 35 minutes, the elution time of ARA.
Figure 3.4-4. HPLC separation of radioactivity in brain total phospholipids of $^3$H-DHA-infused rats.

HPLC separation of radioactivity in brain total phospholipids (TPL) from $^3$H-DHA-infused low and high n-6 PUFA rat brains, 4 days post-infusion (n = 4 in each dietary group). Peaks indicated that radioactivity elutes at 31 minutes, the elution time of DHA.
3.4.3 Baseline Brain Eicosanoid and Docosanoid Concentrations

After 15 weeks of feeding, the low n-6 PUFA rats had significantly lower levels (p < 0.05) of several ARA-derived eicosanoids (PGF2α, 5-HETE, 8-HETE, 11-HETE, 12-HETE and 15-HETE), but not PGE2, 6-keto-PGF1α, 9-HETE, 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET (Figure 3.4-5). The largest difference was seen in PGF2α, which was ~86% lower in the low n-6 PUFA rats than the high n-6 PUFA rats. In contrast, nearly all detectable EPA-derived eicosanoids were found at higher concentrations in the low-n-6 PUFA rats (5-HEPE, 8-HEPE, 9-HEPE, 11-HEPE, 15-HEPE and 18-HEPE, but not 12-HEPE) (Figure 3.4-6). The magnitude of these differences ranged from 1.8 fold (5-HEPE) to 9.9 fold (15-HEPE). Interestingly, there were no significant differences in docosanoid levels between the two dietary groups (Figure 3.4-7). The following eicosanoids and docosanoids were also measured, but their levels were below the detection limit: TXB2, 8-isoprostane, PGD2, 11HDy-TXB2, LXA4, LTB4, PGJ2, TRXA3, TRXB3, D17-6keto-PGF1α, TXB3, 8-isoprostanepG3α, PGF3α, PGE3, PGD3, LXA-5, LTB5, 8(9)-EpETE, 17(18)-EpETE, resolvins D1, resolvins D2, resolvins E1, 10(S),17(S)-DiHDoHE(Protectin), 7(R)Marisin1, and PD1.
Figure 3.4-5. ARA-derived eicosanoid concentrations of baseline un-infused rat brains after 15 weeks of the high or low n-6 PUFA diet.

Data are means ± SEM (n = 8 high n-6 PUFA rats, n= 7 low n-6 PUFA rats). Eicosanoids were measured by LC-MS-MS. Differences were assessed using the student’s t-test. * indicates significant difference from high n-6 PUFA group (p < 0.05).
Figure 3.4-6. EPA-derived eicosanoid concentrations of baseline un-infused rat brains after 15 weeks of the high or low n-6 PUFA diet.

Data are means ± SEM (n = 8 high n-6 PUFA rats, n= 7 low n-6 PUFA rats). Eicosanoids were measured by LC-MS-MS. Differences were assessed using the student’s t-test. * indicates significant difference from high n-6 PUFA group (p < 0.05).
**Figure 3.4-7.** DHA-derived docosanoid concentrations of baseline un-infused rat brains after 15 weeks of the high or low n-6 PUFA diet.

Data are means ± SEM ((n = 8 high n-6 PUFA rats, n= 7 low n-6 PUFA rats). Eicosanoids were measured by LC-MS-MS. Differences were assessed using the student’s t-test. * indicates significant difference from high n-6 PUFA group (p < 0.05).
3.4.4 Baseline Brain Phospholipid Fatty Acid Concentrations

Figure 3.4-8 depicts the concentrations of esterified fatty acids found in the total brain phospholipids of both high and low n-6 PUFA rats after 15 weeks of feeding. There was a 15% lower concentration of ARA in the low versus high n-6 PUFA rats (5191 ± 158 versus 6077 ± 103 nmol/g of brain, p < 0.05). This was accompanied by a 50% lower concentration of DPAn-6 (22:5n-6) (79 ± 2.7 versus 158 ± 3.7 nmol/g of brain, p < 0.05). Conversely, there was an 18% higher concentration of DHA and a 164% higher concentration of DPAn-3 (22:5n-3) in the low n-6 PUFA rats (7323 ± 200 versus 6232 ± 97 and 267 ± 8.5 versus 101 ± 2.4 nmol/g of brain respectively, p < 0.05). Interestingly, the largest percentage difference between the dietary groups was seen in EPA. The low n-6 PUFA rats had a > 10 fold higher level of EPA than the high n-6 PUFA rats (9.2 ± 1.5 versus 94 ± 3.4 nmol/g of brain).

