THE LOSS HALF-LIFE OF ARACHIDONIC ACID IN RAT BRAIN PHOSPHOLIPIDS FOLLOWING 15 WEEKS OF N-3 PUFA ADEQUATE OR DEPRIVED FEEDING

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
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ABSTRACT

Polyunsaturated fatty acids (PUFA) comprise a significant portion of mammalian brain tissue, and are involved in neural signalling and cellular homeostasis. One brain PUFA, arachidonic acid, represents an attractive target for manipulation, with evidence suggesting it plays a role in the pathology of several neurological diseases. In this study, we fed rats a 15-week diet of an n-3 PUFA adequate or deprived diet, and then injected $^3$H arachidonic acid into the right lateral ventricle and measured its rate of loss over time. The half-life was 44 and 46 days for the n-3 PUFA adequate and deprived dietary groups, respectively. We compared the rate of loss with a predicted rate of loss (~45 days). We concluded that plasma unesterified AA is quantitatively a major source of brain phospholipid AA. Furthermore, we demonstrated selective regulation of brain PUFA by showing AA, unlike DHA, is not conserved in n-3 PUFA deprivation.
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Nature does not hurry, yet everything is accomplished.

~Lao Tzu (600-531 BC)
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**Abbreviations:** AA, arachidonic acid; DHA, docosahexaenoic acid; ALA, alpha-linolenic acid; LA, linoleic acid; PLA2, phospholipase A2; cPLA2, calcium-dependant cytosolic phospholipase A2; sPLA2, secretory phospholipase A2; iPLA2, calcium-independent phospholipase A2; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; i.c.v., intracerebroventricular; ChoGpl, choline glycerophospholipids; EtnGpl, ethanolamine glycerophospholipids; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; FAME, fatty acid methyl ester; PUFA, polyunsaturated fatty acid; COX, cyclooxygenase; LOX, lipooxygenase; PGG2, prostaglandin G2; PGH2, prostaglandin H2; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PGI2, prostaglandin I2.
Chapter 1

Introduction and Literature Review

Fatty acids are lipids which comprise a significant portion of caloric intake in the mammalian diet, and due to biochemical adaptations, function as a practical means of storing energy, as well as structural and signalling molecules at a cellular level. All fatty acids are composed of a carboxylic acid with a carbon chain extension; it is the number of carbons in this chain, as well as the presence and position of double bonds in the carbon chain, that define the identity of the fatty acid. FA’s with fewer than six carbons are referred to as “short-chain”; those with six to twelve carbons are referred to as “medium-chain”, and those with thirteen or more are referred to as “long-chain”. The polar carboxyl group increases solubility, which decreases as the length of the neutral carbon chain increases. Those fatty acids with fully saturated carbon chains are denoted “saturated fatty acids”; if the carbon chain contains one or multiple double bonds, the FA is classified as monounsaturated or polyunsaturated, respectively. Fatty acids are stored in the body as triglycerides, a molecule of three fatty acids joined by ester bonds to glycerol. Polyunsaturated fatty acids (PUFA) are of considerable interest in neuroscience, due to their abundance in nervous tissue compared to the rest of the body. One PUFA, arachidonic acid (20:4n-6; AA), is highly enriched in the mammalian brain and is known to be involved in a variety of neural signalling mechanisms and immune responses; thus, it is of great importance in the nervous system. This thesis will attempt to discuss some known functions of AA in the brain, as well as evidence for its regulation. The experiment undertaken sought to define the half-life of AA in the rat brain, and in doing so, may have helped answer several questions about the complex role of this fatty acid in neural tissue.
Brain Polyunsaturated Fatty Acids- Structure and Transport in the Body

Fats ingested in the diet are transported to the stomach, where they are broken down into triglycerides. The triglycerides enter the small intestine, where they form micelles, surrounded by bile salts. Pancreatic lipase degrades the triglycerides into the component fatty acids and glycerol. Short and medium-chain fatty acids, as well as glycerol, are sufficiently hydrophilic and can pass directly though the intestinal capillaries into the bloodstream, traveling through the portal vein. Long-chain fatty acids are too large to pass through the intestinal capillaries; therefore, they are absorbed into the intestinal villi, re-assembled into triglycerides in the smooth endoplasmic reticulum, and packaged into chylomicrons in the Golgi apparatus; these molecules contain the triglycerides, cholesterol esters, and fat soluble vitamins, enveloped by cholesterol and protein. The chylomicron then enters a lymphatic capillary, or lacteal, and is transported to the thoracic duct, where it enters the bloodstream through the left subclavian vein.

During hepatic processing, PUFAs can either be packaged in lipoproteins (HDL, LDL, etc) as phospholipids (lysophosphatidylcholine, etc.) and directed to tissues via the blood, or esterified in adipose tissue. Once released from the adipose tissue, the free fatty acids can be transported through the blood by binding to serum albumin. Upon reaching the blood-brain endothelium (barrier), fatty acids pass through, though there is debate as to how the passage occurs; free fatty acids may pass through the endothelium by passive or facilitated diffusion, and/or active transport (for example, endocytosis of lipoprotein-bound fatty acids into lipoprotein receptors) (Chen et al. 2008a, Ouellet et al. 2009). Additionally, the existence of a selective fatty acid transporter on the BBB has not been disproved, and may represent a viable mode of entry as opposed to passive/facilitated diffusion (Chen et al. 2008a, Chen et al. 2008b).
Within the mammalian brain, PUFA account for ~25% of fatty acids and are known to be involved in signal transduction. The two most common PUFAs in the mammalian brain are AA and docosahexaenoic acid (22:6n-3, DHA). Arachidonic acid is a 20-carbon chain containing four double bonds, with the first double bond occurring at the sixth carbon from the methyl end. DHA is a 22-carbon chain containing six double bonds, with the first double bond occurring at the third carbon from the methyl end (see Figure 1). These fatty acids comprise the majority of PUFA in the brain, and are generally stored (esterified) into the sn-2 position of membrane phospholipids. The release and turnover of DHA and AA is not currently well understood but thought to play a significant role in homeostasis and disease.

DHA and AA are considered “non-essential” dietary fatty acids, with “non-essential” denoting the fact that both can by synthesized by the body using the respective pre-cursor fatty acids, alpha-linolenic acid (18:3n-3, ALA) and linoleic acid (18:2n-6, LA), respectively. Both ALA and LA are considered “essential” dietary fatty acids, as they must be obtained from the diet and cannot be synthesized de novo. Conversion of DHA and AA from ALA and LA primarily takes place in the liver, where the precursors are desaturated and elongated through a sequential process (Scott & Bazan 1989)(Figure 2).
Figure 1. Chemical structure of DHA and AA
Figure 2. Desaturation and elongation of n-6 and n-3 PUFA from LA and ALA, respectively.
**History of Arachidonic Acid**

The name “arachidonic acid”, stemming from the Latin “*arachis*”, or “peanut”, was given to the n-6 PUFA due to its similarity to the 20-carbon saturated fatty acid *arachidic acid*, a component of peanut oil. AA was first isolated from liver tissue in 1907, and identified in a follow-up study in 1909 by Hartley (Hartley 1909). Linoleic acid was proposed as the pre-cursor to AA in 1940; later, in 1943, the chemical structure of both LA and AA were fully described (Dolby *et al*. 1940, Arcus & Smedley-Maclean 1943). AA was first synthesized in 1961 (Osbond, 1961). Bergstrom et al demonstrated the conversion of AA into prostaglandin E2 in 1964 (1981); in 1979, a derivative of AA was identified and named Leukotriene (Samuelsson *et al*. 1979). Further metabolites of AA, Lipoxin A and B, were discovered in 1984 by Serhan et al (Serhan *et al*. 1984).

**AA turnover in the brain**

After crossing the blood-brain endothelium, AA likely enters cells by passive diffusion through the double-layered phospholipid membrane, composed of phospholipids oriented with the polar phosphate group on the outer membrane pointing outwards into the extracellular space, and the phosphate on the inner membrane pointing into the polar cytosol. The neutrally-charged lipids forming the phospholipid tail are aligned inwardly, away from the polar environment (see Figure 3). Crossing the phospholipid membrane, AA is immediately attached to a coenzyme A (CoA) by a membrane-bound acyl-CoA synthetase. This reaction is energetically costly, requiring the consumption of two phosphates from ATP, forming AMP. One consequence of this reaction is the maintenance of an intercellular/extracellular concentration gradient so that new AA is always diffusing into the cell. Binding the fatty acid to a hydrophilic CoA molecule also prevents the fatty acid from diffusing out of the cell. Some of the AA-CoA molecules can be shuttled to the mitochondria for β-oxidation or other metabolic processes, but the majority are esterified into the sn-2 position of membrane phospholipids.
(Robinson et al. 1992). The mechanisms by which the transport and esterification take place are unknown; however, it is thought to be accomplished through the action of an as-yet-uncloned acyl-transferase. The existence of such an enzyme could help explain the specificity by which fatty acids are esterified into phospholipids in a passive diffusion model of entry into the cell, with PUFAs (including AA) normally being esterified in the sn-2 position of phospholipids, while saturated fatty acids are normally esterified into the sn-1 position. Additionally, some fatty acids are noticeably absent in the brain, including the n-3 PUFA eicosapentaenoic acid (20:5n-3, EPA), which further indicates preferential storage of particular fatty acids over others (Chen et al. 2009).

AA in the brain is thought to be consumed at a rate of ~18 mg/day in humans (Rapoport 2008a). Once esterified, AA is released from rat brain phospholipids at a rate of ~170 pmol/g brain/s (Lee et al. 2007a); this rate greatly exceeds the ~3.6 pmol/g brain/s rate of incorporation for new AA entering the cell (Contreras, 2001). The release of esterified brain phospholipid AA is thought to be mediated by the action of phospholipases from the A2 family, and there is evidence suggesting specificity of certain phospholipases for specific brain PUFA. DHA is thought to be preferentially cleaved from the membrane by calcium-independent phospholipase A2 (iPLA2), and AA is thought to be preferentially cleaved by calcium-dependant cytosolic phospholipase A2 (cPLA2). Once cleaved, AA can be utilized for metabolic or signalling functions, β-oxidized in the mitochondria, or reacylated back into the phospholipid membrane. In fact, reacylation is the destination for the vast majority of AA (~97%) cleaved from the membrane (Lee et al. 2008a). AA consumed in cell signalling mechanisms may be converted through the action of several enzymes, including cyclooxygenase 1 and 2 (COX 1 and 2), lipoxygenase (LOX) and cytochrome-P450, as well as through non-enzymatic pathways. AA consumption through these pathways result in products called eicosanoids (DHA consumption products are referred to as docosanoids). Both eicosanoids and docosanoids are thought to mediate a diverse range of cellular
Figure 3. Structure and organization of phospholipid membranes.
Figure 4. Turnover of AA in mammalian brain. (A) Albumin-bound AA disassociates and crosses the blood-brain barrier. (B) AA entering the cell is covalently attached to a CoA molecule via action of a membrane-bound acyl-CoA synthetase. (C) AA-CoA molecules may be transported to the mitochondria where they are beta-oxidized. (D) An unidentified acyl-CoA transferase shuttles the AA-CoA molecule to the phospholipid membrane to be esterified. (E) cPLA₂, following stimulation by neuroreceptors and/or cellular chemical signals, cleaves AA from the sn-2 position of the phospholipid membrane. (F) De-acylated AA may be consumed through a variety of metabolic processes, including eicosanoid production. (G) ~97% of the de-acylated AA is bonded to an acyl-CoA molecule by acyl-CoA synthetase, and re-esterified into the phospholipid membrane.
processes, but are perhaps best known for immune response regulation. As a result, they are being investigated for their potential role in various neurological disorders, including bipolar disorder, Alzheimer’s disease, and others.

**Arachidonic Acid and Eicosanoids**

Arachidonic acid is found ubiquitously throughout the body. It is known to be rich in fetal placental tissue and thought to be important for organogenesis and vascularization (Bitsanis et al. 2005). AA is a significant component of cellular phospholipid membranes. Due to the presence of four double bonds, it is “kinked” in shape, and thought to contribute to cellular “membrane fluidity”; this theory suggests the presence of unsaturated fatty acids in the phospholipid membrane results in greater mobility and less viscosity of the membrane and potentially plays a role in endocytosis and signal transduction (Helmreich 2003). Evidence for this theory is sparse; however, recent studies examining the characteristics of synthetic membranes have demonstrated significant increases in permeability among unsaturated fatty acid/cholesterol “lipid raft” membrane interactions when compared to saturated fatty acid/cholesterol interactions (Simons & Vaz 2004), suggesting further investigation might be useful. More substantial evidence suggests AA is a potent signalling molecule and precursor to a variety of metabolic products known as eicosanoids (*eicosa*, Greek for twenty), twenty-carbon derivatives of AA, which include leukotrienes, thromboxanes, prostacyclins and prostaglandins. After AA is cleaved from the membrane by cPLA₂, it may follow one of several metabolic paths. One potential pathway is via interaction with the cyclooxygenase (COX) enzyme and peroxidase (POX), which converts AA non-reversibly into an intermediary product, prostaglandin H₂ (PGH₂) or prostaglandin G₂ (PGG₂). PGH₂ may then be modified into prostacyclins or other prostaglandins; PGG₂ is the precursor of thromboxane. Prostacyclin, also known as prostaglandin I₂ (PGI₂), acts to prevent clotting and increase vasodilation. Thromboxanes, such as A₂ and B₂, exert the opposite effect of PGI₂, facilitating blood
clotting, vasoconstriction and platelet aggregation. The prostaglandins, most notably prostaglandin D<sub>2</sub> and E<sub>2</sub> (PGD<sub>2</sub> and PGE<sub>2</sub>, respectively), may paradoxically have both neuroprotective and neurotoxic effects; McCullough et al. (2004) showed E-prostanoid 2 receptor (EP2) is activated through binding of PGE<sub>2</sub>, resulting in a neuroprotective increase in cAMP during ischemia (McCullough et al. 2004); D-prostanoid 1 receptor (DP1), activated by PGD<sub>2</sub>, showed a similar neuroprotective ability (Liang et al. 2005). Alternatively, EP2 stimulated by PGE<sub>2</sub> in an inflammatory model has been shown to induce reactive oxygen species (ROS) and nitric oxide, which can damage neurons (Montine et al. 2002).