These changes in total PL concentrations were reflected by changes in the PL fractions, although some fractions changed more than others for certain fatty acids. ARA was about 20% lower (p < 0.05) in the ChoGpl, EtnGpl and PtdSer fractions (Figure 3.4-9,10,12) but not the PtdIns fraction (Figure 3.4-11). DPAn-6 was 50-56% lower (p < 0.05) in all 4 fractions. A 9% and 11% higher concentration of DHA was found in the low n-6 PUFA rat PtdSer and ChoGpl fractions respectively (1940 ± 38 versus 1776 ± 13 and 1137 ± 2 versus 1017 ± 19 nmol/g of brain, p < 0.05). DPAn-3 was higher in the low n-6 PUFA rats by 105%, 159%, 145% and 167% in the ChoGpl, EtnGpl, PtdSer and PtdIns fractions respectively (p < 0.05). EPA was > 10 fold higher in the ChoGpl and EtnGpl fractions of the low n-6 PUFA rats, and was > 1.5 fold higher in the PtdSer and PtdIns fractions compared with the high n-6 PUFA rats.
Figure 3.4-8. Total phospholipid fatty acid concentrations of baseline un-infused brains after 15 weeks of the high or low n-6 PUFA diet.

A)

B)

Total phospholipid (TPL) fatty acid concentrations of baseline un-infused brains after 15 weeks of feeding (n = 8 per dietary group). (A) Saturated and monounsaturated fatty acids. (B) PUFA. Data are means ± SEM. Fatty acid concentrations were measured by GC-FID. Differences were assessed using the student’s t-test. * indicates significant difference from high n-6 PUFA group (p < 0.05).
Figure 3.4-9. ChoGpl fatty acid concentrations of baseline un-infused brains after 15 weeks of the high or low n-6 PUFA diet.

A) Fatty acid concentrations in the ChoGpl pool of baseline un-infused brains after 15 weeks of feeding (n = 8 per dietary group). (A) Saturated and monounsaturated fatty acids. (B) PUFA. Data are means ± SEM. Fatty acid concentrations were measured by GC-FID. Differences were assessed using the student’s t-test. * indicates significant difference from high n-6 PUFA group (p < 0.05).
Figure 3.4-10. EtnGpl fatty acid concentrations of baseline un-infused brains after 15 weeks of the high or low n-6 PUFA diet.

A)

B)

Fatty acid concentrations in the EtnGpl pool of baseline un-infused brains after 15 weeks of feeding (n = 8 per dietary group). (A) Saturated and monounsaturated fatty acids. (B) PUFA. Data are means ± SEM. Fatty acid concentrations were measured by GC-FID. Differences were assessed using the student’s t-test. * indicates significant difference from high n-6 PUFA group (p < 0.05).
Figure 3.4-11. PtdIns fatty acid concentrations of baseline un-infused brains after 15 weeks of the high or low n-6 PUFA diet.

(A) Saturated and monounsaturated fatty acids. (B) PUFA. Fatty acid concentrations in the PtdIns pool of baseline un-infused brains after 15 weeks of feeding (n = 8 per dietary group). (A) Saturated and monounsaturated fatty acids. (B) PUFA. Data are means ± SEM. Fatty acid concentrations were measured by GC-FID. Differences were assessed using the student’s t-test. * indicates significant difference from high n-6 PUFA group (p < 0.05).
Figure 3.4-12. PtdSer fatty acid concentrations of baseline un-infused brains after 15 weeks of the high or low n-6 PUFA diet.

A) Fatty acid concentrations in the PtdSer pool of baseline un-infused brains after 15 weeks of feeding (n = 8 per dietary group). (A) Saturated and monounsaturated fatty acids. (B) PUFA. Data are means ± SEM. Fatty acid concentrations were measured by GC-FID. Differences were assessed using the student’s t-test. * indicates significant difference from high n-6 PUFA group (p < 0.05).
3.4.5 Brain Phospholipid ARA and DHA Rate of Loss

The loss of $^3$H-ARA and $^3$H-DHA from brain phospholipids was plotted from 4 to 128 days post-infusion and then logarithmically transformed for linear regression analysis (Figure 3.4-13 and 14). All slopes were negative and significantly different from zero ($p < 0.0001$) (Table 3.4-1). Linear regression analysis of the slopes for $^3$H-ARA showed significant differences between high and low n-6 PUFA rats in the total PL pool as well as all PL fractions, except for PtdSer ($p < 0.05$). The slopes were more negative in the high n-6 PUFA group than the low n-6 PUFA group, reflective of a shorter loss half-life and a more rapid loss of $^3$H-ARA over time ($p < 0.05$).

To take into account the differences in baseline concentrations of ARA between the two dietary groups, the number of ARA molecules lost per gram of brain each day ($J_{\text{out}}$) was calculated. The difference in $J_{\text{out}}$ between dietary groups was generally even larger than the difference in the $t_{1/2}$, except in the PtdIns fraction; the low n-6 PUFA group had a significantly lower $J_{\text{out}}$ than the high n-6 PUFA group ($p < 0.001$) in the total PL, ChoGpl, EtnGpl and PtdSer fractions. $J_{\text{out}}$ in the total PL pool was $134.7 \pm 2.3$ nmol/g of brain/day in the high n-6 PUFA group versus $85.4 \pm 2.6$ nmol/g of brain/day in the low n-6 PUFA group ($p < 0.05$). This corresponds to a daily fractional loss of 2.2% versus 1.6% in the high versus low n-6 PUFA groups. The $J_{\text{out}}$ (nmol/g of brain/day) in the PL fractions ranged from $6.9 \pm 0.06$ (PtdSer) to $43 \pm 0.6$ (ChoGpl) in the high n-6 PUFA group, and from $4.0 \pm 0.1$ (PtdSer) to $24 \pm 0.3$ (ChoGpl) in the low n-6 PUFA group. Overall, ARA was lost from brain PL at a slower rate in the low n-6 PUFA group.

There were no differences in the slopes and thus, no difference in the loss half-lives for $^3$H-DHA between the high and low n-6 PUFA rats. Due to its higher baseline DHA concentrations, the low n-6 PUFA group appeared to have a higher $J_{\text{out}}$ than the high n-6 PUFA group. This difference was only significant in the total PL pool and the ChoGpl fraction. The $J_{\text{out}}$ for total phospholipids in the high and low n-6 PUFA groups was $129.1 \pm 11.4$ nmol/g of brain/day and $145.7 \pm 15.5$ nmol/g of brain/day respectively ($p < 0.05$). The $J_{\text{out}}$ (nmol/g of brain/day) for the PL fractions ranged from $2.3 \pm 0.3$ (PtdIns) to $113.5 \pm 7.8$ (EtnGpl) in the high n-6 PUFA group, and from $2.4 \pm 0.3$ (PtdIns) to $121 \pm 10.2$ (EtnGpl) in the low n-6 PUFA group. Overall, DHA seemed to be lost from brain PL at a more rapid rate in the low n-6 PUFA rats.
Figure 3.4-13. Loss of $^3$H-ARA from brain total PL and PL fractions over time.

Logarithmically transformed curves of the loss of $^3$H-ARA from brain total PL (TPL) and PL fractions over time with linear regression analysis (untransformed curves are inset). A) TPL. B) ChoGpl. C) EtnGpl. D) PtdIns. E) PtdSer. Data are mean ± SEM (n = 4 independent samples per dietary group per time point, except for the 4 day time-point of the high n-6 PUFA rats where n = 3). All slopes are significantly different from zero (p < 0.0001).
C) EtnGpl

- High n-6 PUFA
- Low n-6 PUFA
- 95% confidence interval

D) PtdIns

- High n-6 PUFA
- Low n-6 PUFA
- 95% confidence interval
E) Brain PtdSer Radioactivity (nCi/g brain)

Days post-\(^3\)H-ARA infusion

- • High n-6 PUFA
- ▲ Low n-6 PUFA
- --- 95% confidence interval

\(\log_{10}\) Brain PtdSer Radioactivity (nCi/g brain)
Figure 3.4-14. Loss of $^3$H-DHA from brain total PL and PL fractions over time.