Lipoxygenase (LOX) enzymes present in the brain include the 5, 12, and 15-isoforms. They act by oxidizing AA into a variety of products; one group of products, known as leukotrienes, is synthesized by 5-LOX. AA interaction with 5-LOX produces hydroperoxyeicosatetraenoic acid (5-HPETE), which spontaneously reduces to 5-hydroxyeicosatetraenoic acid (5-HETE). 5-LOX then converts this product into leukotriene A<sub>4</sub> (LTA<sub>4</sub>). In some immunoregulatory cells, such as neutrophils and monocytes, this unstable intermediary product is then converted primarily into leukotriene B<sub>4</sub> (LTB<sub>4</sub>). LTB<sub>4</sub> is known to act as a potent chemoattractant for neutrophils during an immune response. Other leukotriene isoforms, such as C<sub>4</sub>, D<sub>4</sub>, E<sub>4</sub>, and F<sub>4</sub>, have been shown to have vasoconstrictive and immuno-regulatory properties (Brain & Williams 1990).

Cytochrome P450 epoxygenases (CYP450) are enzymes primarily located in astrocytes, endothelial cells and arteriolar smooth muscle that metabolize AA into oxygenated derivatives in the presence of NADPH (Peng et al., 2004). One product is cis-epoxeyicosatrienoic acid (EET), which is hydrolyzed into cis-trans-conjugated monohydroxyeicosatetraenoic acid (HETE). Isomers of EET have been shown to induce cerebral capillary endothelial cell mitogenesis and tube formation (Munzenmarier and Harder, 2000), and modulate angiogenesis (Wang et al., 2003). AA may also undergo free-radical-catalyzed peroxidation to form isoprostanes. These prostaglandin-like molecules exert a vaso-
constrictive effect in brain tissue, partly through inducement of thromboxane in endothelial cells. They are increased in situations of oxidative stress (Morrow et al. 1991).

One AA-derived eicosanoid of growing interest is anandamide, also known as N-arachidonoyl-ethanolamide, which is chemically similar to \( \Delta^9 \)-tetrahydrocannabinol (THC), the active ingredient of cannabis sativa. This compound is classified as an endocannabinoid, due to its effects on cannabinoid receptors 1 and 2 (CB1 and CB2, respectively). CB1 is a G-protein coupled receptor expressed throughout the nervous system on pre-synaptic axons, thought to be involved in food intake behavior, memory, and pain (Herkenham et al. 1990, Hansen & Artmann 2008). A study by Alvares (2008) observed that injection of anandamide into the dorsal hippocampus increased memory extinction in subsequent testing; this mechanism has been hypothesized to help the brain partition memories into long-term storage, or discarded for good (Marsicano et al. 2002). Anandamide is a retro-grade neurotransmitter released from post-synaptic neurons and binding to CB1; it is thought to exert many of its effects by inhibiting neurotransmitter release, particularly gamma-amino-butyric acid (GABA), the main inhibitory neurotransmitter, and norepinephrine (Fernandez-Solari et al. 2009). Anandamide is a transient molecule, produced when needed and degraded by fatty acid amide hydrolase (FAAH) into AA and ethanolamine. Interestingly, the widely-used drug Acetaminophen was only recently discovered to react with AA through FAAH to form the compound N-arachidonoylphenolamine (AM404), a mild agonist of CB1 and inhibitor of anandamide; this increase in endogenous endocannabinoid levels is thought to explain the analgesic effects of the drug (Bertolini et al. 2006).
Figure 5. Enzymatic metabolism of arachidonic acid into eicosanoids.
AA in Neurological Disorders

AA has been implicated as potentially mediating a variety of neurological diseases, including psychiatric disorders, both as a secondary signalling molecule and promoter of neuroinflammation. The following is a review of some important findings involving AA for each disorder.

Bipolar Disorder

Bipolar disorder is a psychiatric mood disorder involving both depressive episodes and manic episodes, resulting in a fluctuation between periods of low mood and apathy with pronounced euphoria and agitation. Studies of widely-used mood stabilizing drugs, including Lithium, Carbamazepine, and Haloperidol, have shown the brain phospholipid AA cascade to be a common target (Bazinet et al. 2007, Basselin et al. 2007, Bazinet et al. 2006a). Basselin et al (2007) gave Lithium to rats for six weeks, at which time lipopolysaccharide (LPS) was administered to induce a neuroinflammatory response known to involve an up-regulated brain AA cascade, and found Lithium attenuated the inflammation by decreasing AA turnover, as well as (dopamine) D2-like receptor signalling (implicated in the pathogenesis of bipolar disorder) (Basselin et al. 2007). Furthermore, chronic Lithium decreased COX-2 activity and prostaglandin E2 levels (Bosetti et al. 2002), and appears to down-regulate AA turnover by decreasing cPLA₂ activity, which was demonstrated by Rintala et al (Rintala et al. 1999). Similarly, Carbamazepine has been shown to decrease AA turnover by decreasing cPLA₂ activity (Bazinet et al. 2006a). Valproate, an anticonvulsant, has also been shown to target the AA cascade; however, its effects, unlike Lithium and Carbamazepine, appear to be from decreasing acyl-CoA synthetase activity, inhibiting AA re-esterification into the phospholipid membrane (Bazinet et al. 2005b). Conversely, the commonly used selective-serotonin reuptake inhibitor (SSRI) Fluoxetine, was found to increase AA turnover, as well as cPLA₂ and COX-2 activity in susceptible patients; it is prescribed in the management
of unipolar depression and may induce mania in this population (Lee et al. 2007b). Post-mortem analysis of the orbitofrontal cortex of bipolar patients showed significantly lowered AA composition in psychotic and psychosis-free bipolar patients, compared to age-matched normal controls (McNamara et al. 2008); potentially, this suggests an increased turnover and metabolism of regional brain AA in bipolar disorder.

**Schizophrenia**

Schizophrenia, another pervasive psychiatric disorder, may involve disregulated AA metabolism. A study by Maekawa et al. (2009) found that AA supplementation increased prepulse inhibition (PPI), a behavioural characteristic commonly deficient in Schizophrenia whereby the affected individual does not become conditioned to a startle-invoking stimulus, despite the introduction of a mild warning stimulus prior to its activation. One hypothesis for this characteristic is the disruption of fatty-acid binding protein 7 (FABP-7), a gene thought to be involved with neurogenesis and shown to control PPI (Maekawa et al. 2009). A post-mortem study by McNamara (2007) found a significantly increased AA:DHA ratio in the orbitofrontal cortex of male schizophrenic patients (both drug-naive and treated with antipsychotics), as compared to male controls (McNamara et al. 2007). This brain region is thought to be responsible for cognitive and emotional disturbance in schizophrenic patients.

**Parkinson’s Disease**

Parkinson’s disease (PD) is a neurological disease characterized as a “movement disorder”, involving muscular rigidity, tremor, and bradykinesia (slowed movement), thought to be caused by decreased activation of motor neurons by the basal ganglia following a decrease in dopamine production, or loss of dopamine-producing neurons. Levodopa, the standard treatment for Parkinson’s, initiates and/or exacerbates motor dysfunction, resulting in tics and other involuntary movements. A
study by Julien et al (2006) examining the post-mortem brains of twelve PD patients, found significantly higher levels of AA in the brains of PD patients suffering from levodopa-induced motor dysfunction, compared to PD patients without levodopa-induced motor dysfunction, and controls (Julien et al. 2006).

**Multiple Sclerosis**

Multiple sclerosis (MS) is a neuro-degenerative disorder characterized by de-myelination of neuronal axons, resulting in a disruption of nerve signal propagation. Symptoms of this disorder include a variety of physical and cognitive deficits, including muscular weakness/atrophy, and fluctuations in mood. Marusic et al. (2008) found relapses in autoimmune encephalomyelitis (EAE), an animal model of MS, had reduced duration when cPLA₂ was inhibited, implicating AA turnover in the etiology of the disease (Marusic et al. 2008).

**Alzheimer’s Disease**

Alternatively, AA has been implicated in the etiology of AD due to post-mortem brain samples demonstrating increased levels of COX-2 and cPLA₂ compared to controls (Sun et al. 2004). Amyloid-beta peptide (A-beta) aggregation in neural tissue is a common pathohistological finding in post-mortem brain samples of Alzheimer’s patients; studies suggest this aggregation may result in altered neuro-signalling involving AA. Shelat et al. (2008) showed N-methyl-D-aspartic acid (NMDA) and A-beta produced an increase in reactive oxygen species (ROS), which caused a down-stream increase in cPLA₂ activity and AA release (Shelat et al. 2008). Potentially, this increase in AA release could result in eicosanoid-mediated inflammation and neuro-toxicity, two symptoms commonly observed in Alzheimer’s disease.
CHAPTER 3

Introduction to AA paper

With considerable evidence pointing to PLA₂ specificity in cleaving brain PUFA from phospholipids membranes, a new question arises; does alteration of one PUFAs turnover in the brain necessarily affect the turnover of another PUFA? In a study by Rao et al. (2007) rats were fed either an n-3 PUFA adequate or deprived diet for 15 weeks. They observed what could be interpreted as a compensatory reaction (Rao et al. 2007c); rats on an n-3 PUFA deprived diet had increased cPLA₂ and COX-2 protein and mRNA activity, and decreased iPLA₂ protein and mRNA activity, compared to the n-3 PUFA adequate group. This evidence, in addition to AA and DHA competing for the same sn-2 position in brain phospholipids, raises the possibility of an overlapping regulation of turnover of these fatty acids. We chose to use AA in our study for two reasons. A study by DeMar et al. (2004) gave an intracerebroventricular injection of [¹⁴C] DHA to rats following 15 weeks of n-3 PUFA adequate or deprived feeding, and observed a marked prolonging of DHA half-life in the n-3 PUFA deprived group compared to the n-3 PUFA adequate group; this raised the question of whether the half-life of brain AA is affected in such a model, either through increased conservation or loss, following the 15 weeks of n-3 PUFA adequate or deprived diet. Furthermore, we were interested in AA due to the evidence implicating brain AA in psychiatric and neurological disorder, coupled with mixed results from n-3 PUFA trials in treating these disorders (Lee et al. 2007c, Richardson 2006).
**Objective:**

- To quantify the loss half-life of [³H] AA in rat brain phospholipids following 15 weeks of n-3 PUFA adequate or deprived diet.