Logarithmically transformed curves of the loss of $^3$H-DHA from brain total PL (TPL) and PL fractions over time with linear regression analysis (untransformed curves are inset). A) TPL. B) ChoGpl. C) EtnGpl. D) PtdIns. E) PtdSer. Data are mean ± SEM (n = 4 independent samples per dietary group per time point). All slopes are significantly different from zero (p < 0.0001).
C) 

EtnGpl

- High n-6 PUFA
- Low n-6 PUFA

log_{10} EtnGpl Radioactivity (nCi/g brain)

Days post-^{3}H-DHA infusion

D) 

PtdIns

- High n-6 PUFA
- Low n-6 PUFA

log_{10} PtdIns Radioactivity (nCi/g brain)

Days post-^{3}H-DHA infusion
E)

E) 

PtdSer

- High n-6 PUFA
- Low n-6 PUFA
- 95% confidence interval

log_{10} PtdSer Radioactivity (nCi/g brain)

Days post-^{3}H-DHA infusion

Brain PtdSer Radioactivity (nCi/g brain)

- High n-6 PUFA
- Low n-6 PUFA
- 95% confidence interval

Days post-^{3}H-DHA infusion

- High n-6 PUFA
- Low n-6 PUFA
- 95% confidence interval

log_{10} PtdSer Radioactivity (nCi/g brain)

Days post-^{3}H-DHA infusion
Table 3.4-1. Kinetic parameters for ARA and DHA in total PL and PL fractions of high and low n-6 PUFA rats.

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<th>Infusate</th>
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<th>Diet</th>
<th>Slope (per day)</th>
<th>Slope SEM</th>
<th>Loss t1/2 (day)</th>
<th>Jout (nmol/g/day)</th>
<th>Fractional loss (% per day)</th>
<th>p-value</th>
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<td>40.3 ± 4.4</td>
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Loss half-lives were calculated from the slopes of the linear regressions using the equation \( t_{1/2} = \log_{10}2/(\text{slope of the regression line}) \).
Baseline fatty acid concentrations were used to calculate the rates of loss (\( J_{\text{out}} \)) from the different fatty acid pools using the equation \( J_{\text{out}} = 0.693C_{\text{FA}}/t_{1/2} \), where \( C_{\text{FA}} \) is the baseline brain TPL/PL fraction FA concentration after 15 weeks of feeding (Figure 3.4-8 to 12). Data are mean ± SEM. Differences in slope and \( J_{\text{out}} \) were assessed with an ANOVA and student’s t-test respectively. * \( p < 0.05 \) versus high n-6 PUFA.
3.5 Discussion

This study investigated the effect of a low (2% LA) versus high (24% LA) n-6 PUFA diet on the rate of loss of ARA and DHA from rat brain phospholipids. The diets used in this study were the same as those used in previous studies examining body/organ weights, enzyme expression, and brain DHA uptake/turnover (Igarashi, Gao et al. 2009; Kim, Rao et al. 2011; Igarashi, Kim et al. 2012).

The overall growth curves of both the high and low n-6 PUFA rats were similar to those seen in the 2009 Igarashi study, with rats reaching about 350 g in weight after 15 weeks of feeding. This previous study, however, found no differences in the bodyweights of high and low n-6 PUFA rats at these dietary fatty acid % compositions (Igarashi, Gao et al. 2009). In our study, the rats in the ^3^H-ARA infusion group showed a slight difference in body weight starting around 7 weeks of age. By the 15^th^ week of feeding, the mean bodyweight of the low n-6 PUFA rats (356 ± 2.2 g) was 1.9% lower than in the high n-6 PUFA group (363 ± 2.9 g). This difference may be significant due to the large sample size (n= 28 per dietary group) in our study. In addition, it has been reported that low n-6 PUFA rats have a 10% lower testis weight than high n-6 PUFA rats (Igarashi, Gao et al. 2009). This may partially account for the body weight differences seen here, especially since the weights began to differ after 6 weeks of age, the age at which rats reach sexual maturity (Sengupta 2013). However, the difference in bodyweight may also be a chance finding, or be due to an undefined external factor, since the ^3^H-DHA infusion rats did not have any consistent differences in bodyweight between the dietary groups. In addition, the changes in body weight did not reflect changes in food intake.

More severe LA deprivation in rats does cause more noticeable physiological problems. In a study that used a much lower n-6 PUFA diet containing only 0.05% of total fatty acids as LA, there was a 15% reduction in body weight accompanied by skin scaling and hair loss (Cunnane and Anderson 1997). The level used for the high n-6 PUFA diet in our study is based on the level at which ARA concentrations in all tissues has plateaued, and ceases to increase with any further increase in dietary LA (Bourre, Piciotti et al. 1990; Igarashi, Gao et al. 2009). The level of LA in the low n-6 PUFA diet (2%) was set at ~10% of the level in the high n-6 PUFA diet (24%). The concentration of ARA plateaus at different LA levels in different tissues. Thus, even though the heart, liver and lung (the last tissues to plateau) have not plateaued in ARA
concentration with a 2% LA diet, all nervous tissue has, including the brain, retina and sciatic nerves (Bourre, Piciotti et al. 1990). This may be why the low n-6 PUFA diet used in this study does not cause any noticeable physiological problems, whereas the low n-6 PUFA diet with only 0.05% LA study does.

After 15 weeks of feeding, the fatty acid concentrations in the brain PL fractions were comparable to previously reported levels. In the total brain phospholipids, there was a 15% reduction in ARA concentration and an 18% increase in DHA concentration with the low n-6 PUFA diet. These changes are similar to the 28% reduction in ARA and the 11% increase in DHA found previously in brain total lipids (Igarashi, Gao et al. 2009). It may also suggest that there is a greater uptake of DHA into the phospholipids than the neutral lipids. In fact, the incorporation rate ($J_{in}$) of DHA is nearly 10-fold higher in the brain phospholipids than in the brain triacylglycerol pool (Igarashi, Kim et al. 2012). This suggests that the DHA functions associated with brain phospholipids are quantitatively important.

One of the fates of DHA/ARA unesterified from the PL membrane is metabolism into eicosanoids/docosanoids. We reported decreases in the levels of certain ARA-derived eicosanoids in rats fed the low versus high n-6 PUFA diet. The largest reduction was seen in the enzymatically-derived PGF$_{2\alpha}$. As seen in previous studies, a low n-6 PUFA diet causes a reduction in the activity of cPLA$_2$ and COX-2, which are both enzymes required for the synthesis of PGF$_{2\alpha}$ (Kim, Rao et al. 2011). However, this effect was selective for PGF$_{2\alpha}$ since there were no significant reductions in the levels of other non-auto-oxidative eicosanoids that were detected (PGE$_2$ and PGI$_2$). This suggests that the reductions in enzyme activity are somehow able to selectively reduce certain eicosanoids but not others. The mechanism behind this is unknown. In addition, it could be that the differences in the other eicosanoids may only be seen when the brain is responding to an insult. For example, in a neuroinflammatory state, there is an increased production of PGE$_2$, and perhaps the low n-6 PUFA rats would not have as large of an increase as the high n-6 PUFA rats. In addition, there was no significant difference in the expression of COX-1, which often works in conjunction with COX-2 to form certain eicosanoids. This may have accounted for some of the selective decreases in enzymatically-derived eicosanoids. The rest of the eicosanoids that changed are thought to be auto-oxidative, meaning that they are capable of being non-enzymatically derived (Chen, Trepanier et al. 2014). This suggests that
these eicosanoid reductions could, for the most part, be due to the lower ARA concentration in the brain phospholipids of low n-6 PUFA rats.

One thing to note is that eicosanoids function on a picomolar level while concentrations of ARA in the brain are in the nanomolar range. The most concentrated eicosanoid detected was 5-HETE, which dropped from 0.0019 ± 0.00001 nmol/g of brain in the high n-6 PUFA rats to 0.0013 ± 0.000007 nmol/g of brain in the low n-6 PUFA rats. This was after a change in brain PL ARA concentration from 6077 ± 103 nmol/g of brain in the high n-6 PUFA diet to 5191 ± 158 nmol/g of brain in the low n-6 PUFA diet. This raises an interesting question. If these eicosanoids are derived from ARA, why does a reduction of ~1000 nmol of ARA per g of brain cause a ~0.0006 nmol/g of brain reduction in 5-HETE when the amount of ARA available is still > 2 million fold higher than the amount needed to create the 0.0019 nmol of 5-HETE? Perhaps this highlights how important ARA is in its other roles aside from eicosanoid production, seeing as how the brain does not have 0.0006 nmol of ARA per g of brain to spare on maintaining the 5-HETE level at 0.0019 nmol/g of brain. It may also be that there is a set rate at which ARA auto-oxidation occurs, which cannot be altered to compensate for alterations in brain PL ARA concentration.