**Hypothesis:**

- The loss half-life of brain phospholipid AA will approximate the predicted loss half-life based on the rate of unesterified AA entry into the brain.
- Brain phospholipid AA will not be conserved in an n-3 PUFA deprived model following 15 weeks of feeding.
THE LOSS HALF-LIFE OF ARACHIDONIC ACID IN RAT BRAIN PHOSPHOLIPIDS FOLLOWING 15 WEEKS OF N-3 PUFA ADEQUATE OR DEPRIVED DIET

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Green, JT; Liu, Z; Bazinet, RP. The loss half-life of arachidonic acid in rat brain phospholipids following 15 weeks of n-3 PUFA adequate or deprived diet. 2008.
Abstract

Previous studies have infused radiolabelled arachidonic acid (AA) into rat brains and followed AA esterification into phospholipids for up to 24 hours; however, the loss half-life of AA in rat brain phospholipids is unknown. Unesterified radiolabelled AA infused into the femoral vein of rats fed an n-3 PUFA adequate or deprived diet predicted the brain phospholipid AA half life to be 42 and 48 days, respectively. In this study we directly measured the loss half-life of AA in brain phospholipids following 15 weeks of n-3 PUFA adequate or deprived diet. Method: Twenty-one day old Long Evans male pups were fed either an n-3 PUFA adequate or deprived diet for 15 weeks. Following the 15 weeks, 40 µCi of \[^3\text{H}\] AA was injected intracerebroventricularly into the right lateral ventricle using stereotaxic surgery; the rats were then returned to their respective diet for the remainder of the study. From 4-120 days post-[\(^3\text{H}\)] AA administration, brains were collected and phospholipids were isolated by thin-layer chromatography and radioactivity was determined by liquid scintillation counting. Results: The loss half-life of AA in rat brain phospholipids was 44 ± 4 days for the n-3 PUFA adequate group and 46 ± 4 days for the n-3 PUFA deprived group, which closely approximates the predicted loss half-life previously mentioned, based on the rate of entry from the plasma unesterified pool. Conclusion: Direct quantification of AA loss from brain phospholipids approximates what enters the brain from the plasma unesterified fatty acid pool, suggesting the plasma unesterified pool is a major contributor to brain uptake of AA. Furthermore, unlike a previous report in which the loss half-life of brain phospholipid docosahexaenoic acid (DHA) was increased in n-3 PUFA deprived rats, n-3 PUFA deprivation did not significantly alter the AA loss half-life, suggesting different mechanisms exist to maintain brain concentrations of AA and DHA.
Introduction

The polyunsaturated fatty acids (PUFA) docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (AA; 20:4n-6) have been increasingly recognized as biochemically potent compounds in the human diet playing an important role in the nervous system (Salem et al. 2001, Rapoport 2008a, Alessandri et al. 2004). DHA and AA may be consumed directly through the diet or they may be synthesized de novo through hepatic desaturation and elongation of their precursors, alpha-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6), respectively (Rapoport et al. 2007, Demar et al. 2005, Scott & Bazan 1989, DeMar et al. 2006b). Although neurons have the ability to synthesize DHA from ALA (Kaduce et al. 2008), the rate of brain desaturation and elongation of ALA and LA into DHA and AA, respectively, has been shown to be <1% of the brain rate of uptake of preformed DHA and AA from plasma in vivo (Igarashi et al. 2007a, DeMar et al. 2006b). One area of interest in these PUFA is with regards to brain development and function, as the brain is highly enriched in both DHA and AA (Diau et al. 2005). Currently, DHA is being investigated for its potential neuroprotective and anti-inflammatory properties (Lukiw & Bazan 2008, Orr & Bazinet 2008, Kim 2007). Alternatively, understanding AA-mediated signal-transduction and metabolism within the brain may provide insight into a variety of neurological and neurodegenerative disorders. Several studies have shown widely-used mood stabilizers target turnover and enzymatic pathways of AA (Rao et al. 2008, Rapoport & Bosetti 2002). Other studies have implicated upregulated AA signalling cascades in Alzheimer’s disease and vascular dementia (Yagami 2006), Multiple Sclerosis (Harbige & Sharief 2007), and Parkinson’s disease (Minghetti 2004, Julien et al. 2006).

AA entry into the brain may originate from several sources, including the plasma unesterified pool, or via lipoproteins including high-density, low-density, and very low-density lipoproteins (HDL, LDL, and VLDL); lipoprotein receptors have been identified on the blood brain barrier (Meresse et al. 1989).
Chen et al. examined the contribution of the LDL pathway of PUFA entry into the brain by comparing brain phospholipid PUFA levels in LDL-knockout and wild-type mice, and found no significant difference (Chen et al. 2008b). However, it is unknown whether other pathways of entry (HDL, VLDL, unesterified, lysophosphatidylcholine, etc.) compensated for the lack of influx from the LDL pool. Contreras et al. infused unesterified radiolabelled AA into the femoral vein of awake third-generation n-3 PUFA deprived rats and calculated the rate of unesterified AA entry into brain phospholipids $J_{in,i}(\text{plasma unesterified PUFA})$ (Contreras et al. 2001). From this calculation, a mathematical model was used to predict an AA half-life in rat brain phospholipids (equation 1: $\ln(2)*c_{\text{brain},i}(\text{PUFA})/J_{in,i}(\text{plasma unesterified PUFA})$, where $c_{\text{brain},i}(\text{PUFA})$ is the concentration of the PUFA in brain phospholipid i, and $J_{in,i}(\text{plasma unesterified PUFA})$ is the net rate of entry from the plasma unesterified pool into brain phospholipid i.), which was determined to be 42 days and 48 days for n-3 PUFA adequate and n-3 PUFA deprived animals, respectively. However, this rate of AA loss from brain phospholipids was predicted exclusively by the rate of entry of plasma unesterified AA into the brain. Similarly, Positron Emission Tomography (PET) studies in humans using unesterified [1-11C] AA have calculated a brain AA incorporation rate $J_{in,i}(\text{plasma unesterified AA})$ of 17.8 mg/day (Giovacchini et al. 2004, Rapoport 2008a). However, these studies did not take into account other potential sources of AA, including lipoprotein-transported AA or an as-yet-unidentified source.

Upon entry into the brain AA is esterified into, and subsequently released from the sn-2 position of neural phospholipid membranes via the A2 family of phospholipases. DeMar et al. measured the rate of loss of injected radiolabelled DHA in rat brain phospholipids following 15 weeks of n-3 PUFA adequate or deprived diet, and found the rate of loss of DHA in the n-3 PUFA deprived rats to be significantly decreased compared to rats consuming the n-3 PUFA adequate diet (DeMar et al. 2004). Another study by Rao et al, using the same fifteen week rat model of n-3 PUFA adequate or deprived feeding, observed significantly decreased calcium-independent phospholipase A2 (iPLA2) activity in the n-3 PUFA deprived group, while calcium-dependant phospholipase A2 (cPLA2) and secretory phospholipase A2 (sPLA2)
activities were significantly increased, compared to the n-3 adequate group (Rao et al. 2007c). Furthermore, administration of psychiatric medications such as Lithium and Carbamazepine down-regulate cPLA₂ activity and brain AA turnover with no change in iPLA₂ activity or DHA turnover (Weerasinghe et al. 2004, Rao et al. 2007a, Bazinet et al. 2006a). Likewise, chronic Fluoxetine administration up-regulates cPLA₂ activity and AA turnover, but does not affect iPLA₂ activity (Lee et al. 2007b). These findings suggest that brain DHA and AA are predominantly released from brain phospholipid membranes by different enzymes, with iPLA₂ releasing DHA, and cPLA₂ releasing AA (Green et al. 2008).

Two distinct brain phospholipid PUFA half-lives have been described (DeMar et al. 2004, Robinson et al. 1992). In one method, upon infusion of a unesterified radiolabelled PUFA into the plasma, the net rate of entry from the plasma unesterified pool is used to calculate the half-life of replacement in brain phospholipids according to equation 1: \[ \ln(2) \times \frac{c_{\text{brain},i(\text{PUFA})}}{J_{\text{in},i(\text{plasma unesterified PUFA})}} \] (see above). Because brain phospholipid concentrations are relatively stable over time (DeMar et al. 2004), if plasma unesterified PUFA are a major contributor to brain phospholipids then the \( J_{\text{in},i(\text{plasma unesterified PUFA})} \) should closely approximate the net rate of loss of PUFA from brain phospholipids \( J_{\text{out},i(\text{PUFA})} \) which can be measured directly by labelling brain phospholipids with a PUFA radiotracer and measuring the loss over time (DeMar et al. 2004, Rapoport et al. 2007). Indeed, DHA’s loss half-life from brain phospholipids (33 days) approximates the rate of replacement from the plasma unesterified DHA pool, suggesting that for DHA \( J_{\text{in},i(\text{plasma unesterified DHA})} \approx J_{\text{out},i(\text{DHA})} \) (DeMar et al. 2004, Rapoport et al. 2007). By correcting for potential dilution from sources besides the plasma unesterified PUFA pool, Rapoport and colleagues have developed a method to calculate the net rate of PUFA uptake into brain phospholipids from the PUFA-CoA pool \( J_{\text{FA},i(\text{PUFA})} \) (Rapoport et al. 2001, Robinson et al. 1992, Grange et al. 1995). This is accomplished by correcting \( J_{\text{in},i(\text{plasma unesterified PUFA})} \) for the ratio of the specific activity of the brain PUFA-CoA pool to the specific activity of the plasma unesterified PUFA pool according to equation 2: \[ J_{\text{FA},i(\text{PUFA})} = J_{\text{in},i(\text{PUFA})}/(\text{c}^*_{\text{brain}} \times \text{c}_{\text{plasma},i(\text{PUFA})}) \]
PUFA-CoA/[brain PUFA-CoA] /[c* plasma unesterified PUFA/ c* plasma unesterified PUFA]), where c* is the radiolabelled brain PUFA-CoA or plasma unesterified PUFA. Because brain synthesis of PUFA in vivo is slow (DeMar et al. 2006b, Demar et al. 2005), one assumption of $J_{FA,i(PUFA)}$ is that if the plasma unesterified pool is a major contributor to brain uptake, the dilution would be largely attributed to Land’s recycling (Chen et al. 2008a, Lee et al. 2007c, Robinson et al. 1992) and not uptake from other plasma pools (i.e. lipoproteins) (Chen et al. 2008b). In this model, 90-97 % of PUFA released from brain phospholipids is recycled back into brain phospholipids through the PUFA-CoA pool giving half-lives much more rapid (30-33 fold) than the net loss of PUFA from brain phospholipids. Furthermore, because PUFA are largely recycled (90-97 %) and not lost in this model the $J_{FA,i(PUFA)} \neq J_{out,i(PUFA)}$, which as discussed above should $\cong J_{in,i(plasma unesterified PUFA)}$ if plasma unesterified PUFA are a major pool of uptake into brain phospholipids (Chen et al. 2008a, Rapoport et al. 2007). Several experiments have, in regards to AA, demonstrated that $J_{FA,i(AA)}$ can be regulated independently of $J_{in,i(plasma unesterified AA)}$ (Bazinet et al. 2006a, Lee et al. 2008a) but whether or not the $J_{in,i(plasma unesterified AA)} \cong J_{out,i(AA)}$ has not been reported.

AA is recognized as a significant component in neuronal cell membranes not only due to its effects on phospholipid membrane fluidity, but through its role in signal-transduction (Axelrod 1990, Rapoport 2008a). These signalling cascades may play a crucial role in the development and treatment of a variety of psychiatric and neurological disorders. Therefore, it is important to understand how AA enters the brain from the plasma, the dynamics of AA turnover, and kinetic control once in the brain. In the current study we measured the rate of AA loss from rat brain phospholipids directly, by injecting radiolabelled AA into the brains of rats consuming either an n-3 PUFA adequate or deprived diet (DeMar et al. 2004, DeMar et al. 2006c, Rao et al. 2007c). Brain phospholipid radiolabelled AA levels were measured over multiple time points to determine the rate of loss and compared to previously predicted half-lives based on the rate of entry from the plasma unesterified pool (Contreras et al. 2001) in an attempt to determine if the unesterified AA pool is a significant contributor to brain phospholipid AA.
Brain phospholipid AA half-lives calculated in this study closely match the predicted half-lives from the unesterified fatty acid pool.

Materials and Methods

Animals

The protocol was approved by the Department of Comparative Medicine at the University of Toronto. Eighteen-day old male Long Evans pups and their dams were purchased from Charles River Laboratories (Saint -Constant, Quebec). The pups were allowed to nurse for 3 days. When they reached 21 days of age, they were removed from the dams and fed either an n-3 PUFA adequate or n-3 PUFA deprived diet, as described below. The pups were maintained on their assigned diet for the duration of the study. The rats were housed at 22° C under a 12 h light/dark cycle with ad libitum access to food and water.

n-3 PUFA adequate and deficient diets

Rodent diets (prepared by Dyets, Inc., Bethlehem, PA) were designed around a standard AIN-93 formulation (AIN-93 custom saturated fat level [product # 101093] and AIN-93 custom low n-3 [product # 101094], n-3 PUFA adequate and deprived diets, respectively), with carbohydrate, protein, fat, fiber, salt and vitamin/essential amino acid contents at 60, 20, and 10, 5, 3.5, and 1.5% (by weight), respectively (Reeves et al. 1993, Moriguchi et al. 2001, DeMar et al. 2004, Contreras et al. 2001). Dietary fat came from adding select amounts of hydrogenated coconut, safflower or flaxseed oils. Hydrogenated coconut (6% and 6.6% by weight, n-3 PUFA adequate and deprived, respectively) and safflower oil (3.2% and 3.4% by weight, n-3 PUFA adequate and deprived, respectively) were added to both diets as a base. Flaxseed oil (0.8% by weight) was added to the n-3 PUFA adequate diet to provide ALA at approximately 3.3% of total fatty acids. The n-3 PUFA deprived chow was very low in ALA (0.15%
of total fatty acids). Lauric (12:0), myristic (14:0), palmitic (16:0) and stearic (18:0) saturated fatty acids comprised approximately 39, 14, 9, and 7% of the total fatty acids for the n-3 PUFA adequate chow, respectively. The n-3 PUFA deprived chow had a similar saturated fatty acid profile, but contained a higher percentage of lauric acid (42%), compared to the n-3 PUFA adequate diet, to compensate for the lack of n-3 fatty acids. Both diets contained safflower oil to provide LA as approximately 21% of total fatty acids. All 20+ carbon chain fatty acids amounted to less than 0.05% of total fatty acids in both diets. LA and ALA were set at 6% and 1% of total caloric intake (3935 kcal/kg), giving a ratio of ~6:1 in the n-3 PUFA adequate diet. Dietary fatty acid composition was confirmed by GC analysis (see below).

Radiotracer

5,6,8,9,11,12,14,15-³H Arachidonic acid ([³H] AA) in 100% ethanol, specific activity of 200 Ci/mM, was purchased from Moravek Biochemicals (Brea, CA). High performance liquid chromatography (HPLC) with liquid scintillation counting was used to verify radioactive purity at >95%. The [³H] AA was dissolved in 5 mM HEPES buffer (pH 7.4) containing 50 mg/mL fatty acid-free bovine serum albumin and sonicated for 15 min (Gatti et al. 1986, DeMar et al. 2004). The radioactivity of the perfusate was confirmed to be 94 % 20:4n-6 with 6 % of the radioactivity eluting with 16:0.

Intra-cerebroventricular (i.c.v.) injection of [5,6-³H]AA

After 15 weeks of feeding, each rat was weighed using a digital scale. Rats were then anesthetised by isoflurane inhalation, and the head was placed in a stereotaxic instrument (Stoelting Co., IL) and the skull exposed. A 33-gauge bevelled injection needle (World Precision Instruments Inc., Sarasota, FL, U.S.A.) was inserted into the right lateral cerebral ventricle (4 mm ventral to the dura) via a hole that was drilled in the cranium at 1 mm posterior and 1.5 mm lateral to the bregma (Noble et al. 1967, Gatti et al. 1986). An injection (5 µL total volume, 0.175 µL/min; quintessential stereotaxic
injector, Stoelting Co., IL) was made of 40 µCi of [³H]AA. The needle was left in for 5 minutes following the end of the injection, after which it was removed at a rate of 1 mm/min, and the hole sealed with cranioplast cement. The wound was closed with self-dissolving sutures and swabbed with iodine. For pain control, 1% sensocaine solution was injected under the scalp and a subcutaneous injection of 1 mL 0.9% saline was given to prevent dehydration. Animals were placed in a recovery cage with a heat-lamp and then returned to their respective n-3 PUFA adequate or deficient diets, where they remained until the end of the study.

Collection of brains

At 4, 6, 8, 12, 16, 36, 48, 60 and 120 days post-i.c.v. injection of [³H] AA, n-3 PUFA adequate (n=4) and deprived rats (n=4) were euthanized by CO₂ inhalation and decapitation. Brains were removed and stored at -80° C.

Isolation of brain lipids

Total lipids from whole brain were extracted according to the method of Folch (Folch et al. 1957). Isolation of various lipid classes from the total lipid extract was achieved by thin-layer chromatography (TLC). TLC H-plates (Analtech, Newark and DE) were washed in chloroform and methanol (2:1) and activated by heating for 1 hour at 100° C. Brain total lipid extracts were separated into total phospholipids and phospholipid classes (ChoGpl, EtnGpl, PtdIns and PtdSer) using a solvent system of heptane : diethyl ether : glacial acetic acid (60 : 40 : 2 by volume), or chloroform : methanol : 2-propanol : 0.25% w/v M KCl : triethylamine (30 : 9 : 25 : 6 : 18 by volume), respectively. TLC plates were sprayed with 8-anilino-1-naphthalene sulfonic acid (0.1% w/v) and lipid bands were visualized under UV light. The positions of brain ChoGpl, EtnGpl, PtdIns and PtdSer bands were identified using authentic phospholipid standards (Avanti, Alabaster, AL) run on the TLC plates.
Quantitation of phospholipid radioactivity and fatty acid concentrations

Brain phospholipid bands were scraped from TLC plates into scintillation vials with 5 ml of scintillation cocktail (ASC; GE Healthcare Bio-sciences Corp., Piscataway, NJ) and counted using a Packard TRI-CARB2900TR liquid scintillation counter (GMI, Ramsey, MN) with a detector efficiency of 47.7%. Radioactivities (dpm) were adjusted for counting efficiency and converted to curies (Ci). To determine fatty acid concentrations in each phospholipid, TLC scrapes were methylated using 14% boron trifluoride-methanol at 100°C for 1 hour. Prior to methylation, di-17:0 phosphatidylcholine was added as an internal standard to brain phospholipids. Fatty acid methyl esters (FAMEs) from total brain phospholipids, phospholipid fractions, and rodent chow were analyzed using a Varian-430 gas chromatograph (Varian, Lake Forest, CA) equipped with a Varian FactorFour capillary column (VF-23 ms; 30 m x 0.25 mm i.d. x 0.25 μm film thickness) and a flame ionization detector. Samples were injected in splitless mode. The injector and detector ports were set at 250°C. FAMEs were eluted using a temperature program set initially at 50°C for 2 min, increased at 20°C/min and held at 170°C for 1 min, then at 3°C/min and held at 212°C for 5 min to complete the run at 28 min. The carrier gas was helium, set to a constant flow rate of 0.7 ml/min. Peaks were identified by retention times of FAME standards. Fatty acid concentrations (μmol/g wet wt brain) were calculated by proportional comparison of GC peak areas with the area of the 17:0 internal standard (Chen et al. 2009).

Confirmation of Radiotracer Identity

Radiotracer separation and identification was performed according to the method of Aveldano et al., with slight modifications (Aveldano et al. 1983, Igarashi et al. 2006, Lee et al. 2007a, Lee et al. 2007b). FAMEs from total phospholipids were separated by HPLC (Waters 2690, Boston, MS) with a Luna C18 reverse column (4.6 x 250 mm, 100 Å; Phenomenex, Torrance, CA) equipped with an in-line UV photodiode array detector (Waters 996, Boston, MS) and monitored at 242 nm. Initial conditions were
set at 1 ml/min gradient system consisting of (A) 100% H₂O and (B) 100% acetonitrile. The gradient commenced with 85% (B) for 30 min, then increased to 100% (B) over a 10 min period, where it was maintained for 20 min before returning to 85% (B) over a 5 min period. All fractions were collected at 1 min intervals for a total of 55 min and then analyzed separately by liquid scintillation counting. Similar to what has been reported by Igarashi et al., using authentic standards (Nu-Chek-Prep, Elysian, MN), we find that methyl esters of 20:4n-6, 22:4n-6 and 22:5n-6 eluted from the HPLC at 33.5, 42.5 and 38.5 min, respectively (Igarashi et al. 2006).

Calculations and Statistics

Data were expressed as means ± SE. Log₁₀ radioactivity (in total or individual brain phospholipid fractions, nCi/brain) was plotted against time post-i.c.v. injection of [³H] AA, and the data were fit by linear regression to provide slopes in (log₁₀) nCi/brain/day (Figure 6). Linear regression was used to determine if the slopes were significantly different from zero, and was used to determine whether the slope in each phospholipid fraction differed significantly between n-3 PUFA deprived and adequate rats (GraphPad Prism 4, GraphPad Software Inc., La Jolla, CA, U.S.A.). Statistical significance was taken as p≤0.05. Half-lives (days) of [³H] AA were calculated from the measured slopes in total and individual phospholipids by the following equation (Stinson et al. 1991, DeMar et al. 2004):

Equation 3: 

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

Results

Bodyweights

Rat bodyweights (735 ± 57, 716 ± 69 g, n-3 PUFA adequate and deprived groups, respectively) did not differ statistically (p=0.2) after 15 weeks of n-3 PUFA adequate or deprived diet.
Tracer identification and Brain Phospholipid PUFA Concentrations

Two brain samples (Day-4 time point, post-surgery, n-3 PUFA adequate and deprived diet) were analyzed by HPLC to confirm successful infusion of the perfusate into the brain; 20:4n-6 eluded at 33.5 min, and total phospholipid radioactivity was > 94 % AA. Trace amounts of palmitic acid (16:0) (< 6 %) were also found in brain phospholipids. Because the radioactivity associated with 16:0 was also found in the perfusate, it is not possible to associate this to brain metabolism. We also analysed the 20:4n-6 fraction obtained from the HPLC by GC/FID and did not detect any other PUFA in this fraction.

Table 1 shows the values for esterified fatty acids in brain phospholipid classes, from n-3 PUFA adequate and deprived rats, measured after 15 weeks of feeding. DHA concentrations in total brain phospholipids were 27% lower in the n-3 PUFA deprived group, compared to the n-3 PUFA adequate group. DHA was also lower in phospholipid fractions of the n-3 PUFA deprived rats (ChoGpl-29%, EtnGpl-24%, PtdIns-50%, PtdSer-31%), compared to the n-3 PUFA adequate group (p<0.05). The n-6 PUFA docosapentaenoic acid (DPA; 22:5n-6) was significantly elevated in the n-3 PUFA deprived group compared to the n-3 PUFA adequate group across all phospholipid classes (p<0.001). AA was significantly higher in total brain phospholipids and ethanolamine glycerophospholipids from the n-3 PUFA deprived group compared to the n-3 PUFA adequate group, whereas stearic acid (18:0) was significantly higher in PtdSer in the n-3 PUFA deprived group (p<0.05). See Table 1 for other fatty acids.

Brain Phospholipid AA Half-lives

Total brain phospholipid and phospholipid fraction radioactivity was plotted over time (days) following surgery (Figure 6). Slopes for all phospholipid classes among both dietary groups were significantly different from zero (p<0.0001). Linear regression analysis of slopes showed no statistical difference between n-3 PUFA adequate and deprived groups across all phospholipid classes (p>0.05). Loss half-lives are shown in Table 2, ranging from 26 to 51 days for the n-3 PUFA adequate group, and
from 28 to 57 days for the n-3 PUFA deprived group. No significant differences were found for the half-lives between the groups (p>0.05). Within the dietary groups, the half-lives of AA in both EtnGpl and PtdSer were significantly longer than the half-lives of AA in ChoGpl and PtdIns (p<0.0001). No other differences in half-life were observed between phospholipid fractions with a dietary group. The rate of AA loss from brain phospholipids ($J_{out}$) was calculated using Equation 4 (Purdon et al. 2002, DeMar et al. 2004):

\[
J_{out} (\mu\text{mol/brain/day}) = \ln(2)C_{\text{AA}}/T_{1/2}
\]

and ranged from 0.014 (PtdSer) to 0.149 µmol/brain/day (EtnGpl) for the n-3 PUFA adequate group, and from 0.015 (PtdSer) to 0.149 µmol/brain/day (EtnGpl) for the n-3 PUFA deprived group (Table 2).

**Discussion**

Dietary deprivation of n-3 PUFA in rats has previously been shown to result in a 27-37% decrease in brain phospholipid DHA following 15 weeks of feeding, compared to the n-3 PUFA adequate diet (DeMar et al. 2006c, DeMar et al. 2004, Rao et al. 2007c); we observed a significant total brain phospholipid DHA decrease of 27% in our n-3 PUFA deprived group compared to the n-3 PUFA adequate group (from 9.9 to 7.3 µmol/ g brain). Furthermore, after 15 weeks, total phospholipid AA was significantly increased in the n-3 PUFA deprived group; this effect has been previously reported (Igarashi et al. 2007c, Mathieu et al. 2008) and brain PUFA concentrations appear to be stable upon 15 weeks of adequate or deprived feeding (DeMar et al. 2004). We also observed a 2000% increase in total phospholipid n-6 DPA in the n-3 PUFA deprived group compared to the n-3 PUFA adequate group (from 0.2 to 4 µmol/ g brain), which is consistent with observations from other studies (DeMar et al. 2006c, DeMar et al. 2004, Contreras et al. 2001). Past studies have examined brain AA metabolism for ≤ 24 hours following i.c.v. injection in the lateral ventricle (Gatti et al. 1986, Sun & Yau 1976), giving us the opportunity to measure brain phospholipid AA loss over a greater time period (4-120 days). We
calculated the loss half-life of AA in rat brain total phospholipids to be 44 ± 4 and 46 ± 4 days, for the n-3 PUFA adequate and deprived groups, respectively; these half-lives represent the rate of AA metabolic consumption, and not the more rapid kinetic rate of recycling into the phospholipid membrane. The loss half-lives for phospholipid fractions ranged from 26 (PtdIns) to 51 (PtdSer) days in the n-3 PUFA adequate group, and 28 (ChoGpl, PtdIns) to 57 (PtdSer) days in the n-3 PUFA deprived group; however, the differences were not significant between the dietary groups. Aminophospholipids EtnGpl and PtdSer, thought to be asymmetrically located on the cytosolic surface of brain phospholipid membranes (Walton et al. 1997, Ding et al. 2000), had a significantly longer AA loss half-life compared to ChoGpl and PtdIns, within their respective dietary groups. This finding is consistent with slower rates of brain AA incorporation into EtnGpl and PtdSer, compared to ChoGpl and PtdIns, observed in other studies (Sun & Su 1979, DeGeorge et al. 1989). It should be noted, that i.c.v. infusion can induce inflammation and damage to the BBB (Choi et al. 1999). To minimize this we used a 33 gauge needle and allowed the animal to recover for 4-120 days prior to measuring brain phospholipid radioactivity. This procedure has been widely used to deliver compounds including fatty acids to the brain (Gatti et al. 1986, DeMar et al. 2004, Sun & Yau 1976, Thakker et al. 2009, Golovko & Murphy 2006, Choi et al. 2008).