EPA-derived eicosanoids should also be considered. There was a significantly higher level of all detectable EPA-derived eicosanoids in the low n-6 PUFA rats, except for 12-HEPE. These eicosanoids were all auto-oxidation products and their levels are a reflection of the 10 fold increase in brain PL EPA concentration in the low n-6 PUFA rats. A 10-fold increase in brain EPA is very large. The brain maintains very low levels of EPA in many species (Svennerholm 1968; Chen, Ma et al. 2008; Igarashi, Ma et al. 2010; Chen, Liu et al. 2011). Even supplementation of EPA in the diet does not create such large increases in EPA. For example, in fish, a 4-fold increase in the EPA content of the diet only results in a 70% increase in brain EPA concentration (Trushenski, Schwarz et al. 2012). Being able to raise EPA levels by this much may be therapeutic in certain mood disorders including major depression (Martins 2009; Martins, Bentsen et al. 2012).

There were no changes in the levels of docosanoids, though the only 4 docosanoids detected were those that are also considered auto-oxidative (Chen, Trepanier et al. 2014). Perhaps we would have observed increases in the level of protectins, maresins and resolvins in the low n-6
PUFA rats if these enzymatically-derived docosanoids had been detectable. It is interesting here, how even though the concentration of brain PL DHA increased by ~1000 nmol/g of brain in the low n-6 PUFA rats, there was no change in the level of these auto-oxidative docosanoids, unlike what was seen with the eicosanoids. Maybe the extra DHA is largely being shunted towards increasing the levels of enzymatically-derived docosanoids, which we were unable to measure at this time. There was, after all, an increase in the activity of iPLA2 and in the levels of 15-LOX, enzymes that metabolize DHA into resolvins, protectins and maresins (Kim, Rao et al. 2011).

Lowering the amount of n-6 PUFA in the diet leads to longer ARA loss half-lives in the total brain PL, ChoGpl, EtnGpl and PtdIns pools. After factoring in the concentration of ARA in baseline, un-infused rats, low n-6 PUFA rats had a slower rate of loss ($J_{out}$) of ARA from the total brain PL, ChoGpl, EtnGpl and PtdSer pools. This was likely caused by a decrease in cPLA2 and COX-2 activity (Kim, Rao et al. 2011). Decreased cPLA2 activity corresponds to a decreased ARA turnover, and less opportunity for ARA to be lost through eicosanoid production or β-oxidation. The decrease in eicosanoid production may not be enough to account for the decreased loss of ARA, unless the eicosanoids have short half-lives. Thus, there is also likely a decreased amount of β-oxidation.

Conversely to our observations with ARA, there was a more rapid loss of DHA from total brain phospholipids and the ChoGpl pool, but not the other PL fractions in the low n-6 PUFA rats. This was a function of the higher DHA concentrations because the fractional loss of DHA and the loss half-lives for DHA were not significantly different between the two dietary groups in all PL pools. In the low n-6 PUFA rats, the $J_{out}$ of DHA for total PL was smaller than the $J_{in}$ (rate of incorporation) of DHA found previously, whereas the $J_{in}$ approximately matched the $J_{out}$ in the high n-6 PUFA rats (Igarashi, Kim et al. 2012). Having a more rapid daily uptake than loss of DHA may account for the higher concentration of DHA in the low n-6 PUFA rats than in the high n-6 PUFA rats upon 15 weeks of feeding. However, caution should be taken when comparing and combining results for kinetic analyses from two different studies, which were performed at different times, with different rats, in different facilities. Future experiments, testing these hypotheses, should be completed under more similar conditions.