With growing evidence implicating brain AA signalling in neurological and psychiatric disorders (Rapoport 2008a, Yagami 2006, Harbige & Sharief 2007, Minghetti 2004), manipulation of brain phospholipid AA concentration appears to be a likely direction for future research (Igarashi et al. 2008, Rapoport 2008a), and our loss half-life estimate offers insight into brain phospholipid AA depletion over time. One caveat to the use of these calculated half-lives, however, is that the brain appears to be capable of conserving a minimal concentration of both AA and DHA. DeMar et al. measured the rate of loss of i.c.v.-infused radiolabelled DHA from rat brain phospholipids, following 15 weeks of n-3 PUFA adequate or deprived feeding (DeMar et al. 2004). The rates of loss were used to calculate loss half-lives for the two dietary groups, which were reported as 33 and 90 days for the n-3 PUFA adequate and
deprived group, respectively. The prolonged half-life of the n-3 PUFA deprived group was interpreted as brain conservation of DHA. Conversion of ALA to DHA in the brain is slow (<1%), and is not increased in periods of n-3 PUFA deprivation (Igarashi et al. 2007c). Conservation of brain DHA may be occurring, as the enzyme thought to be responsible for cleaving DHA from brain phospholipids (iPLA2) has been shown to be down-regulated during n-3 PUFA deprivation (Rao et al. 2007c). We did not observe a similar conservation of AA in our study, using the same n-3 PUFA dietary model for 15 weeks, suggesting DHA but not AA is selectively conserved in situations of n-3 PUFA deprivation. The difference between AA and DHA may be explained by the specificity of enzymes known to release AA and DHA from brain phospholipids and/or other selective catabolic reactions including β-oxidation and eicosanoid/docosanoid synthesis (Cunnane et al. 2003, Green et al. 2008, Gavino & Gavino 1991). Several studies suggest AA and DHA are preferentially released from the sn-2 position of brain phospholipids by fatty-acid selective PLA2. Strokin et al. demonstrated that addition of 4-bromoenol lactone, an inhibitor of iPLA2, to cultured rat astrocytes significantly reduced DHA release, but had little effect on AA release. Conversely, addition of methyl arachidonyl fluorophosphonate, a general inhibitor of cPLA2, to astrocytes resulted in a total inhibition of AA release, while quenching extracellular calcium significantly down-regulated AA release, but left DHA release unaffected (Strokin et al. 2003). Lee et al observed an up-regulation of both AA turnover and cPLA2 activity in rats chronically administered Fluoxetine, with no discernable change in sPLA2 or iPLA2 activation (Lee et al. 2006). Other studies observed a down-regulation of both AA turnover and cPLA2 activity in rats treated with Carbamazepine, with no change in iPLA2 activity (Ghelardoni et al. 2004) or DHA turnover (Bazinet et al. 2006a). Confirming enzyme specificity would be beneficial for pharmacological interventions of neurodegenerative disorders originating from an up-regulation of the AA signalling cascade, by targeting AA turnover without altering phospholipid DHA release. n-3 PUFA deprivation has also been shown to up-regulate cPLA2 activity (Rao et al. 2007c), therefore, brain phospholipid AA turnover may be
increased in the present study. We did not observe a significantly different brain phospholipid AA loss half-life between the n-3 PUFA adequate and deprived group; however, only absolute loss of brain phospholipid AA was measured, and under normal conditions ~97% of brain phospholipid AA cleaved from the phospholipid membrane is re-esterified after being released (Rapoport 2008a, Green et al. 2008), thus brain phospholipid AA turnover may be increased but recycled back into brain phospholipids. Furthermore, we tested the half-life of AA loss in rodents receiving 21% of fatty acids as LA. It would be of interest to repeat the current study with chow consuming lower levels of n-6 PUFA (Bazinet et al. 2003) to see if n-3 PUFA deprivation increases AA loss when the AA supply to the brain is limited.

A study by Igarashi et al. using an n-6 PUFA adequate and deprived diet, observed a 27% decrease in brain phospholipid AA after 15 weeks of feeding in the n-6 PUFA deprived group, compared to the n-6 PUFA adequate group (Igarashi et al. 2008). While significant, this decrease is short of the ~75% decrease predicted by our loss half-lives of 44 and 46 days (n-3 PUFA adequate and deprived groups, respectively); assuming no change in brain uptake of plasma AA and no brain phospholipid AA conservation, this 27% decrease in brain phospholipid AA translates into a loss half-life of approximately 210 days, compared to our shorter calculated loss half-life. This discrepancy suggests significant compensatory mechanisms are in place during n-6 PUFA deprivation. Future studies using an n-6 PUFA deprived diet could determine if brain phospholipid AA conservation takes place during n-6 PUFA deprivation.

To our knowledge, direct measurement of the AA loss half-life in rat brain phospholipids has not been calculated before; however, brain uptake rates for AA have been calculated (using the same dietary model) based on the rate of radiolabelled AA entry into brain phospholipids from the plasma unesterified pool (Contreras et al. 2001). This study predicted a brain phospholipid loss AA half-life of approximately 42 and 48 days for n-3 PUFA adequate and deprived groups, respectively, which closely
approximates the direct loss half-life found in our study. The similarity of the predicted AA loss half-life and our direct-measure of AA loss half-life support findings suggesting the plasma unesterified pool may contribute a significant percentage of brain phospholipid AA (Rapoport et al. 2001, Chen et al. 2008a, Hamilton & Brunaldi 2007b). If the unesterified pool is supplying the brain with AA, attempts to alter the accretion and/or turnover of brain phospholipid AA through pharmacological manipulation of blood-brain barrier endothelium lipoprotein receptors may prove ineffective. Consequently, plasma unesterified AA influx to the brain could be decreased by lowering plasma unesterified AA concentrations. Furthermore, investigations using unesterified [1-\(^{11}\)C] AA for PET imaging have calculated human brain AA uptake based on the rate of AA incorporation from the plasma unesterified AA pool (Rapoport 2008a, Esposito et al. 2007). However, if other pools (LDL, HDL, VLDL, lysophospholipid, etc.) are also contributing significantly to brain AA concentrations, current estimations of brain AA uptake rate would be underestimated, and the brain phospholipid AA loss half-life therefore overestimated. Future studies are needed to confirm which plasma pool(s) contribute to brain phospholipid AA uptake.

**Conclusion**

In conclusion, we infused \([^{3}\]H\) AA in the right lateral ventricle of male Long Evans rats, following 15 weeks of n-3 PUFA adequate or deprived diet, to determine the loss half-life of AA in rat brain phospholipids. We calculated a loss half-life of 44 and 46 days for the n-3 PUFA adequate and deprived groups, respectively, which is similar to the loss half-life predicted from the incorporation rate of plasma unesterified \([^{3}\]H\) AA into brain phospholipids, using the same dietary model (Contreras et al. 2001). This similarity suggests the unesterified AA pool is a significant contributor to brain phospholipids. The calculated half-lives may be used as a guideline in future studies attempting to manipulate brain phospholipid AA using a dietary strategy; however, our half-lives may represent the upper-limit rate of
loss, as a study using an n-6 PUFA adequate and deprived diet for 15 weeks reported a much slower rate of loss (Igarashi et al. 2008). Finally, we did not observe a conservation of AA as was observed for DHA in a similar n-3 PUFA adequate and deprived 15 week feeding model (DeMar et al. 2004). This result could be explained by the selectivity of enzymes (iPLA₂, cPLA₂ and sPLA₂) known to release AA and DHA from brain phospholipids; a better understanding of selectivity will be useful for pharmacological targeting of brain AA turnover without disruption of the brain DHA signalling cascade. This may prove beneficial for the treatment of various neurological and psychiatric diseases, such as Bipolar Disorder, in which evidence points to a pathological up-regulation of brain AA signalling (Rao et al. 2008).
Acknowledgements

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References


Igarashi, M., Gao, F., Kim, H. W., Ma, K., Bell, J. M. and Rapoport, S. I. (2008) Dietary n-6 PUFA deprivation for 15 weeks reduces arachidonic acid concentrations while increasing n-3 PUFA concentrations in organs of post-weaning male rats. *Biochimica et biophysica acta.*


Scott, B. L. and Bazan, N. G. (1989) Membrane docosahexaenoate is supplied to the developing brain and retina by the liver. *Proceedings of the National Academy of Sciences of the United States of America, 86*, 2903-2907.


Table 1. Esterified fatty acid concentrations in brain phospholipid classes from n-3 PUFA adequate and deprived rats, following 15 weeks of feeding

<table>
<thead>
<tr>
<th>Esterified Fatty Acid</th>
<th>ChoGpl</th>
<th>EtnGpl</th>
<th>PtdIns</th>
<th>PtdSer</th>
<th>Total PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.1 ± 0.09</td>
<td>0.12 ± 0.02</td>
<td>0.3 ± 0.09</td>
<td>0.3 ± 0.17</td>
<td>0.02 ± 0.003</td>
</tr>
<tr>
<td>16:0</td>
<td>17.7 ± 0.9</td>
<td>20.3 ± 1.8</td>
<td>3.7 ± 0.2</td>
<td>4.3 ± 0.2*</td>
<td>0.6 ± 0.03</td>
</tr>
<tr>
<td>18:0</td>
<td>5.8 ± 0.3</td>
<td>6.8 ± 0.6</td>
<td>8.1 ± 0.4</td>
<td>9.2 ± 0.4</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>20:0</td>
<td>0.1 ± 0.06</td>
<td>0.1 ± 0.01</td>
<td>0.2 ± 0.05</td>
<td>0.1 ± 0.009</td>
<td>0.02 ± 0.003</td>
</tr>
<tr>
<td>22:0</td>
<td>0.1 ± 0.01</td>
<td>0.2 ± 0.02</td>
<td>ND</td>
<td>0.05 ± 0.005</td>
<td>ND</td>
</tr>
<tr>
<td>16:1n-9</td>
<td>0.3 ± 0.08</td>
<td>0.60 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>0.8 ± 0.3</td>
<td>0.02 ± 0.008</td>
</tr>
<tr>
<td>18:1n-(7+9)</td>
<td>11.3 ± 1</td>
<td>13.0 ± 1.6</td>
<td>8.0 ± 0.9</td>
<td>9.8 ± 0.9</td>
<td>0.7 ± 0.05</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>0.5 ± 0.01</td>
<td>0.6 ± 0.05</td>
<td>1.4 ± 0.09</td>
<td>1.5 ± 0.06</td>
<td>0.06 ± 0.009</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>0.08 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.08 ± 0.005</td>
<td>0.09 ± 0.005</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>24:1n-9</td>
<td>0.1 ± 0.009</td>
<td>0.2 ± 0.02*</td>
<td>0.03 ± 0.002</td>
<td>0.06 ± 0.006**</td>
<td>0.01 ± 0.002</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>0.3 ± 0.02</td>
<td>0.3 ± 0.03</td>
<td>0.13 ± 0.01</td>
<td>0.27 ± 0.04***</td>
<td>0.02 ± 0.002</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.027 ± 0.004</td>
<td>0.05 ± 0.008*</td>
<td>0.02 ± 0.0007</td>
<td>0.04 ± 0.007*</td>
<td>ND</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>ND</td>
<td>ND</td>
<td>0.008 ± 0.001</td>
<td>0.01 ± 0.006</td>
<td>ND</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>0.05 ± 0.004</td>
<td>0.06 ± 0.007</td>
<td>0.09 ± 0.01</td>
<td>0.07 ± 0.004</td>
<td>ND</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>2.0 ± 0.1</td>
<td>2.4 ± 0.2</td>
<td>4.4 ± 0.03</td>
<td>5.2 ± 0.03</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>0.3 ± 0.01</td>
<td>0.4 ± 0.04***</td>
<td>1.9 ± 0.1</td>
<td>2.5 ± 0.09***</td>
<td>0.09 ± 0.015</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.04 ± 0.003</td>
<td>0.6 ± 0.05***</td>
<td>0.1 ± 0.01</td>
<td>2.3 ± 0.1***</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.03 ± 0.0001</td>
<td>0.01 ± 0.002***</td>
<td>0.09 ± 0.0005</td>
<td>0.04 ± 0.001***</td>
<td>ND</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>1.5 ± 0.1</td>
<td>1.0 ± 0.09***</td>
<td>6.2 ± 0.5</td>
<td>4.5 ± 0.09***</td>
<td>0.2 ± 0.04</td>
</tr>
</tbody>
</table>

**Data are means ± SE (n=5 independent samples per group). Choline glycerophospholipids (ChoGpl); Ethanolamine glycerophospholipids (EtnGpl); Phosphatidylinositol (PtdIns); Phosphatidylserine (PtdSer); Total phospholipids (Total PL). ND, not detected. *p < 0.05, **p < 0.01, ***p < 0.001; significant difference between n-3 PUFA deprived (Dep.) and n-3 PUFA adequate (Adq.) means. Fatty acids: 14:0, myristic; 16:0, palmitic; 18:0, stearic; 20:0, arachidic; 22:0, docosanoic; 16:1n-9, palmitoleic; 18:1n-(7+9), vaccenic/oleic; 20:1n-9, eicosenoic; 22:1n-9, erucic; 24:1n-9, nervonic; 18:2n-6, linoleic 18:3n-3, alpha-linolenic; 18:3n-6, gamma-linolenic; 20:3n-3, eicosatrienoic; 20:4n-6, arachidonic; 22:4n-6, docosatetraenoic; 22:5n-6, docosapentaenoic 22:6n-3, docosahexaenoic. ‡ No significant difference between respective n-3 PUFA adequate and deprived lipid pools.**
Table 2. Loss half-lives of [5,6-³H]AA from rat brain phospholipids

<table>
<thead>
<tr>
<th>n-3 PUFA diet group</th>
<th>Brain Lipid</th>
<th>Slope, days⁻¹</th>
<th>SE, days⁻¹</th>
<th>p value</th>
<th>T₁/₂, days</th>
<th>Jₐₒᵤᵗ, μmol/brain/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adequate</td>
<td>Total PL</td>
<td>-0.006830</td>
<td>0.0006142</td>
<td>&lt;0.0001</td>
<td>44</td>
<td>0.248</td>
</tr>
<tr>
<td></td>
<td>ChoGpl</td>
<td>-0.01110</td>
<td>0.0007045</td>
<td>&lt;0.0001</td>
<td>27</td>
<td>0.103</td>
</tr>
<tr>
<td></td>
<td>EtnGpl</td>
<td>-0.006694</td>
<td>0.0006154</td>
<td>&lt;0.0001</td>
<td>45</td>
<td>0.149</td>
</tr>
<tr>
<td></td>
<td>PtdIns</td>
<td>-0.01137</td>
<td>0.0008117</td>
<td>&lt;0.0001</td>
<td>26</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>PtdSer</td>
<td>-0.005847</td>
<td>0.0007621</td>
<td>&lt;0.0001</td>
<td>51</td>
<td>0.014</td>
</tr>
<tr>
<td>Deprived</td>
<td>Total PL</td>
<td>-0.006581</td>
<td>0.0005942</td>
<td>&lt;0.0001</td>
<td>46</td>
<td>0.281</td>
</tr>
<tr>
<td></td>
<td>ChoGpl</td>
<td>-0.01067</td>
<td>0.0006158</td>
<td>&lt;0.0001</td>
<td>28</td>
<td>0.123</td>
</tr>
<tr>
<td></td>
<td>EtnGpl</td>
<td>-0.005873</td>
<td>0.0006609</td>
<td>&lt;0.0001</td>
<td>51</td>
<td>0.149</td>
</tr>
<tr>
<td></td>
<td>PtdIns</td>
<td>-0.01071</td>
<td>0.0007731</td>
<td>&lt;0.0001</td>
<td>28</td>
<td>0.088</td>
</tr>
<tr>
<td></td>
<td>PtdSer</td>
<td>-0.005276</td>
<td>0.0007224</td>
<td>&lt;0.0001</td>
<td>57</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Slopes for decline of [³H] AA radioactivity in brain phospholipids, and corresponding calculated half-lives (T₁/₂) and rates of AA loss (Jₐₒᵤᵗ). The latter were calculated using equations 3 and 4. p-values designate significance of difference of slope from 0. ChoGpl and PtdIns were significantly different compared to EtnGpl and PtdSer within the same dietary group; p < 0.05. Mean ± SE brain weight; 2.02 ± 0.34 and 2.10 ± 0.52 g, n-3 PUFA adequate and deprived groups, respectively. Abbreviations, see Table 1.
**Figure 6.** Select brain phospholipid fraction AA radioactivity over time

Data are mean ± SE; n=4 independent samples per group per time point. Linear regression slopes for (log_{10}) phospholipid radioactivity (nCi/brain) over time (days). Adequate, n-3 PUFA adequate group; Deprived, n-3 PUFA deprived group; Day, time (days) following 15 weeks of feeding, post-i.c.v. [^{3}H] AA injection. (Note differences in y-axis scales.)
Chapter 4

General Discussion

Brain AA turnover kinetics

In the previous study we determined the half-life of AA in rat brain phospholipids following fifteen weeks of feeding, among rats consuming either an n-3 PUFA adequate or deprived diet. We found the AA half-life did not differ significantly between the two groups, unlike the significant difference between groups observed by DeMar et al (2004) when injecting [14C] DHA, under the same fifteen-week feeding model of n-3 PUFA adequate or deprived diet (DeMar et al. 2004). In that study, the n-3 PUFA adequate group had a brain phospholipid DHA half-life of 33 days, while the deprived group had a significantly greater DHA half-life of 90 days, suggesting DHA was being conserved in the n-3 PUFA deprived group. Using AA, we hypothesized the opposite effect; brain phospholipid AA was expected to be turned over faster in the n-3 PUFA deprived group. However, we did not observe a significantly different brain phospholipid AA half-live between the groups. Seemingly, this result would suggest brain AA turnover is not dependent upon brain DHA concentration or turnover; however, brain phospholipid AA turnover may be upregulated under conditions of DHA deprivation, and not detected by the previous study. Our determination of brain AA half-life was dependant on measuring the rate of actual AA “loss”, or consumption by metabolism. The rate of AA being cleaved from the phospholipid membrane and “recycled” back into the membrane, was not measured, and could possibly be altered. One limitation of measuring brain PUFA half-life rates of loss, as was done in the previous study, is the exclusion of this “recycling” rate in the calculation. It is known ~97% of AA is, in fact, not metabolically consumed when it is cleaved from the phospholipid membrane, but returned to the membrane and re-esterified (Rapoport 2008a) (Figure 4). This process is energetically costly, requiring 2 ATP molecules for
an acyl-CoA synthetase to convert the unesterified AA into arachidonoyl-CoA in the endoplasmic reticulum, and re-esterify it back into the phospholipid membrane via an acyl-CoA transferase. Re-acylation of AA into the sn-2 position of the phospholipid membrane has been estimated to consume ~0.2% of total rat brain ATP production, suggesting this turnover cycle is a significant metabolic process in the brain (Purdon et al. 2002). It is possible the rate of AA cleavage from the phospholipid membrane, and subsequent re-esterification, is altered according to the availability of DHA in the brain. Elucidating the function of brain AA cleaved from the phospholipid membrane, but not consumed through enzymatic re-structuring, may be important for the understanding of neural cellular signalling.
Figure 7. Incorporation, turnover and loss rates and half-lives (T1/2) of brain phospholipid AA. (1) Half-life and incorporation rate \( J_{in,i}(AA) \) of AA from the plasma unesterified pool. (2) Half-life and AA turnover rate \( J_{FA,i}(AA) \) from the phospholipid membrane. (3) Half-life and rate \( J_{out,i}(AA) \) of AA metabolic consumption from brain phospholipids.
**Docosapentaenoic Acid**

In the previous study, one result of the 15-week n-3 PUFA dietary manipulation was the significant increase (2000%) in n-6 docosapentaenoic acid (DPA, 22:5n-6) observed in the n-3 PUFA deprived group, compared to the n-3 PUFA adequate group. This increase has been observed in several studies of n-3 PUFA deprivation (Contreras et al. 2001, DeMar et al. 2004, Mathieu et al. 2008); however, the significance of the accretion of this fatty acid in mammalian brain phospholipids is not well understood. DPA is a twenty-two carbon n-6 fatty acid, with a structure similar to the n-3 DHA, and is thought to selectively replace DHA during situations of prolonged DHA deprivation. This raises the question: does DPA play a role in neurological functioning and disease; and, if so, how significantly? Presently, very few studies have examined its effect; however, a study by Lim et al (2005) used n-3 PUFA deprived rats separated at birth, and groups were fed either a DHA-rich diet, a DPA-rich diet, or a diet with both DHA and DPA until adulthood. They were given tests of spatial task performance (Morris water maze). The DPA-fed rats showed increased escape latency, and decreased spatial retention, compared to the other groups (Lim et al. 2005). Post-mortem brain analysis showed an increased DPA:DHA ratio in the orbito-frontal cortex of drug-naive schizophrenics compared to normal controls (McNamara et al. 2009). These findings suggest that, in terms of functioning, n-6 DPA does not fully substitute for DHA in the brain. However, there is evidence n-6 DPA may have anti-inflammatory properties in the brain, following metabolic synthesis into resolvin-like compounds. A study by Dangi et al. (2009) exposed n-6 DPA, n-3 DPA, and DHA to 5, 12, and 15-lipoxygenase in vitro, and collected and analyzed the compounds formed. The products formed by these fatty acids were then injected into rats around a surgically-implanted air pouch, followed immediately by an injection of TNF-α into the pouch. The TNF-α produced a four-fold increase in leukocyte migration, which was significantly decreased by the n-6 DPA products to the level of the negative control. Furthermore, the n-6 DPA product was
observed to cause a shift from an active immune response to the resolution phase through the significant proportional increase in macrophages at the site of inflammation.

In light of evidence suggesting increased brain n-6 DPA may be both detrimental (insofar as it is not as functionally efficacious as DHA in the brain) and beneficial, further investigation is warranted. Numerous studies have documented harmful effects of an n-3 PUFA deficient diet, including, but not limited to, decreased learning, attention and memory, behavioural disruption including increased depression and aggression, decreased brain-derived neurotropic factor in the frontal cortex, decreased hippocampal cholinesterase neurotransmission and nerve growth factor, increased eicosanoid production, and increased cellular damage following ischemia (Yamamoto et al. 1988, Mathieu et al. 2008, Chung et al. 2008, Rao et al. 2007d, DeMar et al. 2006c, Aid et al. 2003, Logue et al. 2000, Ikemoto et al. 2000, Moreira et al. 2009). It is possible that some of these effects are not attributable to brain DHA deficiency per se, but rather to increased brain levels of n-6 DPA. The significance of this distinction is, at least in part, due to the reality of dwindling global fish stocks and a concurrent call for increased fish consumption (Ervin et al. 2004, Pauly et al. 2002), which may lead to the scarcity of a viable source of n-3 PUFA. One solution to this problem may lie in differentiating the physiological consequences of increased brain n-6 DPA and the actual consequences of brain DHA deficiency; clinical intervention to alter brain n-6 DPA levels could offer some measure of improvement in symptoms currently associated with n-3 PUFA deprivation, without necessarily requiring greater n-3 PUFA intake. In this thesis project, as well as numerous other studies, significantly increased brain n-6 DPA levels during brain n-3 PUFA deprivation were observed, revealing an evolutionary adaptation in the mammalian brain not fully understood (DeMar et al. 2006c, DeMar et al. 2004, Galli et al. 1970). The chemical properties of this compound are deserving of greater scrutiny in the future.
Implications

Growing evidence suggests the polyunsaturated fatty acids, particularly AA and DHA, are of significant importance in the mammalian brain. Originally thought to simply provide structural integrity to phospholipid membranes, we now understand these lipids have a complex and central role in a large variety of chemical reactions in the brain involved in both homeostasis and disease. AA is abundant in mammalian brain tissue, and is regulated through the action of a variety of enzymes. Although brain AA signalling is not fully understood, we know the products formed from AA, the eicosanoids, are potent molecules for inducing immune response and facilitating neural function. In the present thesis, we have presented evidence to further define the regulation and role of AA in brain. Cleavage of brain AA from the phospholipid membrane is understood to represent the initial stage of transformation of AA into a variety of eicosanoids; therefore, it is important to understand how the de-acylation takes place. Evidence from several studies suggests cPLA₂ preferentially releases brain AA from the phospholipid membrane (Green et al. 2008). Evidence from the thesis experiment further supports this theory of specificity; we did not observe a significant difference in brain phospholipid AA half-life, following 15 weeks of feeding an n-3 PUFA adequate or deprived diet and i.c.v. injection of [³H] AA, between the two groups. DeMar et al. (2004) used the same dietary model, but i.c.v. injected radio-labelled DHA, and observed a significant increase in brain phospholipid DHA half-life in the n-3 PUFA deprived group, compared to the adequate group. We interpret these different findings as evidence of PLA₂ specificity, due to evidence relating brain specific brain PUFA turnover with specific PLA₂ activity (Rao et al. 2008, Chen et al. 2008a). Establishing PLA₂ specificity for PUFA release from brain phospholipids is of considerable importance if the two primary (or other PUFA present, such as n-6 DPA) brain polyunsaturates, AA and DHA, are to be targeted as potential mediators of a variety of neurological illnesses (Lee et al. 2007c, Rapoport 2008a, Rapoport 2008b).
One result of this thesis project was to determine the rate of loss of brain phospholipid AA from rat brain, following a fifteen-week n-3 PUFA adequate or deprived diet. The rate we determined has several implications. First, it allows us to compare our calculated rate of AA loss against an experimentally-derived rate of AA entry into the brain from the unesterified pool (Contreras et al. 2001). We hypothesized that the rate of entry ($J_{\text{in,AA}}$) would approximate the rate of metabolic loss ($J_{\text{out,AA}}$), if the plasma unesterified pool supplies a significant portion of brain phospholipid AA. We calculated a half-life of ~45 days for the two dietary groups, which closely resembles the predicted half-life of brain AA loss derived from the measured rate of unesterified AA entry into the brain (~42 and 48 days, n-3 PUFA adequate and deprived diets, respectively). We can therefore conclude the plasma unesterified AA pool is a significant contributor of brain phospholipid AA; if this is the case, brain phospholipid AA concentration can be targeted through targeting of the plasma unesterified pool, rather than pursuing brain AA level changes through lipoprotein-based therapies. Furthermore, the half-life of brain AA loss is significant for emerging PET studies using a radio-labelled AA tracer, as the current studies assume unesterified plasma AA is the major source of brain phospholipid AA in mapping uptake of the unesterified tracer (Giovacchini et al. 2004). Finally, quantifying a rate of brain AA loss offers a guideline for future studies attempting to alter brain AA levels in vivo. Our calculated half-life of ~45 days likely represents the minimum length for which a dietary intervention could significantly deplete brain AA concentration in rats; other studies suggest the time needed may be much longer (Igarashi et al. 2008).