In rats, the level of LA at which ARA concentrations plateau is 1200 mg of LA per 100 g of diet, and this intake is considered the recommended minimum LA intake (Bourre, Piciotti et al. 1990).
This level is comparable to the recommended level of LA for humans: 1000-1500 mg of LA per 100 g of food, which equates to around 2-3% of energy (Collins, Sinclair et al. 1971; Wene, Connor et al. 1975; Goodgame, Lowry et al. 1978). However, more recent studies are suggesting that these are overestimations of the actual LA requirement, especially since there is the growing concern that the amount of LA in the average human diet is too high. For rats, the level at which tissue ARA levels plateau cannot necessarily be substituted for the LA requirement level, as there is no evidence that ARA at this peak level is critical for health. For instance, the low n-6 PUFA diet used in our study and in previous studies had a LA level that was 10% of the suggested requirement, and rats did not show significant signs of LA deficiency (Igarashi, Gao et al. 2009). One of the problems with earlier experiments that formed the basis of LA requirements is that many of them did not ensure an adequate level of ALA (Burr and Burr 1929; Burr and Burr 1930; Holman 1958; Cunnane 2003). The effects of an inadequate level of ALA were attributed to having an inadequate level of LA, and thus lead to an overestimation of LA requirement. The low n-6 PUFA diet in our study contained an adequate amount of ALA, allowing for the effects here to be attributed to changes in dietary n-6 PUFA, without the influence of n-3 PUFA. Still, the search for the actual LA requirement continues, and the characterization of changes in metabolism that result from changes in dietary LA is an important piece of the puzzle.

3.6 Conclusion

Rats fed a low n-6 PUFA diet (2% LA) or a high n-6 PUFA diet (24% LA) for 15 weeks were infused with \(^3\)H-ARA or \(^3\)H-DHA, which allowed for the determination of the rate of loss of ARA and DHA from their brain phospholipids. ARA was lost at a slower rate in the total PL, ChoGpl, EtnGpl and PtdSer pools of the low n-6 PUFA rats, illustrating the brain’s ability to conserve ARA in response to lower dietary LA. In contrast, DHA was lost more rapidly in the total PL and ChoGpl pools of the low n-6 PUFA rats, but not the other PL fractions. These effects are approximately opposite of what was observed with the low versus high n-3 PUFA diets in previous studies (DeMar, Ma et al. 2004; Green, Liu et al. 2010). Characterizing the metabolic responses to changes in the amount of n-6 and n-3 PUFA in the diet is important for determining the optimal n-6:n-3 PUFA ratio that should be targeted by a healthy diet. In addition, it is critical for the development of potential therapies for neurological disorders that are associated with altered PUFA metabolism.
CHAPTER 4:
General Discussion and Conclusions
4.1 Limitations

In order to create the low n-6 PUFA diet, the n-6 PUFA (mainly LA) that was removed from the diet had to be replaced by other fatty acids to ensure that the high and low diets were iso-caloric, and that they provided the same % of energy from fat. Due to the direct and complex interactions between n-3 and n-6 PUFA, as well as some of the similarities between the theorized effects of raising n-3 PUFA and lowering n-6 PUFA, we could not use n-3 PUFA to replace the n-6 PUFA in the low n-6 PUFA diet. Otherwise, we would not have been able to differentiate between the effect of lowering n-6 PUFA and the effect of raising n-3 PUFA. The type of fatty acids thought to be least involved in the pathways of PUFA metabolism are saturated fatty acids. Thus, the majority of the n-6 PUFA (safflower oil) that was removed was replaced by saturated fatty acids (hydrogenated coconut oil). This resulted in a 40% higher amount of saturated fatty acid in the low n-6 PUFA diet. There was also a 30% lower amount of monounsaturated fatty acid. Although these are significant differences, the 92% lower amount of n-6 PUFA is a larger difference. We also chose not to alter the diet from previous studies that were directly related to our study in the interest of being able to make comparisons (Igarashi, Gao et al. 2009; Kim, Rao et al. 2011; Igarashi, Kim et al. 2012). In addition, the Igarashi study in 2009 had validated that the diet did not cause any major physiological, unlike other studies using lower levels of LA (Cunnane and Anderson 1997). Nonetheless, it is possible that a portion of the effects seen in this study are due to differences in saturated and monounsaturated fatty acids, though based on our theoretical knowledge of the functions of different fatty acids, it is likely that the majority of the effects are due to changes in n-6 PUFA levels.