Another significant implication of the thesis project is in a result not observed; that is, we did not observe a difference in brain AA loss half-lives comparing the n-3 PUFA adequate and deprived groups. n-3 PUFA deprivation could result in increased brain phospholipid AA turnover, due to findings by Rao et al. (2007), who observed both a decrease in iPLA$_2$ activity and an increase in cPLA$_2$ activity following 15 weeks of n-3 PUFA adequate or deprived feeding (Rao et al. 2007c). In our study, we did not observe a difference in rate of AA metabolic loss; however, we did not measure brain phospholipid
AA de-acylation/re-acylation rates from the phospholipid membrane. As the majority (~97%) of AA in this process is re-esterified, the potential exists for this rate of turnover to be increased in an n-3 PUFA deprivation model while not increasing brain phospholipid AA metabolic loss appreciably. In effect, our finding appears to disprove up-regulated brain AA metabolic loss in n-3 PUFA deprivation, narrowing the consequences of prolonged n-3 PUFA deprivation.

**Future Directions**

We did not measure brain phospholipid AA turnover rates in our 15-week dietary models; as the potential exists for increased brain AA turnover rates in an n-3 PUFA deprived feeding model, future studies could examine the dietary effects using rapid high-energy microwave fixation to capture basal turnover rates without ischemia-induced PUFA release and eicosanoid production (Bazinet et al. 2005a, Anton et al. 1983).

In our study, we used an n-3 PUFA adequate and deprived diet to compare rates of brain phospholipid AA loss over time. The n-3 PUFA adequate diet contained ALA as 3.3% of fatty acids, with LA accounting for 21% of fatty acids for both the n-3 PUFA adequate and deprived diet. While these diets have been used previously to examine effects of n-3 deprivation (DeMar et al. 2006c, DeMar et al. 2004, Rao et al. 2007c), future studies could use diets with different fatty acid profiles. For instance, brain phospholipid AA half-life does not appear to be affected by 15 weeks of n-3 PUFA deprivation, however, the effects of n-6 PUFA deprivation on brain AA half-life is unknown. Few studies have implemented an n-6 PUFA deprived, n-3 PUFA adequate diet, though one study by Igarashi et al. (2008) compared such a diet to an n-6 and n-3 PUFA adequate diet fed to rats, and found significantly decreased tissue concentrations of AA, with concomitant increases in DHA, in the n-6 PUFA deprived group (Igarashi et al. 2008). It is therefore possible that prolonged n-6 PUFA deprivation could affect brain phospholipid AA turnover and/or loss; such a finding could be meaningful given the potential
involvement of brain AA in a variety of neurological illnesses. Conversely, the n-3 PUFA diets used in the thesis project and elsewhere contained concentrations of n-3 PUFA currently accepted as “adequate” and “deprived”. However, there is currently no clear guideline to define the minimum or maximum limits of n-3 PUFA in the mammalian diet. Potentially, there exists a spectrum of “adequate” consumption of n-3 PUFA, where the lower end on consumption maintains some degree of basal functioning, while a higher end promotes disease resistance, improved cognition, etc. The opposite is also likely to be true; n-3 PUFA “deprivation”, best measured by clinical effects, may range from somewhat compromised immune function to full pathology. Future work could repeat this study, and others using this dietary model, and manipulate the n-3 PUFA fatty acids to better define the effects of varying consumption.

One conclusion from the thesis project was that because our measured brain phospholipid AA half-life approximates the half-life predicted through measurement of influx of unesterified AA, the unesterified plasma pool is likely the major contributor of brain AA. Implications of this finding may be important for properly targeting the major source of brain AA in disease treatment/prevention; therefore, future studies could attempt to replicate our brain AA half-life using different lipoprotein-receptor knockout animals to help confirm or discount the importance of the unesterified AA pool. This experimental model could also be used to determine the source of other brain fatty acids.
Conclusions

Arachidonic acid is a polyunsaturated fatty acid of great importance in the mammalian body, particularly in the brain. Once thought of as merely a structural constituent of phospholipid membranes, research has demonstrated the complex and far-reaching role of this fatty acid in cellular signalling and immune response. Regulation and physiological effects of AA, and its synthesis into eicosanoids, is not fully understood; however, we do know they are potent components of brain function. Because substantial evidence points to AA playing a role in a variety of neurological diseases and conditions, it is therefore crucial to define the mechanisms by which it is controlled and metabolized. We have presented evidence which points to brain phospholipid AA being released from the membrane preferentially by cPLA2, suggesting brain AA and DHA release from the phospholipid membrane is accomplished through different processes. This concept is important because it suggests the brain utilizes these fatty acids for separate purposes, and allows for potential pharmacological manipulation of one brain PUFA without disturbing the other. The purpose of the thesis project was to measure the brain phospholipid AA rate of loss, and compare findings to other studies. We calculated a half-life of ~45 days for both the n-3 PUFA adequate and deprived groups, which was comparable to the half-life predicted in a previous study based on the rate of unesterified AA entry into the brain. Thus we were able to conclude

1. The unesterified pool is likely the major contributing pool to brain AA.
2. n-3 PUFA deprivation does not alter brain phospholipid AA rates of loss, unlike the prolonged half-life of brain phospholipid DHA in n-3 PUFA deprivation.

Whether n-3 PUFA deprivation, or n-6 PUFA deprivation, alter AA turnover from the phospholipid membrane, remains to be answered.
Brain AA is an intriguing molecule for study, not only because it is increasingly recognized as an important neuronal signalling molecule, but because it is consumed in the diet. We do not fully understand how AA is recycled and what determines its metabolic fate, but the fact that we can, to some degree, affect its accretion in the body through dietary manipulation offers a modicum of control over the exceedingly complex biochemical reactions within our bodies. Present evidence suggests that as our knowledge of this fatty acid increases, it will only be regarded with greater respect. Millions and perhaps billions of years of evolution have selected this fatty acid to perform a variety of complex regulatory reactions in the mammalian brain, and for all of the insight gained into AA in the last century, it is likely to be proven primitive compared to the future potential for discovery. The mammalian brain is perhaps the most complex and poorly understood organ in the animal kingdom, and the key to understanding it lies, at least in part, in uncovering the function of AA.
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APPENDIX

Introduction to appendix

Regulation of brain AA and DHA turnover is important for understanding both normal brain function and neurological disease. The issue is complex due to the fact both of these PUFA occupy the same location in the phospholipid membrane when esterified (sn-2 position), but once cleaved, are metabolized into different products; AA is metabolized into eicosanoids, and DHA is metabolized into docosanoids. Several studies have found evidence to suggest the PLA2s, which cleave the PUFA from the membrane, may be preferential for one PUFA over the other (Ghelardoni et al. 2004, Lee et al. 2008b, Lee et al. 2007b, Rao et al. 2007c) Thus, it is now believed that cPLA2 preferentially cleaves AA, and iPLA2 preferentially cleaves DHA. One important aspect of this possible specificity is in understanding the role of PUFA in neurological disease. For instance, the etiology of bipolar disorder, a common psychiatric disorder, may involve dysregulation of AA turnover (Basselin et al. 2006, Basselin et al. 2005a, Basselin et al. 2005b, Basselin et al. 2003, Bazinet et al. 2006a, Bazinet et al. 2006b, Bosetti et al. 2002, Lee et al. 2008b, Lee et al. 2007b). If the PLA2 are preferential for cleaving specific PUFA from the phospholipid membrane, this could offer an attractive target for manipulation of specific brain PUFA turnover using pharmacological approaches. Neurological diseases such as bipolar disorder, in which evidence suggests brain AA turnover and signalling may play a significant role, could potentially be treated by manipulation of brain AA; the benefit of PLA2 specificity being the potential to do so without disruption of other brain PUFA metabolism, particularly DHA. Conversely, if DHA metabolic products prove to be neuroprotective, brain DHA levels could be increased theoretically without increasing pro-inflammatory AA metabolism. Thus, the following review was written to summarize the current evidence for PLA2 specificity, citing studies of DHA and iPLA2.
THE EMERGING ROLE OF GROUP VI CALCIUM-INDEPENDENT PHOSPHOLIPASE A2 IN RELEASING DOCOSAHEXAENOIC ACID FROM BRAIN PHOSPHOLIPIDS

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Abstract

Brain phospholipids are highly enriched in docosahexaenoic acid (22:6n-3). Recent advances indicate that 22:6n-3 is released from brain phospholipids via the action of phospholipase A2 (PLA2), in response to several stimuli including neurotransmission, where it then acts as a secondary messenger. Furthermore, it is now known that released 22:6n-3 is a substrate for several oxygenation enzymes whose products are potent signaling molecules. One emerging candidate PLA2 involved in the release of 22:6n-3 from brain phospholipids is the group VI calcium-independent (i)PLA2. After a brief review of brain 22:6n-3 metabolism, cell culture and rodent studies facilitating the hypothesis that group VI iPLA2 releases 22:6n-3 from brain phospholipids are discussed. The identification of PLA2’s involved in cleaving 22:6n-3 from brain phospholipids could lead to the development of novel therapeutics for brain disorders in which 22:6n-3 signaling is disordered.
Brain uptake and utilization of docosahexaenoic acid

The mammalian brain is particularly enriched with the polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA; 22:6n-3) (Diau et al. 2005). Within the brain 22:6n-3 helps maintain membrane fluidity (Salem et al. 2001), promotes cell survival (Bazan 2005, Kim 2007, Rao et al. 2007d), acts as a secondary messenger via coupling to neuroreceptors (Jones et al. 1997, Garcia & Kim 1997, DeGeorge et al. 1991) and is converted, via oxygenation, to a variety of signaling molecules some of which have potent anti-inflammatory properties (Ariel & Serhan 2007, Schwab et al. 2007). Thus, it is not surprising that 22:6n-3 is important in neural development (Neuringer et al. 1986, Gibson & Makrides 1998) and has been implicated in a variety of neurological disorders, including stroke (Marcheselli et al. 2003), Alzheimer’s disease (Calon et al. 2004) and major depression (Sinclair et al. 2007).

The brain cannot synthesize n-3 PUFA de novo, therefore it must either uptake preformed 22:6n-3 or desaturate and elongate one of its precursors. With regards to the latter, although brain cells and in particular astrocytes have the capacity to desaturate and elongate α-linolenic acid (18:3n-3) to 22:6n-3 (Williard et al. 2001), in vivo tracer studies suggest that brain desaturation/elongation is a relatively minor pathway (0.024 pmol/g brain/s) (Demar et al. 2005), as compared to brain uptake of preformed 22:6n-3 (13-15 pmol/g brain/s) (Demar et al. 2006a, Demar et al. 2005, Garattini et al. 1999). Furthermore, unlike the liver, the brain does not upregulate its ability to desaturate and elongate 18:3n-3 to 22:6n-6 when dietary n-3 PUFA are limited (Igarashi et al. 2007b, Igarashi et al. 2007a, Igarashi et al. 2007d).

Plasma unesterified 22:6n-3 rapidly disassociates from albumin, passes through the blood-brain barrier and enters the brain (Figure 8, step A) (Robinson et al. 1992, Hamilton & Brunaldi 2007a). Upon its entry, 22:6n-3 is activated by an acyl-CoA synthetase (Bazinet et al. 2006b, Mashek et al. 2006). A
small portion of the newly formed docosahexaenoyl-CoA is β–oxidized (Cunnane et al. 2003, Lee et al. 2005), and the remainder is esterified via an acyl-CoA transferase to the stereospecifically numbered (sn)-2 position of phospholipids. The 22:6n-3 that enters the brain and passes through the docosahexaenoyl-CoA pool is esterified into brain phospholipids at a rate of 13-15 pmol/g brain/s. This 22:6n-3 pool is predominately esterified to ethanolamine (6-7 pmol/g brain/s) and choline glycerophospholipids (4-5 pmol/g brain/s) (Bazinet et al. 2005b, Bazinet et al. 2006a, Cunnane et al. 2003, Lee et al. 2005) and subsequent remodeling and de novo phospholipid synthesis likely explain its relative mass distribution within various phospholipid species (Kim et al. 2004). Phospholipid 22:6n-3 (sn-2 esterified) is then released by PLA2 (see below) at a net rate of 102-131 pmol/g brain/s (Bazinet et al. 2005b, Bazinet et al. 2006a, Lee et al. 2005). A portion of the released 22:6n-3 is available for the synthesis of oxygenated derivatives via cyclooxygenase-2 (Serhan et al. 2002) or a putative 15-lipoxygenase (Serhan & Savill 2005) while the remainder is activated by an acyl-CoA synthetase where again a small portion of it is available for β-oxidation and the remainder is reesterified into the sn-2 position of brain phospholipids. Kinetic studies estimate that under basal conditions approximately 90% of the 22:6n-3 that is released via PLA2 is reesterified into the sn-2 position of brain phospholipids (Bazinet et al. 2005b, Bazinet et al. 2006a, Lee et al. 2005) while the 10% that is lost is replaced by uptake from the plasma unesterified 22:6n-3 pool (Rapoport et al. 2007).

**Brain phospholipase A2**

To date at least 22 genes that encode PLA2 proteins have been identified in mammals. PLA2’s cleave fatty acids from the sn-2 position of glycerophospholipids, resulting in an unesterified “free fatty acid” and a 2-lysophospholipid. On a broad level, the mammalian PLA2 isoforms differ in respect to their tissue and cellular distribution, substrate specificities, calcium requirements and interested readers should consult other reviews for more details (Ghosh et al. 2007, Farooqui et al. 2006, Bonventre &
Sapirstein 2002, Farooqui & Horrocks 2004, Balsinde & Balboa 2005, Schaloske & Dennis 2006) including PLA2 nomenclature/classification (Schaloske & Dennis 2006, Six & Dennis 2000). The isoforms identified in the brain thus far include groups IVA, IVB and IVC calcium-dependent cytosolic (c)PLA2, groups IIA, IIC, IIE, V and X calcium-dependent secretory (s)PLA2, and groups VIA and VIB calcium-independent (i)PLA2 (Molloy et al. 1998, Pickard et al. 1999, Hamaguchi et al. 2003, Kolko et al. 2006, Rao et al. 2007b, Strokin et al. 2007, Mancuso et al. 2000). It is worth noting that the mouse strains C57BL/6, 129/Sv, and B10.RIII have a naturally occurring missense mutation in the gene encoding for the group IIA sPLA2 (Sapirstein & Bonventre 2000, Hamaguchi et al. 2003, Bosetti 2007, Kennedy et al. 1995). As new proteins with PLA2-like activity are identified (Jenkins et al. 2004, Manevich et al. 2007) and specific antibodies and reagents are developed, it is possible that more isoforms will be detected within the brain. Another, yet to be cloned, PLA2 whose activity has been detected in the brain is the plasmalogen-selective PLA2 (Hirashima et al. 1992). This enzyme is capable of cleaving fatty acids from plasmanyl-ethanolamine and because in the brain this glycerophospholipid is rich in 22:6n-3 it is possible that this enzyme may also play a role in 22:6n-3 release from brain phospholipids (Farooqui & Horrocks 2004, Farooqui et al. 2007).

**Group VI iPLA2: Overview**

There are two distinct members of the iPLA2 family, group VIA iPLA2 and group VIB iPLA2. The 85-88 kDa group VIA iPLA2 was first isolated from macrophages in 1994 (Ackermann et al. 1994) and independently cloned by two laboratories in 1997 (Balboa et al. 1997, Tang et al. 1997), whereas the group VIB iPLA2 was cloned in 2000 (Mancuso et al. 2000). The group VIA iPLA2 shares several conserved regions at the C-terminus but has very little homology at the N-terminus with the group VIB iPLA2. The group VI iPLA2’s do not require calcium for their activity but do utilize ATP for stabilization and are stimulated by thrombin and bradykinin (Balsinde & Balboa 2005, Strokin et al. 2003), while inhibited by
bromoenolactone (Ackermann et al. 1995). However, very little is known about the molecular/genetic regulation of group VI iPLA₂, especially within the brain (Chakraborti 2003). Initial studies suggested a homeostatic role for group VI iPLA₂ in regulating membrane turnover (for review see (Winstead et al. 2000)). However, several studies have now shown that group VI iPLA₂ plays an important role in signal transduction (for review see (Balsinde & Balboa 2005, Leslie 2004a)).

In the brain, the basal expression and activity of group VI iPLA₂ is higher than other PLA₂’s (Molloy et al. 1998, Yang et al. 1999b, Yang et al. 1999a, Lee et al. 2006) and its protein expression decreases during aging (Aid & Bosetti 2007). Whereas brain cPLA₂ and sPLA₂ are commonly thought be selective for arachidonic acid (20:4n-6) release (Alonso et al. 1986, Farooqui et al. 2000, Rapoport & Bosetti 2002, Lee et al. 2007d, Leslie 2004a, Leslie 2004b), the specific activity of group VI iPLA₂ using 1-palmitoyl 2-R-phosphatidylcholine when linoleic acid (18:2n-6), palmitic acid (16:0), oleic acid (18:1n-9) or 20:4n-6 were esterified in the sn-2 position (R) was 10.0, 4.3, 3.0 and 2.0 μmol/min/mg protein, respectively (Yang et al. 1999b). However, it is important to consider cellular localization and substrate availability when trying to determine in vivo selectivity. The net rate of release of 18:2n-6 from brain phospholipids (19 pmol/g brain/s) is at least five times lower than that of 22:6n-3 (102-131 pmol/g brain/s) and when preformed 22:6n-3 is present in chow, the concentration of phospholipid esterified 18:2n-6 (987 nmol/g brain) is almost 15 times lower than that of 22:6n-3 (13 844 nmol/g brain) (Demar et al. 2006a). Although these latter observations do not suggest that group VI iPLA₂ is not involved in cleaving 18:2n-6 from brain phospholipids, they do raise the question of why so much group VI iPLA₂ is present in the brain when so little 18:2n-6 is present?
Group VI iPLA₂ and docosahexaenoic acid release from brain phospholipids: Evidence from cell culture studies

The first suggestion that brain group VI iPLA₂ may be selective for DHA release came from Strokin et al., (2003) when they observed that bromoenolactone inhibited 22:6n-3 but not 20:4n-6 release from phospholipids of astrocytes stimulated with ATP (Strokin et al. 2003). This study was followed up by showing that bromoenolactone also inhibited oxygen/glucose deprivation-induced 22:6n-3 release from hippocampal phospholipids (Strokin et al. 2006). Strokin et al., (2007) repeated their 2003 finding in which ATP stimulated 22:6n-3 release from astrocyte phospholipids was inhibited with bromoenolactone and reproduced this effect with siRNA silencing of group VIB iPLA₂. This latter study addressed the issue of bromoenolactone inhibition selectivity and was followed up by further demonstrating that bromoenolactone inhibition of 22:6n-3 release from astrocyte phospholipids was absent upon silencing group VIB iPLA₂ (Strokin et al. 2007).

Group VI iPLA₂ and docosahexaenoic acid release from brain phospholipids: Evidence from in vivo studies

DeMar et al., (2004) tested the half-life of [4,5-³H]22:6n-3 upon its intracerebroventricular administration to rats that had consumed a diet either adequate or deprived of n-3 PUFA for 15 weeks post-weaning (DeMar et al. 2004). The half-life of [4,5-³H]22:6n-3 in brain phospholipids of rats consuming the n-3 PUFA adequate diet was 33 days, whereas it was increased to 90 days in the deprived rats. This conservation of [4,5-³H]22:6n-3 suggested that enzymes involved in the catabolism of 22:6n-3 must be downregulated in the brains of n-3 PUFA deprived rats and in a subsequent experiment candidate brain phospholipase A₂ isoforms (IIA sPLA₂, IVA cPLA₂, and VIA iPLA₂) were examined (Rao et al. 2007b). Whereas activities, protein and mRNA of group IVA cPLA₂ and group IIA sPLA₂ were upregulated in the brains of rats consuming the n-3 PUFA deprived diet, only the group VIA iPLA₂...
isoform was downregulated making it a candidate mechanism by which the brain half-life of $[4,5^3$H]22:6n-3 was prolonged (DeMar et al. 2004, Rao et al. 2007b). The activity, protein and mRNA of group VIA iPLA$_2$ were downregulated, however, the mechanism by which group VIA iPLA$_2$ mRNA was downregulated was not explored and whether or not there was a downregulation of group VIB iPLA$_2$ was not tested.

**Consequences of docosahexaenoic acid release from brain phospholipids**

Although many important functions have been attributed to 22:6n-3 within the brain (Salem et al. 2001, Heird & Lapillonne 2005, Freemantle et al. 2006, Calon & Cole 2007), few studies have attempted to test if these functions are related to released 22:6n-3. Rats deprived of dietary n-3 PUFA to reduce brain concentrations have behavioral deficits (Moriguchi et al. 2000, DeMar et al. 2006c), altered neurotransmission (Aid et al. 2005, Aid et al. 2003), decreased iPLA$_2$ activity and expression (Rao et al. 2007b) and decreased 22:6n-3 release from brain phospholipids (Contreras et al. 2000, DeMar et al. 2004). A portion of released 22:6n-3 can be converted to neuroprotectin D1 (NPD1) or resolvin D1 (RvD1; Figure 8, step F), two signaling molecules involved in brain cell survival and the resolution of inflammation (Lukiw et al. 2005, Marcheselli et al. 2003, Schwab et al. 2007). Docosahexaenoyl-CoA is a HNF-4α ligand (Hertz et al. 1998) while unesterified 22:6n-3 is a PPAR receptor ligand (Keller et al. 1993). Unesterified 22:6n-3 inhibits protein kinase C activity (Seung Kim et al. 2001) and has been implicated in the regulation of brain NFκB (Marcheselli et al. 2003), p38 MAPK (Rao et al. 2007d), Bcl-2 (Lukiw et al. 2005) and Akt (Akbar et al. 2005) signaling pathways. Furthermore, the activation of 22:6n-3 by acyl-CoA synthetase (102-131 pmol/g brain/s) requires the utilization of two high-energy phosphates from one ATP (22:6n-3 + CoA + ATP $\rightarrow$ 22:6n-3-CoA + AMP + PP$_i$; Figure 6, step G) consuming about 0.1% of the rodent brains 208 nmol/g brain/s ATP (Purdon et al. 2002, Hisanaga et al. 2004, Purdon & Rapoport...
Future studies are needed to determine the consequences of this energetically expensive 22:6n-3 release and reesterification.

Summary and conclusions

Recent studies have identified 22:6n-3 as an important secondary messenger within the brain. The PLA2 isoform involved in 22:6n-3 release from neural phospholipids upon cholinergic (M1) (Jones et al. 1997, DeGeorge et al. 1991) and serotonergic (SHT-2A) (Garcia & Kim 1997, Garcia et al. 1997) stimulation is not known. Unesterified 22:6n-3 likely directly, as well as through its oxygenated derivatives, participates in signal transduction. Cell culture studies using small molecule inhibitors and siRNA as well as kinetic studies in the rodent brain suggest that the group VI iPLA2 is involved in cleaving 22:6n-3 from brain phospholipids (Figure 8). Future studies are needed to identify the specific roles of group VIA and VIB iPLA2 in 22:6n-3 release from brain phospholipids. Further approaches to answering these questions could include the study of sn-2 radiolabeled 22:6n-3 glycerophospholipids in group VI iPLA2-specific assay systems, generation and characterization of groups VIA and VIB iPLA2 brain-specific knockouts or assessing 22:6n-3 kinetics in other models with altered brain group VI iPLA2 activity. To date it is known that group VIA iPLA2 decreases in the hippocampus in response to aging (Aid & Bosetti 2007) and in the cortex in response to dietary n-3 PUFA deprivation (Rao et al. 2007b). Whether or not decreased group VI iPLA2, and its potential ability to regulate 22:6n-3 release from brain phospholipids are contributing factors in the susceptibility of the aged or the n-3 PUFA deprived brain to disease remains to be tested.
Acknowledgements

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and learning ability of rats. II. Discrimination process, extinction process, and glycolipid compositions. *Journal of lipid research, 29*, 1013-1021.


Figure 8 Legend

Docosahexaenoic acid (DHA; 22:6n-3) rapidly disassociates from plasma albumin and passes through the blood-brain barrier (A), into the brain where it is activated by an acyl-CoA synthetase (B). Upon its activation, a small proportion of 22:6n-3 can be ß–oxidized (C) whereas the remainder is esterified to a 2-lysophospholipid via acyl-CoA transferase (D). In this model, group VI calcium-independent phospholipase A2 (iPLA2) releases 22:6n-3 from neural phospholipids (creating the 2-lysophospholipid) (E) where a portion can be converted to oxygenated derivatives (F) or activated by acyl-CoA synthetase (G). A small portion of the docosahexaenoyl-CoA can be subjected to ß-oxidation (C) whereas the remainder is reesterified to a 2-lysophospholipid via acyl-CoA transferase (D). ATP, thrombin and bradykinin are known activators of iPLA2. Although cholinergic (M1) and serotonergic (5-HT2A) receptor activation releases 22:6n-3 from neural phospholipids, the phospholipase A2 isoform involved is not known. Plasma unesterified 22:6n-3 enters the brain and is esterified to a 2-lysophospholipid at a rate of 13-15 pmol/g brain/s. However, the net rate of 22:6n-3 esterification to brain phospholipids also includes recycled 22:6n-3 leading to a net rate of 22:6n-3 entry into phospholipids from the docosahexaenoyl-CoA pool (D) of 102-131 pmol/g brain/s, which at steady state approximates the rate of 22:6n-3 release from brain phospholipids (E). NPD1, Neuroprotectin D1; RvD1, resolvin D1.
Figure 8. Incorporation and turnover of DHA in brain phospholipids.