Another limitation of this study was that measuring the radioactivity of the rat brains required euthanization of the rats and removal of their brains. Thus, different rats had to be used at each time-point post-infusion, and the actual loss of radioactivity in the each individual rat brain could not be tracked over 128 days. This posed a problem when trying to calculate the SEM of \( J_{\text{out}} \) because the \( J_{\text{out}} \) calculation was the quotient of 2 measurements – the baseline ARA/DHA concentration, and the loss half-life of ARA/DHA. It is difficult to combine the individual SEM values from each of these measurements because the SEM for the loss half-life of ARA/DHA comes from the error in the slope of the linear regression. There is not really a discernable sample size for this error, and when combining errors, the SEM of the measurement that has the larger sample size should likely be given a larger weighting. Since the SEM and sample size
could only be clearly determined for the measure of baseline ARA/DHA concentration, this was the SEM that was used to calculate the SEM of $J_{out}$. The slopes of the linear regressions as well as the calculated loss-half-lives were treated as constants. It is noteworthy, however, that since the SEM values for the slopes are quite a bit higher than the SEM of the concentrations, incorporation of the slope SEM would result in more conservative analyses of the difference between $J_{out}$ values of the high and low n-6 PUFA rats. For ARA, it does not change the overall conclusion, as the differences in ARA $J_{out}$ between the dietary groups are significant up to a very high SEM. For DHA however, an incorporation of the slope error would likely point towards no significant differences between the DHA $J_{out}$ values of the high and low n-6 PUFA rats in any of the phospholipids. Thus, the conclusions made about DHA metabolism must be taken with this in mind.

### 4.2 Future Directions

With the addition of this study, the effect of a low n-6 PUFA diet on the three major kinetic parameters of DHA (the rate of incorporation, turnover and loss from rat brain phospholipids) has been investigated. We have now also determined its effects on the rate of loss of ARA. It would be useful to measure the rate of incorporation and turnover of ARA in the same high vs. low n-6 PUFA diet system to complete the picture of the balance between ARA and DHA metabolism and how this balance is affected by alterations in the level of n-6 PUFA. In addition, it would provide further support for or against the direct relationship between $J_{in}$ and $J_{out}$. So far studies, including this one, have shown that $J_{in}$ approximates $J_{out}$. Since $J_{in}$ is a measure of fatty acid uptake from the plasma unesterified fatty acid pool, it suggests that the plasma unesterified pool is a major source of fatty acids for brain phospholipids. In addition, it would be useful to measure other aspects of metabolism, including β-oxidation. Understanding how n-6 PUFA alters ARA/DHA β-oxidation may help explain differences in the loss of these fatty acids from the brain that is not accounted for by changes in eicosanoid production.

As mentioned briefly in the discussion of lipid mediators, it would be useful to evaluate the effects of a low n-6 PUFA diet on the production of eicosanoids/docosanoids in a non-homeostatic, neuroinflammatory state. Many of the ARA/DHA-derived mediators elicit effects during inflammation. It is possible that low n-6 PUFA rats will have a significantly lower production of prostaglandins or thromboxanes, and a significantly higher production of
docosanoids that can only be seen when triggered by inflammation. Perhaps studies can be done in animals, using established models of neuroinflammation, such as the infusion of lipopolysaccharide (Orr, Palumbo et al. 2013). It may also be interesting to investigate the effects of a low n-6 PUFA diet on the production of other ARA metabolites, such as the endocannabinoids, which are also important signaling molecules that regulate many processes in the brain.

4.3 Significance

This thesis has shown, for the first time, that alterations in n-6 PUFA at the level of the diet are capable of directly targeting ARA and DHA metabolism – specifically, the rate of loss of these fatty acids from brain phospholipids. It was also shown, for the first time, that lowering the amount of n-6 PUFA in the diet can lower the amount of eicosanoids produced, even in non-inflammatory conditions. This knowledge provides some insight into the potential therapeutic mechanisms of low n-6 PUFA diets seen in clinical trials (eg. trials on chronic headache syndrome), and it supports further development of dietary fatty acid-driven therapies for neurological disorders like Alzheimer’s disease, bipolar disorder, and Parkinson’s disease, where there is altered n-6 PUFA metabolism. It will also help to provide the basis for updated recommendations on how much LA we should be consuming on a daily basis, and on the optimal n-6:n-3 PUFA ratio that we should be aiming for in our diets.

4.4 Conclusions

After investigating the effects of a low versus high n-6 PUFA diet on brain metabolism of ARA and DHA, we can conclude the following:

1. A low n-6 PUFA diet leads to a conservation of ARA in brain phospholipids, reflected by a slower loss of ARA from brain total phospholipid, ChoGpl, EtnGpl, and PtdSer pools, post $^3$H-ARA infusion.

2. A low n-6 PUFA diet leads to a more rapid loss of DHA from total brain phospholipids and ChoGpl, but not from the other phospholipid fractions (EtnGpl, PtdSer and PtdIns).
CHAPTER 5: References


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